

DOUTORAMENTO

CIÊNCIA ANIMAL- MORFOLOGIA E FISIOLOGIA

Factors Affecting Early Life
Patterns of the European Flounder *Platichthys
flesus* in a Nursery
Habitat

Cláudia Vinhas Mendes

D

2018

Factors Affecting Early Life Patterns of the European Flounder
Platichthys flesus in a Nursery Habitat

Cláudia Vinhas Mendes

Instituto Ciências Biomédicas Abel Salazar

CLÁUDIA VINHAS MENDES

**FACTORS AFFECTING EARLY LIFE PATTERNS OF THE
EUROPEAN FLOUNDER *PLATICHTHYS FLESUS* IN A NURSERY
HABITAT**

Tese de Candidatura ao grau de Doutor em Ciência Animal, Especialidade em: Morfologia e Fisiologia submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador

Doutor Adriano A. Bordalo e Sá

Professor Associado com agregação do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Co-orientador

Doutora Sandra Ramos

Investigadora no Centro Interdisciplinar de Investigação Marinha e Ambiental

Co-orientador

Professor Michael Elliott

Professor no Institute of Estuarine and Coastal Studies, University of Hull, UK

The research reported in this thesis was conducted at:

Laboratório de Hidrobiologia e Ecologia do Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto

Institute of Estuarine and Coastal Studies of the University of Hull, UK

Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Universidade do Porto



The research reported in this thesis was funded by Fundação para a Ciência e Tecnologia (FCT) through the PhD grant SFRH/BD/86325/2012.



“In the end, we will conserve only what we love,
we will love only what we understand,
and we will understand only what we are taught.”

Baba Dioum

“Mar, metade da minha alma é feita de maresia.”

Sophia de Mello Breyner Andresen

ACKNOWLEDGEMENTS/AGRADECIMENTOS

I would like to express my gratitude for all the people that in various ways have contributed to this fascinating journey into the flounder life.

Agradeço ao Professor Adriano Bordalo e Sá, por todo o apoio prestado, desde a disponibilização das condições necessárias à realização do trabalho, orientação, preocupação, compreensão, motivação e entusiasmo.

Agradeço à Doutora Sandra Ramos por me ter proposto o tema da tese, pelo apoio e compreensão ao longo do meu trabalho e por todas as discussões científicas acerca da intrigante vida da solha.

I would like to express my gratitude to Professor Michael Elliott for all the support provided by sharing his scientific knowledge, motivation and kindness. Thank you for receiving me in Hull where I had the opportunity to learn so much.

Um grande obrigado à Eva Amorim, companheira de aventuras e desventuras, no barco e em terra firme, no Porto e em Hull. Foram momentos de grande trabalho, mas também de companheirismo e boa disposição.

Agradeço a todos que participaram nas amostragens e triagens do Lima, incluindo voluntários nas triagens e identificação de zooplâncton e macroinvertebrados, ao Rui e ao Sr. Pinto. Agradeço ao Sr. José Manuel por nos receber na sua embarcação, por se mostrar sempre prestável, pela boa disposição e partilha do seu vasto conhecimento acerca do Rio Lima. Um agradecimento à Marta Ferreira, Virgínia e Sandrine pelo apoio prestado na quantificação de ácidos nucleicos. Agradeço ao Filipe Martinho pela disponibilidade em transmitir o seu conhecimento acerca da análise de otólitos. Agradeço ao laboratório de Biologia Celular do ICBAS pela disponibilização do microscópio óptico. Um obrigado especial à Sónia, Elsa e Ângela pelo apoio e boa disposição nesse período.

Este trabalho não teria sido possível sem os elementos do laboratório de Hidrobiologia. Um agradecimento especial à Dona Lurdinhas que sempre me acompanhou desde que cheguei ao laboratório, com os seus conselhos, auxílio e boa disposição; à Fernanda pela sua preocupação, curiosidade, bom-humor e perseverança na “caça aos otólitos”. Obrigada à Élia pelas conversas, risadas e

boleias; à Sérgia pelo incentivo e pelas viagens entre a terrinha e o Porto, à Ana pelo companheirismo, partilha de conversas e bom-humor no dia a dia, e à Catarina e Raquel pelo apoio.

Ao longo destes anos, foram muitos os que entre cafés, chocolate e risadas me deram o carinho, apoio e amizade necessários para concluir esta jornada.

Thank you Bartek and Moni, the best neighbours in the world, for all the special moments in everyday life at our “Bonjardim Society”. Dziękuję bardzo Paulisia for the laughs, support and “surykatka” moments, and Wojtek for the friendship and solidarity in this PhD life. Thank you all “Boredom Busters” for bringing the fun to everyday life in Porto. A special thanks to Kate and Vinnie for receiving me in Hull and making me feel at home. Agradeço também a todos que me deram apoio nesta recta final, em especial Paula, Jacinto, António, João e Hugo. Thank you Sarah for being so present and supportive, and for introducing me to my writing retreat in Flores island.

Quero agradecer à minha “família” da Ilha das Flores por todo o carinho com que me receberam no cantinho mais ocidental da Europa, e por me darem a paz de espírito tão fundamental nesta etapa da escrita – um obrigado muito especial à Sandrina, Guilherme, Ivo e Jaen por toda a amizade e companheirismo, e à Isabel por todo o carinho e por me acolher na sua cozinha/escritório com a esplêndida vista que inspirou a escrita desta tese.

Muito obrigada ao meu pai, mãe e mano Nuno, por me ouvirem e proporcionarem o apoio e afecto incondicional tão importantes, estando sempre presentes.

CONTENTS

ACKNOWLEDGEMENTS/AGRADECIMENTOS	v
CONTENTS	vii
LIST OF FIGURES	xi
LIST OF TABLES	xiii
LIST OF PAPERS	xiv
ABSTRACT	1
RESUMO	3
CHAPTER 1	5
General introduction	5
1.1. Estuaries: complex ecosystems	6
1.2. Living in estuaries	9
1.3. Fishes in estuaries	10
1.4. The estuarine nursery value	16
1.5. The European flounder <i>Platichthys flesus</i>	18
1.6. The study area	22
1.7. Motivation and main objectives	25
CHAPTER 2	29
Early life of European flounder <i>Platichthys flesus</i> in an estuarine nursery	29
Abstract	30
2.1. Introduction	31
2.2. Material and Methods	32
2.2.1. Study area	32
2.2.2. Data Collection	33
2.2.3. Otolith preparation and analysis	33
<u>Early life traits</u>	34
<u>Otolith growth rates</u>	34
2.2.4. Stomach content analysis	35
2.2.5. Data analysis	35

2.3. Results	36
2.3.1. Physical-chemical parameters	36
2.3.2. Spatial and temporal distribution of 0-group European flounder	36
2.3.3. Early life traits and growth rates of 0-group European flounder	39
2.3.4. Diet patterns of 0-group European flounder	42
2.4. Discussion	45
2.4.1. The early life traits of European flounder	45
2.4.2. Growth rates	46
2.5. Conclusions	50
CHAPTER 3	51
Feeding ecology of juvenile flounder <i>Platichthys flesus</i> in an estuarine nursery habitat: influence of prey-predator interactions	51
Abstract	52
3.1. Introduction	53
3.2. Material and Methods	55
3.2.1. Study area	55
3.2.2. Data Collection	56
<u>Macroinvertebrates</u>	56
<u>Fishes</u>	57
3.2.3. Laboratory Procedures	57
<u>Sediment characterization</u>	57
<u>Macroinvertebrates</u>	57
<u>Fish</u>	57
3.2.4. Data Analysis	58
<u>Macroinvertebrates community</u>	58
<u>Flounder diet</u>	58
<u>Prey-predator interactions</u>	59
3.3. Results	61
3.3.1. Environmental parameters	61

3.3.2.	Macroinvertebrate community	62
3.3.3.	<i>P. flesus</i> juvenile distribution.....	64
3.3.4.	Diet of <i>P. flesus</i> juveniles	65
3.3.5.	Prey selection.....	70
3.4.	Discussion	73
3.5.	Conclusions.....	78
CHAPTER 4		79
Feeding strategies and condition of juvenile European flounder <i>Platichthys flesus</i> in a nursery habitat		79
Abstract.....		80
4.1.	Introduction	81
4.2.	Material and Methods.....	84
4.2.1.	Study area.....	84
4.2.2.	Data collection.....	84
4.2.3.	Stomach content analysis	85
4.2.4.	Stable isotope analysis.....	85
4.2.5.	Condition analysis	86
4.2.6.	Data analysis	87
4.3.	Results	88
4.3.1.	Stomach contents analysis	88
4.3.2.	Stable isotope analysis.....	91
	<u>Organic matter sources</u>	91
	<u>Prey</u>	92
	<u>European flounder juveniles</u>	93
4.3.3.	SIAR outputs	94
4.3.4.	Condition analysis	96
4.4.	Discussion	98
4.4.1.	Integrating stomach contents and stable isotope analysis	98
4.4.2.	European flounder juvenile movements and main feeding areas within the Lima estuary	99

4.4.3. Feeding strategies promoting European flounder condition in the Lima estuary and management implications	100
4.5. Conclusions	102
CHAPTER 5.....	103
Condition and growth of 0-group European flounder <i>Platichthys flesus</i> in a nursery habitat	103
Abstract.....	104
5.1. Introduction	105
5.2. Material and Methods	107
5.2.1. Study area	107
5.2.2. Collection and sorting of the 0-group European flounder	108
5.2.3. Condition and growth analysis	108
5.3. Results	109
5.3.1. Condition and growth of 0-group European flounder	109
5.4. Discussion	112
5.5. Conclusions	115
CHAPTER 6.....	117
Final Considerations and Suggestions for Future Research	117
6.1. Final Considerations	118
6.2. Suggestions for future research	122
References.....	125
APPENDICES	161
Published papers.....	161

LIST OF FIGURES

Figure 1.1. The estuarine ecosystem as the sum of relationships between physicochemical attributes, the fundamental ecological niche and the community ecological niche with the superimposed human impact (adapted from Wolanski and Elliott, 2015).	8
Figure 1.2. The guilds of estuarine fish according to estuarine usage functional guilds (EUGF) sorted into four categories: marine, estuarine, freshwater and diadromous (adapted from Potter <i>et al.</i> , 2015).	12
Figure 1.3. Components of nursery ground value (adapted from Sheaves <i>et al.</i> , 2015).	18
Figure 1.4. The life cycle of the European flounder <i>Platichthys flesus</i> (Linnaeus, 1758).	20
Figure 1.5. The Lima estuary with the lower, middle and upper estuarine sections (adapted from Google Earth, 2018).	23
Figure 1.6. The early life stages of flounder in the lower, middle and upper stretches of the Lima estuary (adapted from Ramos <i>et al.</i> , 2010). 1- metamorphosing larvae; 2 – newly-settled and 3 – adults.; *possible settlement area.	26
Figure 2.1. The Lima estuary with the location of the sampling points (adapted from Amorim <i>et al.</i> , 2018).	33
Figure 2.2. a) Spatial covering the lower, middle and upper sections, and b) temporal abundances of 0-group European flounder (individuals 1,000 m ⁻²) per size class (10 mm TL) in the Lima estuary.	37
Figure 2.3. Back-calculated a) hatch and b) settlement dates of 0-group European flounder in the Lima estuary based on otolith daily ring counts.	40
Figure 2.4. Relationship between total length and a) age (days); b) post-settlement and recent growth rates (µm.day ⁻¹) of 0-group European flounder based on otolith daily increments.	41
Figure 2.5. Growth rates of 0-group European flounder in the Lima estuary: a) temporal variation, and b) relationship between post-settlement and recent growth rates (µm.day ⁻¹) based on otolith daily increment widths. Number of otolith samples (n) represented above bars.	42
Figure 2.6. Index of Relative Importance (IRI) for the stomach contents of 0-group European flounder: a) temporal variation, and b) ontogenetic variation between size classes (10 mm total length). Number of counted prey (n _{prey}), and number of full stomachs analyzed (n) are presented below the graphs.	44
Figure 3.1. a) Lima estuary with the location of sampling stations, (L1, L2, L3- lower estuary; L4, L5, L6- middle estuary; L7, L8, L9- upper estuary); and b) average (individuals 1, 000 m ⁻²) and relative abundances (%) of <i>P. flesus</i> juveniles of the lower, middle and upper sections of the Lima estuary; (in brackets: total number of fishes sampled per size class).	56

Figure 3.2. Average abundance (individuals m ⁻²) per estuarine section (lower, middle and upper) of the main taxa of the Lima estuary macroinvertebrate community including main prey of juvenile flounder.	64
Figure 3.3. Numerical (NI), occurrence (OI) and weight (WI) indices for stomach contents of <i>P. flesus</i> juveniles for each size class; (in brackets: number of prey items per size class).	67
Figure 3.4. Cluster analysis of the four <i>P. flesus</i> size classes, based on numerical index (NI)(A), occurrence index (OI)(B) and weight index (WI)(C). Significant clusters according to SIMPROF are shown in red.	69
Figure 3.5. Seasonal abundance of macrobenthos prey in the Lima estuary (A); Strauss linear index values for the main prey items of <i>P. flesus</i> size classes: 1 (B), 2 (C), 3 (D) and 4 (E)(W- winter; Sp- spring; Su- summer; A- autumn).	71
Figure 4.1. Lima estuary with the location of the sampling stations (1- lower estuary; 2 and 3 – middle estuary; 4 and 5 - upper estuary).	84
Figure 4.2. Numerical (NI) and weight (WI) indices for stomach contents of a) 0-group and b) 1-group European flounder in the Lima estuary.	90
Figure 4.3. Ordination diagrams for the Principal Coordinate Analysis (PCO) performed on carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) stable isotope signatures of European flounder prey in the Lima estuary.	92
Figure 4.4. Carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) stable isotopes (‰) of a) 0-group and 1-group European flounder, and respective upstream and downstream prey; b) sediment (SOM, upstream and downstream) and water particulate (POM, upstream and downstream) organic matter sources. Trophic enrichment factors were applied to sources ($\delta^{13}\text{C}$: $\pm 2\text{‰}$), and to prey ($\delta^{13}\text{C}$: $\pm 1\text{‰}$, $\delta^{15}\text{N}$: $\pm 3.4\text{‰}$).	94
Figure 4.5. Boxplots of the mixing models estimates of prey contribution to the diet of a) 0-group and b) 1-group European flounder. Prey groups divided according to upstream and downstream areas.	96
Figure 4.6. Relationship between Fulton K and a) carbon stable isotopes ($\delta^{13}\text{C}$) and b) nitrogen stable isotopes ($\delta^{15}\text{N}$); RNA:DNA and c) carbon ($\delta^{13}\text{C}$) and d) nitrogen ($\delta^{15}\text{N}$) stable isotopes for 0-group and 1-group European flounder.	97
Figure 5.1. The Lima estuary and sampling locations along the lower (station 1), middle (stations 2 and 3) and upper (stations 4 and 5) estuaries.	107
Figure 5.2. Relationship between total length and a) Fulton K, b) RNA:DNA, c) Post-settlement growth rates ($\mu\text{m}.\text{day}^{-1}$), and d) Recent Growth index ($\mu\text{m}.\text{day}^{-1}$) of 0-group European flounder <i>Platichthys flesus</i>	111
Figure 5.3. Relationship between a) total length (mm) and age (days), and b) mean otolith growth rates at the post-settlement stage ($\mu\text{m}.\text{day}^{-1}$) and the Recent Growth index ($\mu\text{m}.\text{day}^{-1}$) of the 0-group European flounder <i>Platichthys flesus</i>	112

LIST OF TABLES

Table 1.1. The estuarine usage functional groups (EUFG) with definitions of the different categories and guilds.	15
Table 2.1. Number of 0-group European flounder sampled (n), abundance (individuals 1,000 m ⁻²), mean age (days), vacuity (%), Shannon-Wiener diet diversity (H') and mean post-settlement (PSGR) and recent (RG) growth rates per size class (10 mm TL). In brackets: number of 0-group flounder selected for otolith analysis.	38
Table 3.1. Results of ANOSIM (R values and significance levels) and SIMPER analyses on abundance of macroinvertebrate taxa (SIMPER results for the three most important taxa contributing to dissimilarities are shown). ni = not identified.	63
Table 3.2. Number of <i>P. flesus</i> juveniles sampled per size class, mean total length (mm) and mean total weight (g).	65
Table 3.3. Statistics for linear regression analysis (R ² and p value) on minimum, mean and maximum prey length and juvenile flounder total length, according to fish size class.	68
Table 3.4. Statistics for the Gamma regression models fitted to 0-group <i>P. flesus</i> densities in the Lima estuary (residual deviance, deviance, percentage of the total deviance explained by each factor and p value).	72
Table 4.1. Number (n) of 0-group and 1-group European flounder sampled in the Lima estuary, mean total length (mm), total weight (g), Fulton's K, RNA:DNA and muscle carbon (δ ¹³ C) and nitrogen (δ ¹⁵ N) stable isotope signatures (‰).	89
Table 4.2. Mean carbon stable isotope δ ¹³ C (‰) of particulate organic matter (POM) and sediment organic matter (SOM) sources in the upstream and downstream areas of the Lima estuary.	91
Table 4.3. Mean carbon (δ ¹³ C) and nitrogen (δ ¹⁵ N) stable isotope signatures of the main prey groups of European flounder juveniles in the upstream and downstream areas of Lima estuary.	93
Table 5.1. Number (n) of 0-group European flounder <i>Platichthys flesus</i> sampled in the Lima estuary, mean total length (mm), total weight (g), age (days) and condition indices Fulton K, RNA:DNA, otolith post-settlement growth rates (PSGR, μm.day ⁻¹) and recent growth index (RG, μm.day ⁻¹).	110

LIST OF PAPERS

This thesis resulted in the publication of the following papers:

Mendes, C., Ramos, S. & Bordalo, A.A. (2014). Feeding ecology of juvenile flounder *Platichthys flesus* in a nursery habitat: influence of prey-predator interactions. *Journal of Experimental Biology and Ecology* 461, 458-468. DOI: 10.1016/j.jembe.2014.09.016.

ABSTRACT

Estuaries provide nursery function for marine fishes and play a key role in the replenishment of adult populations. However, estuaries suffer heavy anthropogenic pressure that may compromise nursery function. In this perspective, knowledge on habitat use patterns sustaining carrying capacity and nursery value for different early life stages is required towards conservation of nursery function. The European flounder *Platichthys flesus* is a typical estuarine nursery user with early juveniles often concentrating in upstream low salinity habitats. However, there are still various questions regarding major drivers of flounder nursery use patterns. This thesis aimed to investigate key factors of flounder lifecycle in an estuarine nursery habitat. The Lima River estuary was chosen as a study system to investigate the role of feeding patterns as major drivers of nursery use by flounder juveniles. Estuarine colonization, abundance, feeding and growth patterns of 0-group flounder (with less than one year old) were integrated towards understanding habitat use patterns underlying nursery habitat quality. The 0-group flounder were most abundant in June. The 0-group flounder hatched from February to June, settled between March and July, with peak settlement occurring in May according to otolith microstructure analysis. The diet focused on upper estuarine prey justifying the concentration of 0-group flounder in this estuarine area. There was an ontogenetic diet shift at 50 mm TL from Chironomidae to *Corophium*. In general, the 0-group flounder presented high growth rates, indicating that these habitat use strategies promoted the growth of the early life stages, reflecting the good nursery value of the Lima estuary. Moreover, the main prey together with salinity were the main factors associated to the distribution of 0-group flounder in the Lima estuary. The diet became more diverse as juveniles grew and there was another ontogenetic diet shift from 0-group to 1-group flounder (flounder juveniles that have completed 1 year old). Accordingly, combined use of stomach contents and stable isotope analysis showed that main feeding locations varied between these age groups: 0-group flounder concentrated upstream explaining the abundance patterns mentioned above, while 1-group flounder dispersed throughout the estuary feeding both upstream and downstream. Feeding strategies affected 0-group flounder recent condition, since juveniles with higher condition fed mostly in downstream areas. Therefore, a trade-off between physiological costs associated to low salinity, and high prey availability and less competition with older 1-group flounder, may explain why 0-

group flounder remained in the upstream area. Overall, 0-group and 1-group flounder with these feeding strategies presented good recent (RNA:DNA) and long-term (Fulton K) individual condition. These findings highlighted the importance of ontogenetic changes in feeding strategies and how they shaped flounder nursery use and individual condition. Moreover, condition indices (Fulton K and RNA:DNA) and otolith based growth rates of 0-group flounder were integrated towards a more comprehensive overview of nursery habitat quality. All indices showed good individual condition and fast growth of 0-group flounder in the Lima nursery. Overall, this study showed that the European flounder uses different strategies throughout the early life stages that enabled good individual condition and high growth rates, showing that the nursery value of the Lima estuary for this species. Moreover, foraging patterns may drive differential occupation of habitat units by early life stages within the same species, explaining the concentration of 0-group flounder in the upper estuary. Therefore, management measures should preserve connectivity between these habitat units composing the seascape towards sustaining carrying capacity and nursery value.

RESUMO

A função viveiro (*nursery* em inglês) é umas das principais funções dos estuários, desempenhando um papel chave na renovação das populações adultas de peixes marinhos. Contudo, os estuários estão sujeitos a elevadas pressões antropogénicas que podem comprometer a função viveiro. Nesta perspectiva, é importante aprofundar o conhecimento relativo aos padrões de utilização de habitat que controlam a capacidade e o valor dos viveiros para diferentes estados iniciais de vida, tendo em vista a conservação da função viveiro. A solha-das-pedras *Platichthys flesus* é uma espécie que utiliza os estuários como local de viveiro, e os estados juvenis iniciais tendem a concentrar-se nos locais mais a montante, geralmente com baixa salinidade. Contudo, várias questões relacionadas com os principais factores que controlam estes padrões de utilização do habitat viveiro encontram-se ainda por resolver. Esta tese teve como objectivo principal investigar factores chave do ciclo de vida da solha-das-pedras num local de viveiro estuarino. O estuário do Rio Lima foi escolhido como local de estudo para investigar a importância das estratégias alimentares como factores chave que controlam a utilização do habitat viveiro pelos juvenis da solha-das-pedras. Assim, estudou-se a colonização estuarina, padrões de abundância, alimentação e crescimento dos juvenis 0⁺ (com menos de um ano de idade) cujos resultados foram integrados para uma melhor compreensão dos padrões de utilização do habitat que determinam a sua qualidade como viveiro. Os juvenis do ano (juvenis 0⁺) foram mais abundantes em Junho. Segundo a análise da microestrutura dos otólitos, verificou-se que estes juvenis 0⁺ eclodiram entre Fevereiro e Junho e o assentamento ocorreu entre Março e Julho, com um pico em Maio. A dieta dos juvenis 0⁺ foi composta predominantemente por presas do estuário superior, justificando a concentração destes juvenis nesta área. Foi observada uma alteração ontogenética da dieta, registando-se uma transição de Chironomidae para *Corophium* aos 50 mm de comprimento total. Em geral, os juvenis 0⁺ apresentaram elevadas taxas de crescimento, indicando que estas estratégias de utilização de habitat promoveram o crescimento das fases iniciais de vida, reflectindo assim a qualidade do estuário do Lima como local de viveiro. Além disso, a distribuição dos juvenis 0⁺ esteve associada à salinidade e à distribuição das principais presas Chironomidae e *Corophium*. A dieta diversificou-se ao longo do crescimento, ocorrendo outra alteração ontogenética da dieta dos juvenis 0⁺ para os juvenis 1⁺ (completaram um ano de idade). De facto,

segundo a análise de conteúdos estomacais e isótopos estáveis verificou-se que os principais locais de alimentação variaram entre grupos etários, sendo que os juvenis 0⁺ concentraram-se a montante (estuário superior) explicando os padrões de abundância mencionados acima, enquanto que os juvenis 1⁺ dispersaram-se ao longo do estuário, alimentando-se quer a montante, quer a jusante (estuários inferior e médio). A utilização de recursos alimentares afectou a condição recente dos juvenis 0⁺, sendo que juvenis com condição mais elevada alimentaram-se nas áreas a jusante. Assim, a permanência dos juvenis 0⁺ na área a montante poderá representar um compromisso entre os desafios fisiológicos impostos pela baixa salinidade desse local e, por outro lado, a elevada disponibilidade de presas e reduzida competição com os juvenis 1⁺ nesta zona do estuário. Em geral, juvenis 0⁺ e 1⁺ que apresentaram as estratégias de alimentação acima mencionadas apresentaram boa condição individual recente (RNA:DNA) e a longo prazo (Fulton K). Estas conclusões evidenciam a importância das alterações ontogenéticas nas estratégias de alimentação e a forma como moldam a utilização do viveiro e a condição individual das solhas-das-pedras. Para além disso, os índices de condição (Fulton K e RNA:DNA) e taxas de crescimento dos otólitos foram integrados para uma melhor compreensão da qualidade do habitat viveiro. Todos os índices evidenciaram a boa condição individual e taxas de crescimento elevadas dos juvenis 0⁺ no estuário do Lima. Este estudo demonstrou que a solha-das-pedras utiliza diferentes estratégias ao longo das suas fases iniciais de vida, estratégias essas que permitiram que os juvenis atingissem boa condição individual e elevadas taxas de crescimento, evidenciando assim o valor do estuário do Lima como local de viveiro para a solha-das-pedras. Este estudo demonstrou que os padrões de alimentação podem ser responsáveis pela ocupação diferencial de habitats por diferentes estados iniciais de vida da mesma espécie, explicando a concentração dos juvenis 0⁺ no estuário superior. Em conclusão, as medidas de gestão devem assegurar a conectividade entre estes habitats de forma a preservar a capacidade e valor dos habitats de viveiro.

CHAPTER 1

General introduction

1.1. Estuaries: complex ecosystems

Estuaries are among the most productive ecosystems in the world (McLusky and Elliott, 2004) due to the high nutrient input in the sediment and water column. Several definitions of an estuary have been proposed through the years. In 1959, Odum defined an estuary as “a river mouth where tidal action brings about a mixing of freshwater and saltwater”. A later definition by Pritchard (1967) proposed that an estuary is “a semi-enclosed body of water which has a free connection with the open sea and within which sea water is measurably diluted with fresh water derived from land drainage”. While still widely used, this definition disregards the tidal influence and excludes coastal water bodies such as hypersaline lagoons (Wolanski and Elliott, 2015). More recently, Dyer (1997) developed the concept proposed by Pritchard (1967) by considering the tidal influence: “an estuary is a semi-enclosed coastal body of water which has a free connection to the open sea, extending into the river as far as the limit of the tidal influence, and within which sea water is measurably diluted with fresh water derived from land drainage”. However, these definitions focused in estuaries from temperate regions of the northern hemisphere. Therefore, Potter *et al.* (2010) suggested a new definition that also included the periodic closure of mouths of estuaries and hypersaline conditions during the dry period, common characteristics in some estuaries of Southern Hemisphere regions, such as South Africa and South Australia. Accordingly, an estuary is “a partially enclosed coastal body of water that is either permanently or periodically open to the sea and which receives at least periodic discharge from a river(s), and thus, while its salinity is typically less than that of natural sea water and varies temporally and along its length, it can become hypersaline in regions when evaporative water loss is high and freshwater and tidal inputs are negligible”. A more global definition of “transitional waters” has been proposed by the Water Framework Directive (WFD) (European Communities 2000), as “bodies of surface water in the vicinity of river mouths which are partly saline”. This definition was created for management purposes to ensure water quality control in all water bodies within Europe. Therefore, it does not apply exclusively to estuaries, but also to other intermediate water bodies such as rias, fjords and lagoons (McLusky and Elliott, 2007; Wolanski and Elliott, 2015).

Estuaries are typically sheltered (e.g. by reefs or fingers of land) from ocean extremes resulting from waves, winds, and storms, and there is accumulation of fine sediment carried from the sea and from the rivers that results in the formation of mudflats (McLusky and Elliott, 2004). Estuaries are characterized by a gradient of conditions that results from the mixing between freshwater and seawater, and constant oscillations in abiotic conditions, such as salinity, temperature and turbidity, and biotic conditions (McLusky and Elliott, 2004). In temperate estuaries, there is usually a gradient of salinity from seawater (salinity 35*) to freshwater (salinity 0) and estuarine waters are classified as brackish waters (salinity 0.5-35). It is challenging to define where an estuary ends due to these gradual geomorphological changes (Wolanski and Elliott, 2015) across the transition between river, estuary and coastline. Both the upstream limit of salt penetration (Pritchard, 1967) and the upstream limit of tidal penetration (Fairbridge, 1980) have been considered as the upstream limits of estuaries (Wolanski and Elliott 2015).

Estuaries are never at a steady-state and the hydromorphology determines the temporal and spatial variability in estuaries. The hydrodynamics results from the combination of the currents and mixing processes caused by the interaction between freshwater and seawater, tides, wind, rainfall and evaporation, oceanic events such as upwelling, and the spatial and temporally variable bathymetry and geomorphology (Elliott and Whitfield, 2011; Wolanski and Elliott, 2015). Connectivity plays a key role where several ecotones, as transition/gradient between systems, are present, from freshwater catchment, out of the estuary to coastal and marine areas, laterally from the supratidal region into littoral margins, vertically from the water column into the estuary bed, and with depth/stratification throughout the water column (Elliott and Whitfield, 2011). The estuarine ecosystem (Figure 1.1.) is then driven by links between physicochemical properties, the fundamental niche i.e. the habitats, and the community structural and functional attributes (McLusky and Elliott 2004, Wolanski and Elliot 2015). Physicochemical attributes shape the fundamental niche that supports colonization by a complement of species to given community structure i.e. environment to biology links. The biological community that is then formed is the base for the biology-to-biology relationships that include all biological interactions, such as prey-predator links and competition, creating the ecological functioning. In turn, the

*Throughout this thesis, salinity is expressed as a dimensionless variable according to the widely adopted UNESCO Practical Salinity Scale (1978).

organisms also influence the structure of the physicochemical system in a process termed biology-to-environment links.

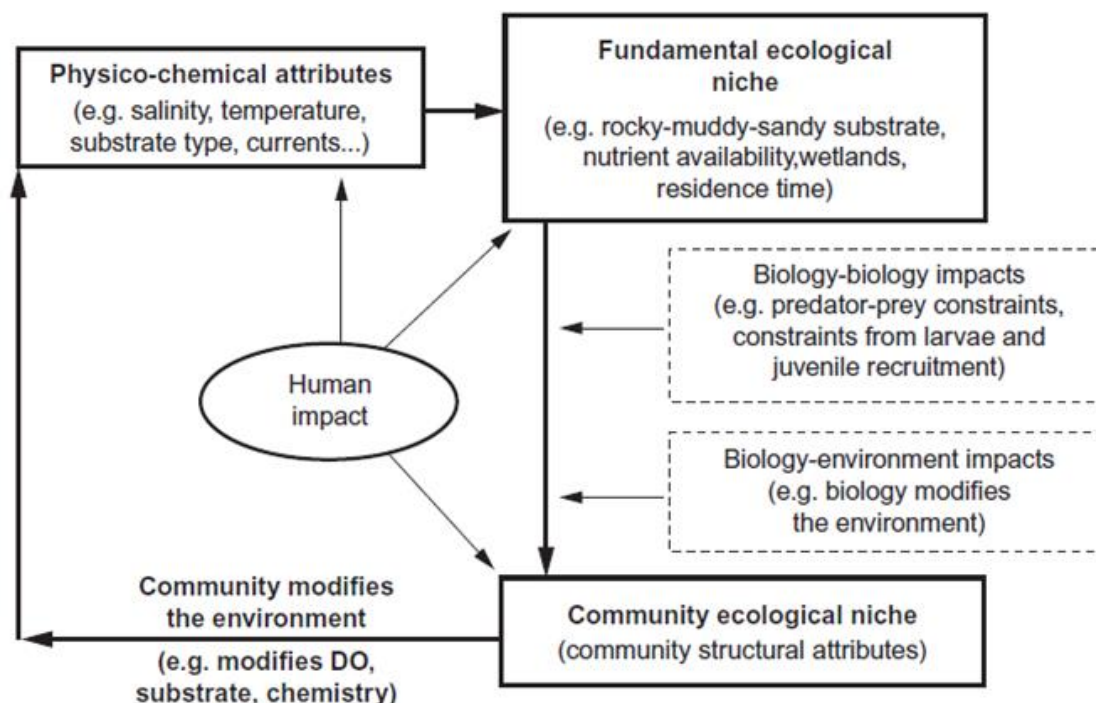


Figure 1.1. The estuarine ecosystem as the sum of relationships between physicochemical attributes, the fundamental ecological niche and the community ecological niche with the superimposed human impact (adapted from Wolanski and Elliott, 2015).

Estuarine areas are also heavily populated and estuarine ecosystems suffer from the pressures of anthropogenic activities, that can be listed into three categories: materials that are put into estuaries (e.g. pollutants and contaminants in waste waters), materials removed from the estuaries (salt, fish, sediments and space), and external influences such as the impacts of global climate change (Wolanski and Elliott, 2015). The full understanding of the estuarine ecosystems at all physical and biological levels is fundamental considering their ecological and human relevance, as well as the constant threats to these environments.

Estuaries comprise several interlinked habitats. Some of these habitats are also common among other ecosystems, such as sandbanks and sandy beaches, while others are typically estuarine, such as the tidal wetlands which are habitats dominated

by a major type of vegetation, with underlying mud owing to the sheltered nature, and usually tolerant to brackish conditions (Wolanski and Elliott, 2015). Tidal wetlands include salt marshes, mangrove areas and intertidal mudflats that will be colonized by the vegetation (McLusky and Elliott, 2004; Wolanski and Elliott, 2015). Salt marshes are among the most productive systems in the world, providing abundant food supply for the detritus decomposers and primary consumers in temperate estuaries. These are also key habitat for animals such as juvenile shrimp and fish, as well as breeding areas for birds. Mangroves dominate the intertidal vegetation of tropical and subtropical estuaries presenting an equivalent role of saltmarshes in temperate estuaries. The intertidal mudflats generally cover a small proportion of estuarine area but may contribute substantially to the total primary production of the estuary.

1.2. Living in estuaries

Estuarine primary producers and different levels of consumers are present within two fundamental niches, the water column and the substratum, in constant interplay (benthic-pelagic coupling) at the sediment-water interface (Wolanski and Elliott, 2015). Estuaries receive large inputs of organic matter from allochthonous sources through tidal import from the sea, river catchment and adjacent wetlands, and anthropogenic waste that adds to the autochthonous production (e.g. seagrass meadows, mangroves and saltmarshes). This high load of nutrients supports high primary productivity by the benthic algae, phytoplankton and saltmarshes (Odum *et al.*, 1971; McLusky and Elliott, 2004; Day *et al.*, 2012). In fact, most estuaries are detrital based systems (Elliott and Whitfield, 2011) where detritus is defined as “all types of biogenic material in various stages of microbial decomposition that represents a potential energy source for consumer species” (McLusky and Elliott, 2004). The phytoplankton, benthic microalgae, plant material and their decomposers form the particulate organic matter (POM) which is the food for the primary consumer animals (McLusky and Elliott, 2004).

The physiological challenges imposed by the mixing of salt and freshwater and the resulting extremes and oscillations in abiotic conditions are responsible for the low diversity of estuarine ecosystems (McLusky and Elliott, 2004). However, estuaries provide abundant resources to the organisms that can adapt to these conditions,

promoting the recruitment of species with diverse physical and trophic structures. Thus, estuaries support high abundances of individual species. While abiotic factors limit the distribution of individual species, the biomass and productivity of estuarine animals are controlled by food supply, supply of colonizing larvae and interspecific competition (McLusky and Elliott, 2004). The estuarine fauna is comprised of sea, river and true estuarine animals. The dominant heterotrophic organisms comprise zooplankton, benthic invertebrates and fish, as well as fungi and bacteria.

Estuarine primary consumers are mainly found in the benthic community, as zooplankton faces flushing by river flow and strong tidal currents, as well as limitations imposed on phytoplankton by turbidity and flushing out of the estuary. Benthic consumers may be classified into macrobenthos i.e. animals retained by a 0.5 mm sieve, meiobenthos i.e. animals passing a 0.5 mm mesh and retained by a 40-60 μm , while microbenthos comprises animals passing a 40-60 μm sieve. Macrobenthic populations are one of the most relevant groups of estuarine communities, including freshwater and marine species (Edgar and Shaw, 1995). They provide fundamental food sources for several demersal fish and invertebrate species and represent an important link in the energy flow to higher trophic levels, recycling organic matter in marine and estuarine ecosystems (Edgar and Shaw, 1995).

1.3. Fishes in estuaries

The estuarine fish communities are dominated by few species in terms of number and biomass (Whitfield, 1994; McLusky and Elliott, 2004). These species have adapted to constant environmental oscillations and thrive in the estuarine environment (Elliott and Quintino, 2007). The set of hydrophysical and biogeochemical factors creates the niches, habitats and conditions available for colonization and the resources to create and support the fish assemblage (Wolanski and Elliott, 2015). The available space, food and shelter in different habitats within estuaries then allow the different uses.

The estuarine fish fauna includes resident fish communities, and transient species at different life stages (Able and Fahay, 1998) and with a variety of history patterns (Haedrich, 1983). The way fishes use estuaries has been categorized by several

authors through the years (Haedrich, 1983; Potter *et al.*, 1990; Elliott and Dewailly, 1995; Whitfield, 1999; Elliott *et al.*, 2007b). The functional guild approach assigns fishes of estuarine assemblages into different functional groups according to their estuarine use, mode of feeding and reproductive success (Franco *et al.*, 2008). The review of Elliott *et al.* (2007b) integrated different terminologies so they could be applied to estuarine ichthyofauna worldwide and it has been recently refined by Potter *et al.* (2015). Fishes of estuarine assemblages were assigned to different estuarine usage functional groups (EUFG) integrated into four major categories: marine, estuarine, diadromous and freshwater. Each group depicted in Figure 1.2. represents characteristics associated to spawning, feeding and/or refuge and migratory movements that may occur between estuaries and other ecosystems.

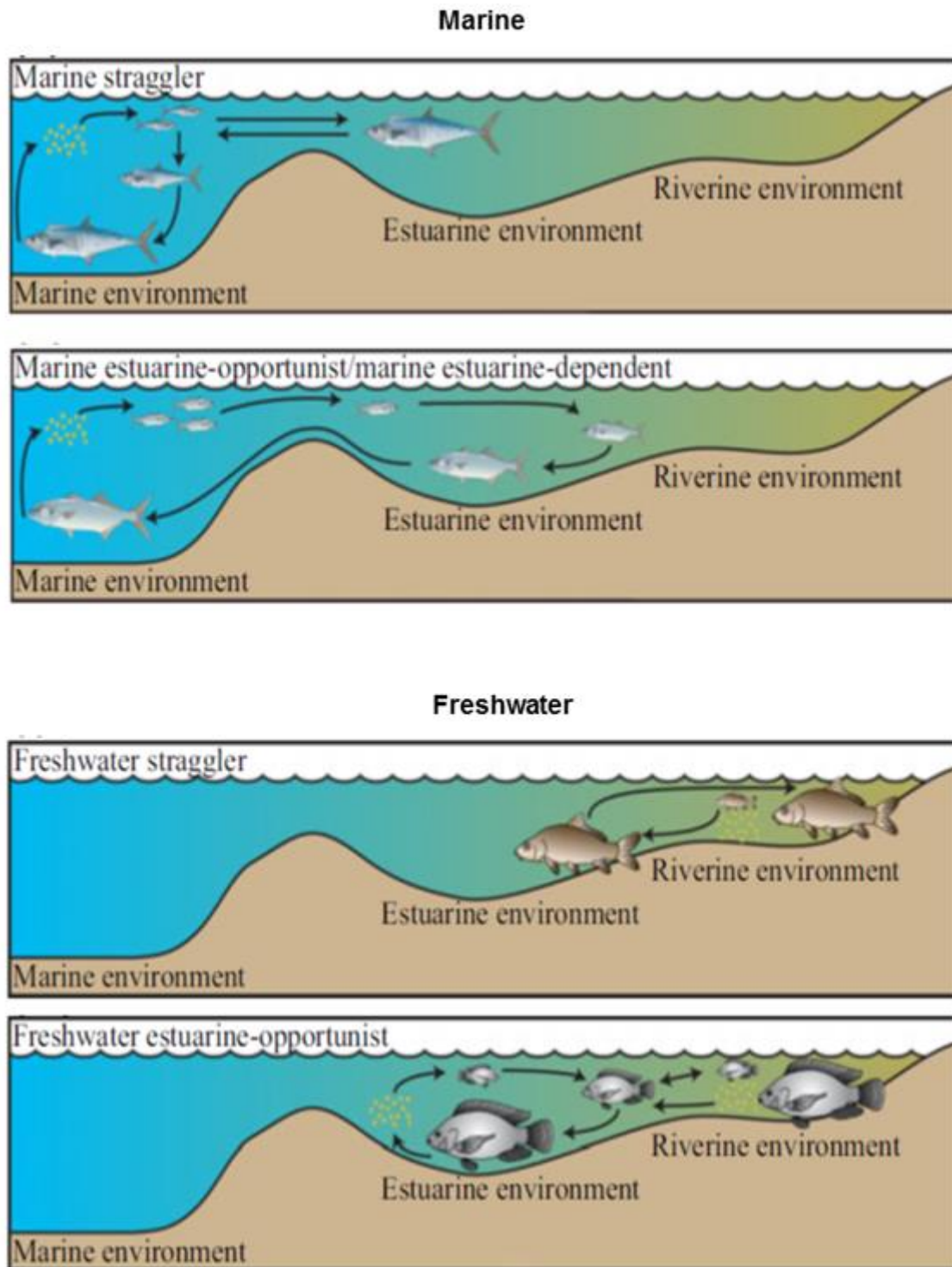


Figure 1.2. The guilds of estuarine fish according to estuarine usage functional guilds (EUFG) sorted into four categories: marine, estuarine, freshwater and diadromous (adapted from Potter *et al.*, 2015).

Estuarine

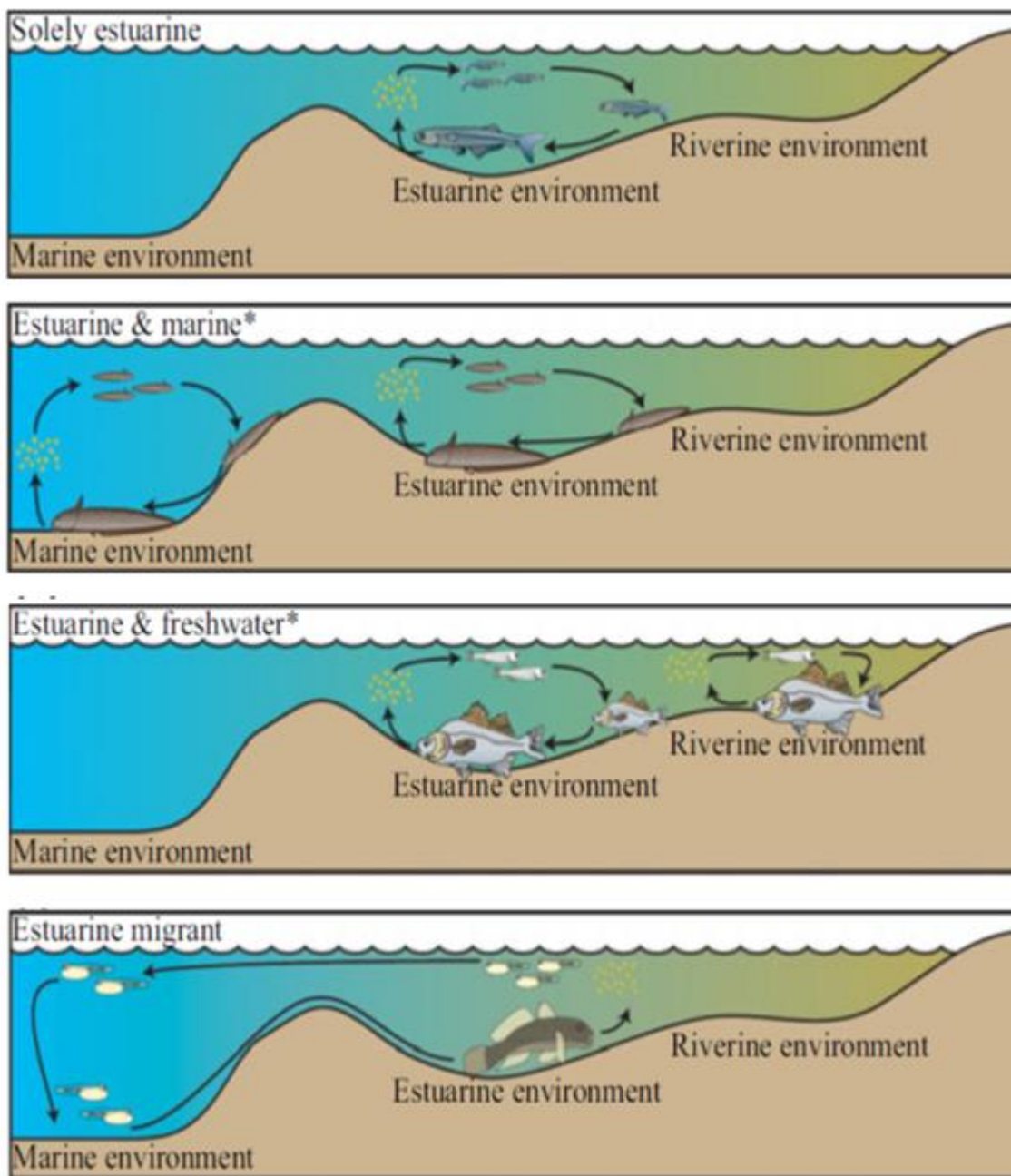


Figure 1.2. (cont.) The guilds of estuarine fish according to estuarine usage functional guilds (EUFG) sorted into four categories: marine, estuarine, freshwater and diadromous (adapted from Potter *et al.*, 2015).

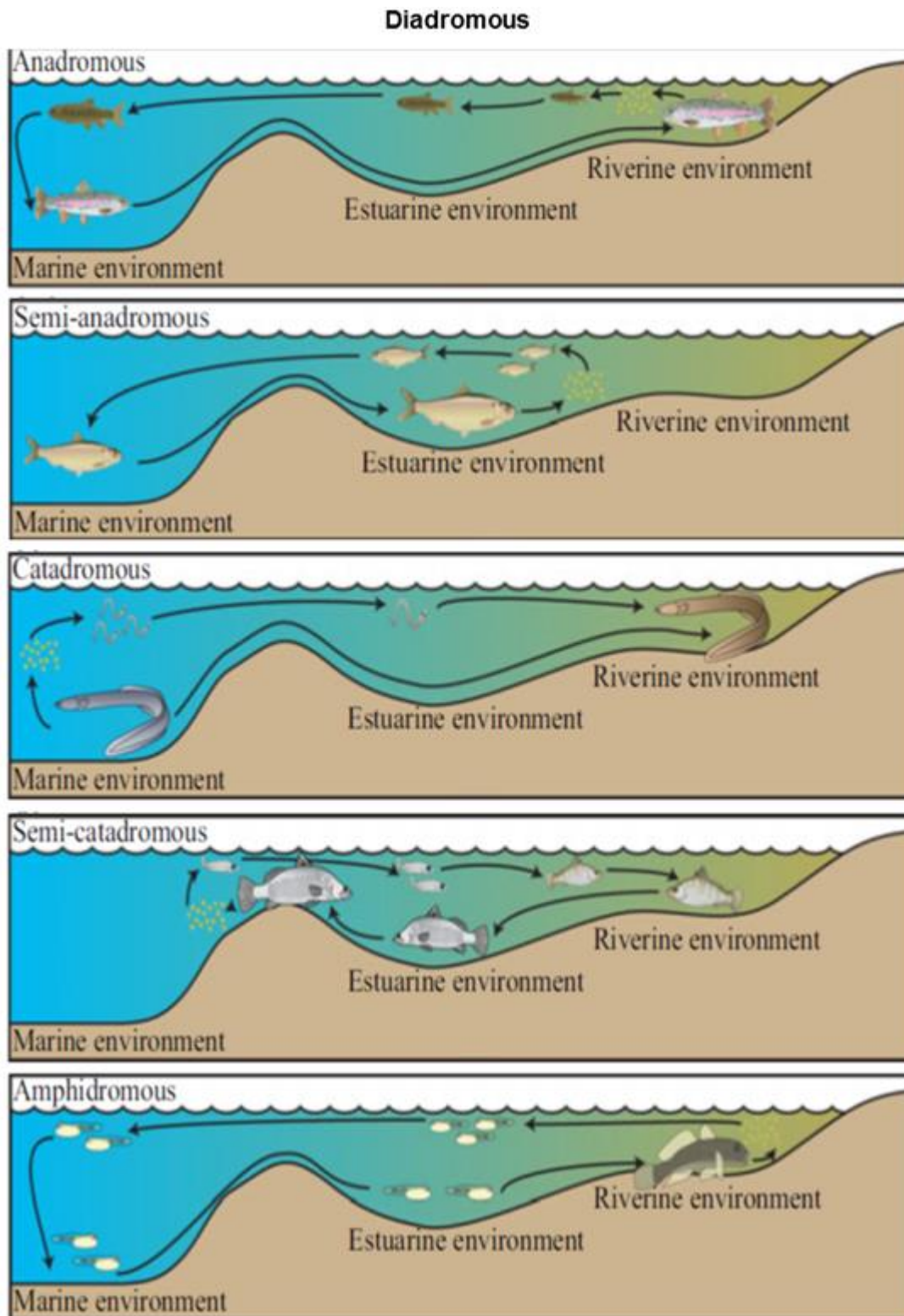


Figure 1.2. (cont.) The guilds of estuarine fish according to estuarine usage functional guilds (EUFG) sorted into four categories: marine, estuarine, freshwater and diadromous (adapted from Potter *et al.*, 2015).

Table 1.1. The estuarine usage functional groups (EUFG) with definitions of the different categories and guilds.

Category	Guild	Definition
Marine Species that spawn at sea	Marine straggler	Typically enter the estuaries sporadically and in low numbers and are most common in the lower reaches where salinities typically do not decline far below 35. Often stenohaline.
	Marine estuarine-opportunist	Regularly enter estuaries in substantial numbers, particularly as juveniles, but use, to varying degrees, coastal marine waters as alternative nursery areas.
	Marine estuarine-dependent	Juveniles require sheltered estuarine habitats and are thus not present along exposed coasts where they spend the rest of their life.
Estuarine Species with populations in which the individuals complete their life cycles within the estuary	Solely estuarine	Found only in estuaries.
	Estuarine & marine	Also represented by marine populations.
	Estuarine & freshwater	Also represented by freshwater populations.
	Estuarine migrant	Spawn in estuaries but may be flushed out to sea as larvae and later return at some stage to the estuary.
Diadromous Species that migrate between the sea and freshwater	Anadromous	Most growth is at sea before migration into rivers to spawn.
	Semi-anadromous	Spawning run from the sea extends only as far as the upper estuary rather than into freshwater.
	Catadromous	Spend their trophic life in freshwater and subsequently migrate out to sea to spawn.
	Semi-catadromous	Spawning run extends only as far as downstream estuarine areas rather than into the marine environment.
	Amphidromous	Spawn in fresh water, with the larvae flushed out to sea, where feeding occurs, followed by a migration back into freshwater, where most somatic growth and spawning occurs.

Table 1.1. (continued) The estuarine usage functional groups (EUFG) with definitions of the different categories and guilds.

Category	Guild	Definition
Freshwater Species that spawn in freshwater	Freshwater straggler	Found in low numbers in estuaries and whose distribution is usually limited to the low salinity, upper reaches of estuaries.
	Freshwater estuarine-opportunist	Found regularly and in moderate numbers in estuaries and whose distribution can extend well beyond the oligohaline sections of these systems.

1.4. The estuarine nursery value

Estuaries provide several functions including food, reproduction, growth and refuge for fish species (Able and Fahay, 1998; Elliott and Hemingway, 2002). The nursery function is among most relevant roles of estuaries and is provided to early life stages, including larvae and juveniles of marine and migratory (e.g. catadromous and anadromous) species (Beck *et al.*, 2001; Pihl *et al.*, 2007). A nursery is a habitat where early life stages are temporal and spatially separated from adults even though a degree of spatial overlap is possible (Pihl *et al.*, 2007).

According to Beck *et al.* (2001), the contribution of juveniles to recruiting adult populations per unit area is on average greater in nursery habitats than in other habitats occupied by the juveniles. This contribution is enabled by optimal feeding and refuge conditions (Beck *et al.*, 2001; Pihl *et al.*, 2007; Nagelkerken *et al.*, 2015) promoting a combination of higher density, growth, survival and movement to adult populations (Beck *et al.*, 2001). However, this concept underestimates habitats with a large area despite a small per-unit-area contribution, but still essential to sustaining adult populations (Dahlgren *et al.*, 2006). Therefore, Dahlgren *et al.* (2006) proposed the concept of Effective juvenile habitat (EJH) for juvenile habitats that present an above average proportional contribution to adult populations in comparison to other juvenile habitats, and regardless of per-unit-area contribution. However, these approaches did not capture dynamic processes, such ontogenetic habitat shifts, hence the differential role of adjacent habitats to different early life stages and the importance of connectivity between these habitat units. Hence, Nagelkerken *et al.* (2015) introduced the concept of seascape nursery consisting of a dynamic mosaic of habitat

units functionally connected. These habitat units are hotspots of animal abundance and productivity spatially restricted by the home range of its occupants and connected by migration pathways, including ontogenetic habitat shifts and inshore-offshore migrations (Nagelkerken *et al.*, 2015). This approach is especially relevant for management of critical areas in situations where it is not possible to protect the entire water bodies due to social-economical or practical constraints. Moreover, previous approaches did not emphasize sufficiently the complex ecological interactions supporting nursery ground occupation (Sheaves *et al.*, 2015). Accordingly, two main aspects contributing to nursery value were identified 1) factors supporting successful nursery occupation and 2) juveniles output to adult populations (Sheaves *et al.*, 2015). Successful nursery occupation (Figure 1.3.) depends on 1) physiological factors (e.g. ecotone effects, eco-physiological factors, food-predation trade-offs and food webs), 2) resource dynamics (resource availability, ontogenetic diet shifts and allochthonous inputs), and 3) connectivity and population dynamics (seascape and ontogenetic migrations, and connectivity). Conservation measures to maintain nursery production and stock replenishment depend on a comprehensive understanding of nursery value, including identification of habitat mosaics and connectivity/ecological interactions between them.

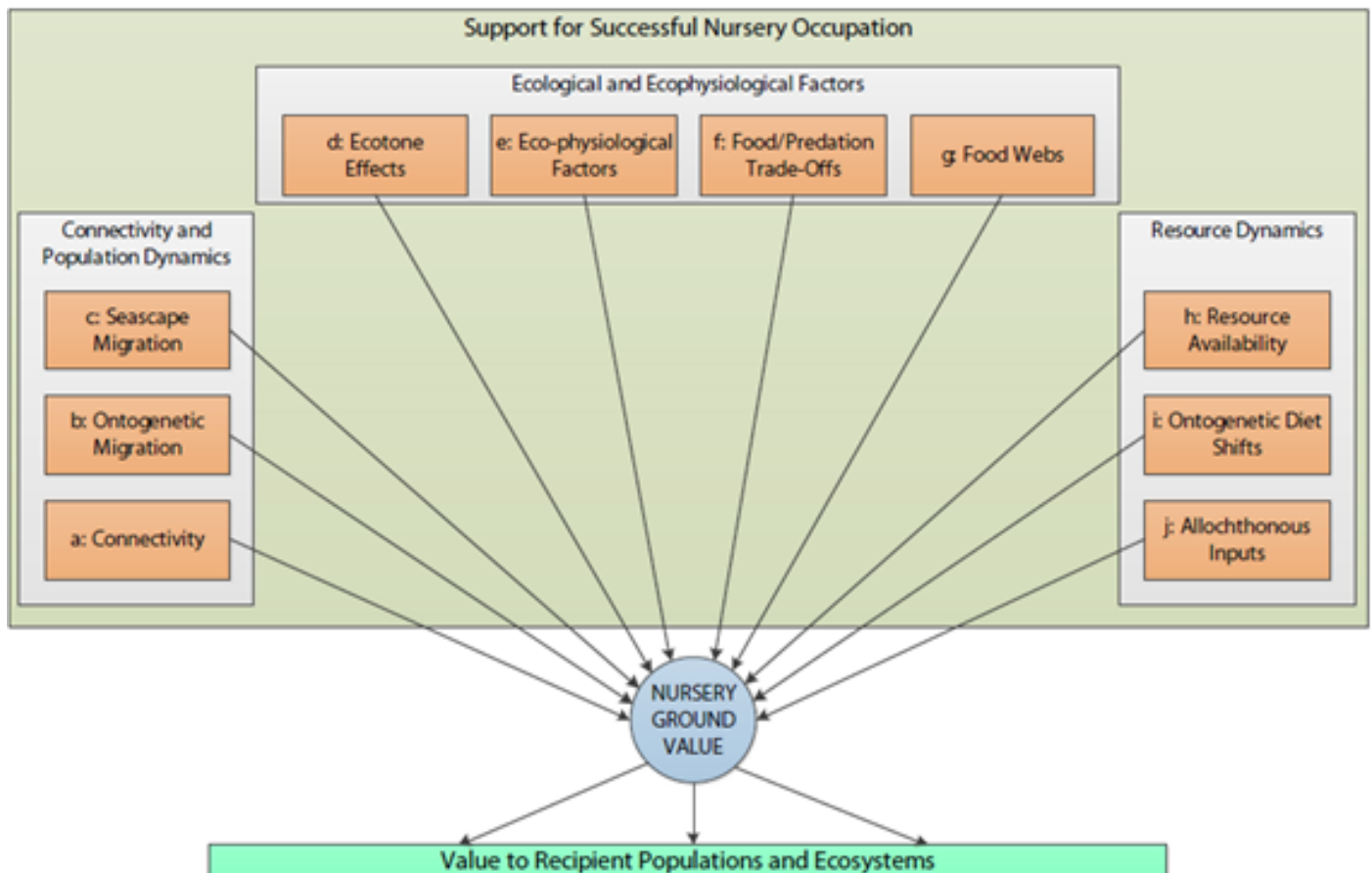


Figure 1.3. Components of nursery ground value (adapted from Sheaves *et al.*, 2015).

1.5. The European flounder *Platichthys flesus*

The European flounder *Platichthys flesus* (Linnaeus, 1758) (Figure 1.4.) is a ray finned (Class Actinopterygii) flatfish (Order Pleuronectiformes), right-eyed flounder (Family Pleuronectidae), although up to 30% of the population can be left-eyed (Munk and Nielsen, 2005). The adults can reach up to 60 cm and 2.5 kg (Munk and Nielsen, 2005). Small bony knobs are present along the lateral line and the body often has red spots. A rough scale is present at the basis of each dorsal and anal fin ray.

The geographical distribution of European flounder ranges from the White Sea in the North Atlantic to the Mediterranean and Black Sea. The Portuguese coast is considered the Southern limit of distribution for this species (Nielsen, 1986). In these areas, the European flounder represents a key component of demersal fish

communities (Thiel and Potter, 2001; Ramos *et al.*, 2009). The landings of this species have increased globally from 1950 to 2014 (FAO, 2018). A minimum of 7,500 tonnes was registered in 1954 and peaked to a maximum of 24,467 tonnes in 2005 (FAO, 2018). The commercial exploitation of the European flounder is important in the Portuguese estuaries where it represents one of the dominant flatfish species (Cabral *et al.*, 2007; Martinho *et al.*, 2007; Ramos *et al.*, 2009).

The lifecycle of the European flounder (Figure 1.4.) generally includes offshore spawning, transition from pelagic larvae to benthic juvenile phase at settlement (Modin and Pihl, 1996; Jager, 1999; Ramos *et al.*, 2010), and use of estuarine and coastal nurseries by the early life stages (Kerstan, 1991; van der Veer *et al.*, 1991; Ramos *et al.*, 2010). Plasticity in flounder life strategies may represent an adaptive behavior to different local environmental conditions (Daverat *et al.*, 2012), as well as genetic divergence between flounder populations (Nissling *et al.*, 2002; Hemmer-Hansen *et al.*, 2007). For example, offshore spawning is generally assumed for the European flounder (Jager, 1998; Ramos *et al.*, 2009; Primo *et al.*, 2013). However, coastal spawning occurs in the Baltic Sea (Nissling *et al.*, 2002) and Gironde (Daverat *et al.*, 2012), while flounder from Minho, Gironde and Seine estuaries may spawn in brackish water (Daverat *et al.*, 2012) according to otolith microchemistry studies. These studies have also suggested that freshwater spawning may also occur in the Minho estuary (Morais *et al.*, 2011; Daverat *et al.*, 2012). Moreover, the flounder eggs are mainly pelagic, but some populations in the Baltic Sea also produce demersal eggs (Nissling *et al.*, 2002; Nissling and Dahlman, 2010). The classification of flounder into an ecological functional guild according to its estuarine use is controversial. In the past, some authors have regarded the species as catadromous (Summers, 1979; McDowall, 1988). Recently, flounder has been considered a marine migrant (Elliott *et al.*, 2007b; Franco *et al.*, 2008; Ramos *et al.*, 2017) or marine estuarine-opportunist according to the latest guild classification proposed by Potter *et al.* (2015), as the freshwater phase is not obligatory (Elliott *et al.*, 2007b).

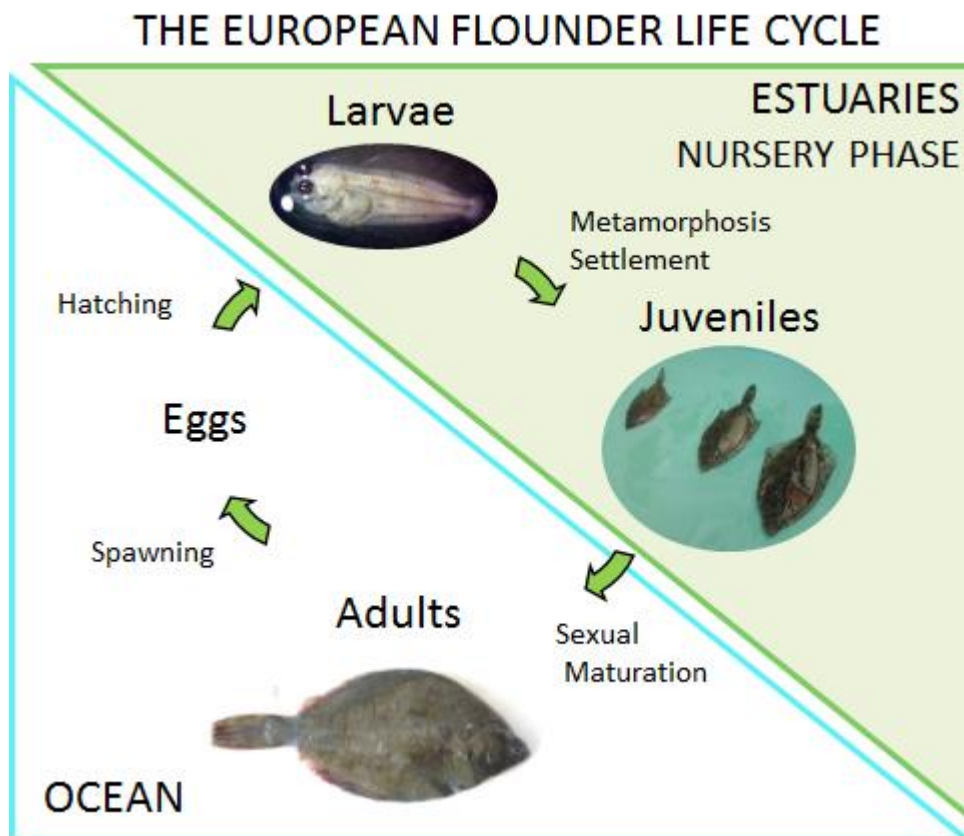


Figure 1.4. The life cycle of the European flounder *Platichthys flesus* (Linnaeus, 1758).

The spawning generally occurs from winter to early spring (Summers, 1979; Muus and Nielsen, 1999; Martinho *et al.*, 2013; Amorim *et al.*, 2016) and the pelagic larval stage lasts between 30 and 60 days (Martinho *et al.*, 2013). The larvae migrate from offshore to coastal and estuarine nurseries (Kerstan, 1991; van der Veer *et al.*, 1991; Ramos *et al.*, 2017) in spring and early summer (Koubbi *et al.*, 2006; Martinho *et al.*, 2013; Amorim *et al.*, 2016). Some studies suggested that young larvae use passive transport (Grioche *et al.*, 1997; Bos and Thiel, 2006). The late larval stages may also use selective tidal stream transport (Jager, 1999) to promote retention in estuarine nurseries (Jager, 1998). In estuaries, upstream migrations of the late larvae may be driven by low salinity (Bos and Thiel, 2006) and prey availability (Bos, 1999). The settlement process of flounder is assumed to be coupled with metamorphosis (van der Veer *et al.*, 1991; ICES, 2008). Although the settlement patterns of flounder are not well understood, many authors have suggested direct settlement (Jager, 1999; Ramos *et al.*, 2010; Morais *et al.*, 2011; Amorim *et al.*, 2016), i.e. the transition from the larval

pelagic phase to juvenile benthic phase occurs inside the estuarine nurseries. However, indirect settlement is also assumed in some areas, such as the Mondego estuary (Primo *et al.*, 2013).

In estuarine nurseries, the distribution of flounder juveniles is determined by abiotic factors, including depth (Cabral *et al.*, 2007; Vasconcelos *et al.*, 2010), salinity (Vinagre *et al.*, 2005; Ramos *et al.*, 2009; Zucchetta *et al.*, 2010), temperature (Power *et al.*, 2000), dissolved oxygen (Power *et al.*, 2000), sediment (Amezcuca and Nash, 2001; Vinagre *et al.*, 2005; Zucchetta *et al.*, 2010) and turbidity (Zucchetta *et al.*, 2010), and biotic factors, such as prey availability (Vasconcelos *et al.*, 2010). Flounder is a euryhaline species, i.e. it can occur in salinities between 0 and 35, but newly-settled show a preference for low salinity areas, while older juveniles tend to disperse to other areas of the estuary (Kerstan, 1991; van der Veer *et al.*, 1991; Andersen *et al.*, 2005a; Freitas *et al.*, 2009). Moreover, flounder juveniles show a preference for shallow (Wirjoatmodjo and Pitcher, 1984; Vinagre *et al.*, 2005; Le Pichon *et al.*, 2014) with sandy and muddy bottoms (Riley *et al.*, 1981; Greenwood and Hill, 2003), typical of sheltered areas (Gibson, 1994), which may be related to prey availability.

Flounder feeds on intertidal mudflats of estuarine or coastal areas during the day (De Groot, 1971; Mattila and Bonsdorff, 1998) with peak feeding at dawn and dusk (Muus, 1967; De Groot, 1971). It is an opportunist feeder (De Groot, 1971; Hampel *et al.*, 2005; Martinho *et al.*, 2008) and its main prey are highly available macroinvertebrates, including amphipods (Aarnio *et al.*, 1996; Andersen *et al.*, 2005b; Selleslagh and Amara, 2015) and other small crustaceans such as isopods (Andersen *et al.*, 2005b; Hampel *et al.*, 2005), polychaetes (Summers, 1980; Hampel *et al.*, 2005; Vinagre *et al.*, 2008) and chironomid insect larvae (Weatherley, 1989; Nissling *et al.*, 2007; Florin and Lavados, 2010). The diet can vary seasonally according to prey availability (Aarnio *et al.*, 1996), habitat structure (Andersen *et al.*, 2005b; Florin and Lavados, 2010), and ontogenetically (Aarnio *et al.*, 1996; Andersen *et al.*, 2005b). Ontogenetic diet shifts may be driven by changes in prey availability (Beaumont and Mann, 1984; Besyst *et al.*, 1999; Selleslagh and Amara, 2015) or fish size (Keast and Webb, 1966; Dörner and Wagner, 2003; Selleslagh and Amara, 2015).

Nursery habitat quality based on condition and growth indices of flounder juveniles varied across spatial (Amara *et al.*, 2009) and temporal scales (Vasconcelos *et al.*,

2009) discriminating between heavy polluted and less polluted nurseries (Amara *et al.*, 2009). Reduced flesh condition was observed in the most southern estuaries at the distribution limit of the species where environmental conditions approached the species tolerance limits (Vasconcelos *et al.*, 2009) in accordance with the decrease in abundance of flounder in these areas in recent decades (Cabral *et al.*, 2001).

1.6. The study area

The Lima River is an international water body located in the north western region of the Iberian Peninsula, with a water basin covering approximately 2,446 km². The river flows from the Ourense province in Spanish territory with 47% of the water basin covering Portuguese territory. It has two hydroelectric dams Alto Lindoso and Touvedo, operating since 1992. The river drains into the Atlantic Ocean towards the town of Viana do Castelo, NW Portugal with 32, 000 inhabitants. The climate of the region is wet with the average precipitation ranging from 1300 to 4200 mm per year (APA, 2016), mainly due to the proximity of the Atlantic Ocean and the presence of mountains surrounding the Lima river basin.

The Lima estuary (Figure 1.5.) presents a tidally dominated (Falcão *et al.*, 2013), small open estuary that extends up to 20 km from the river mouth. The river mouth was obstructed by a 2 km long jetty deflecting the river flow to south (Ramos *et al.*, 2010). The estuary is characterized by a semidiurnal and mesotidal regime, with an average flushing rate of 0.40 m.s⁻¹, the river flow is 70 m.s⁻¹ and a residence time of 9 days (Ramos *et al.*, 2010). The partially mixed estuary presents a seasonal vertical stratification of salinity with a sharp increase of salinity with depth (Ramos *et al.*, 2010). The estuary is further divided into three main stretches: the lower estuary which is heavily modified, and the less disturbed middle and upper estuaries. The lower estuary comprises the first 2.5 km stretch of the estuary and is a narrow channel used for navigation and therefore constantly dredged to a depth of 10 m. It also includes a large shipyard, a commercial seaport, and a fishing harbour (Ramos *et al.*, 2006b). The middle estuary is a saltmarsh zone, mainly colonized by *Juncus* spp., that encompasses several sand islands and intertidal channels (Ramos *et al.*, 2006b). The

upper estuary is a narrow and shallow channel with intertidal banks and sand islands (Ramos et al. 2010).



Figure 1.5. The Lima estuary with the lower, middle and upper estuarine sections (adapted from Google Earth, 2018).

Sources of anthropogenic pressure include urban, industrial and agriculture discharge, dredging activities of the navigation channel (Costa-Dias *et al.*, 2010; Azevedo *et al.*, 2013), and introduction of non-indigenous invasive species, such as the Asian clam *Corbicula fluminea* (Sousa *et al.*, 2006a). Heavy metals (Cardoso *et al.*, 2008; Gravato *et al.*, 2010) and polycyclic aromatic hydrocarbons (PAHs) (Gravato *et al.*, 2010, Ribeiro *et al.*, 2018) were detected in the Lima estuarine sediments at low levels within the sediment quality guideline values (SQGV, Simpson and Batley, 2016). A marked nitrogen enrichment in pelagic zooplankton and common goby *Pomatoschistus microps* larvae were indicative of higher anthropogenic inputs of nitrogen (e.g. sewage and industrial discharges, agriculture) into the Lima estuary (Baeta *et al.*, 2017). Moreover, the lower Lima estuary suffered heavy modifications in dominating habitats, from intertidal and shallow subtidal with soft sediment habitats and saltmarsh to

moderately deep/deep subtidal habitats (Amorim *et al.*, 2017). As a result of this, the most attractive physiotopes to benthic and demersal fish communities were lost, decreasing overall attractiveness of the estuarine areas and potentially impacting nursery carrying capacity and functioning of the fish community (Amorim *et al.*, 2017). Despite this, both macroinvertebrate and fish communities presented good ecological status according to macroinvertebrate (M-AMBI) and demersal (EDI- Estuarine Demersal Indicators) community based indices (Azevedo *et al.*, 2013). The estuary presents an ecological relevance of as an important area for birds nesting and foraging (APA 2016) and it has been integrated in Natura 2000 as a Site of Community Importance (SCI) under the Habitats Directive (EU, 1992).

The macroinvertebrate community varied along the estuarine gradient in terms of abundance, biomass and diversity (Sousa *et al.*, 2006b). The lower estuary with stable salinity and fine sediments, and richer in organic matter supported higher abundance and biomass of macroinvertebrate species than the upper estuary with coarser sediment and variable salinity (Sousa *et al.*, 2006b). The bivalves *Abra alba* and *Cerastoderme edule* were predominant in biomass, while *A. alba* and polychaete *Hediste diversicolor* were the most abundant macroinvertebrate species (Sousa *et al.*, 2006b). These species may represent key food resources for higher trophic levels. However, macroinvertebrate abundance and biomass data showed that the lower estuary is under environmental stress and dominated by opportunistic species such as *Capitella capitata* (Sousa *et al.*, 2007a).

The fish larvae assemblage was composed by 50 taxa belonging to 20 families (Ramos *et al.*, 2006b). The six dominant taxa included *Pomatochistus* spp., *Sardina pilchardus*, *Ammodytes tobianus*, unidentified Clupeidae, *Symphodus melops* and *Solea senegalensis*, and represented 91% of the total catch (Ramos *et al.*, 2006b). Temporal differences in the fish larval assemblages resulted from seasonal variations in temperature and precipitation (Ramos *et al.*, 2006a). Spatial patterns were determined by distance to the river mouth and salinity gradient (Ramos *et al.*, 2006b), with larvae showing a high affinity to the salt marsh zones (Ramos *et al.*, 2006b). The estuarine fish fauna was composed by 41 species and dominated by resident Gobiidae species *Pomatochistus microps* and *P. minutus*. The density, diversity and structure of the benthic fish community were seasonally stable (Ramos, 2007). The spatial structure of

the benthic fish assemblage was controlled by salinity, distance to mouth, sediment composition, and qualitative habitat characteristics such as saltmarsh presence and dredging activity (Ramos, 2007). Marine juveniles were also an important component of the Lima estuary fish community, including the seabass *Dicentrarchus labrax*, common sole *S. solea* and flounder *P. flesus*. In fact, these are the main target species of local commercial fisheries supported by the Lima estuary. Seasonal fisheries targeted highly priced migratory species such as the European eel *Anguilla Anguilla*, shads *Alosa alosa* and *A. fallax*, and the sea lamprey *Petromyzan marinus* (Ramos 2007).

1.7. Motivation and main objectives

The estuarine nursery function has been greatly acknowledged in fisheries research (Kerstan, 1991; Beck *et al.*, 2001; Potter *et al.*, 2015) providing a vital link between early life stages and recruitment to adult populations (Gibson, 1994; Rijnsdorp *et al.*, 1995; Beck *et al.*, 2001). The nursery value is the net result of a mosaic of interacting habitats (Sheaves *et al.*, 2015) serving different ontogenetic stages, including complex ecological interactions and resource dynamics, as well as connectivity between these units. In fact, nursery habitat use is driven by trade-offs between optimal foraging habitats, predation risk and favourable physiological conditions (Sogard, 1992; Kimirei *et al.*, 2013; Tableau *et al.*, 2016; Amorim *et al.*, 2018). Among these factors, resource use, including prey availability, distribution and quality, and ontogenetic diet shifts affect growth (Karakiri *et al.*, 1989; Sogard, 1992; van der Veer *et al.*, 2001; Andersen *et al.*, 2005b) as a result of energetic contribution of different prey (Sheaves *et al.*, 2015). However, anthropogenic pressures in estuarine nurseries have resulted in habitat loss and modification (Courrat *et al.*, 2009; Wolanski and Elliott, 2015; Amorim *et al.*, 2017) potentially compromising food and space resources, hence nursery value. Therefore, there is the need to fully understand the ecological components underpinning the nursery value to help preserve systems carrying capacity and support effective conservation and management programs.

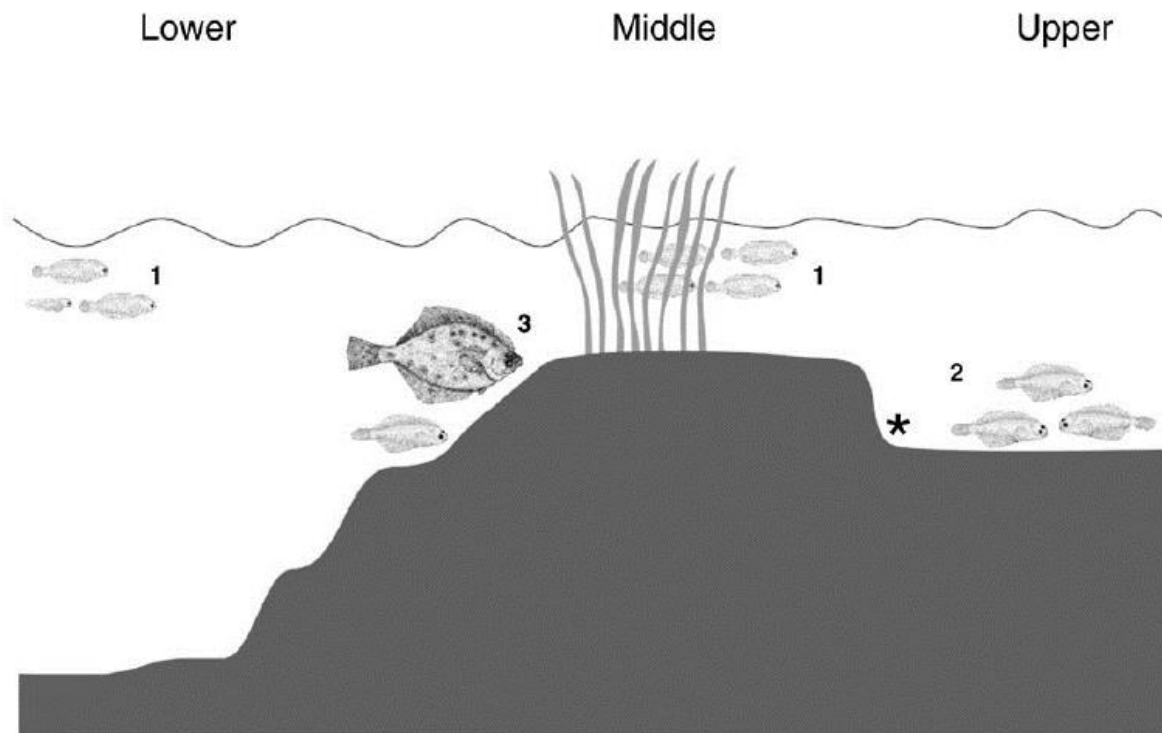


Figure 1.6. The early life stages of flounder in the lower, middle and upper stretches of the Lima estuary (adapted from Ramos *et al.*, 2010). 1- metamorphosing larvae; 2 – newly-settled and 3 – adults.; *possible settlement area.

The European flounder *Platichthys flesus* is a typical estuarine nursery user (Kerstan, 1991; Martinho *et al.*, 2008). The flounder is a key component of the fish community of the Lima estuary that provides a nursery function for this species early life stages (Ramos *et al.*, 2010; Amorim *et al.*, 2016; Ramos *et al.*, 2017). According to Amorim *et al.* (2016), late-stage larvae enter the estuary between February and July, and post-settlement flounder occurred between April and October. Variables associated to spawning and larval growth and survival (e.g. sea surface temperature, chlorophyll a) were the major drivers of flounder occurrence in the Lima estuarine nursery (Amorim *et al.*, 2016). The larval abundance typically increased from offshore towards the upper estuary (Ramos *et al.*, 2017). Moreover, the post-settled flounder were constrained to the shallow upstream area (Amorim *et al.*, 2018) (Figure 1.6.). There were ontogenetic habitat shifts since as the juveniles grew they tended to migrate downstream to the middle estuary (Figure 1.6.) (Amorim *et al.*, 2018). However, the major drivers for these habitat use patterns are still poorly understood. Interestingly, low salinities did not

enhance flounder newly-settled condition (O'Neill *et al.*, 2011) and growth (Gutt, 1985). Hence, these upstream migrations may represent a physiological cost for the flounder juveniles. It is of note that the estuary is located at southern limit of distribution for this species (Nielsen 1986), where a recent decline in abundance has been associated to the rise of seawater temperatures (Cabral *et al.*, 2007). Moreover, reduced growth rates at these lower latitudes may be linked to food limitation through interspecific competition and higher metabolic demands comparatively to other areas (Freitas *et al.*, 2012). Habitat changes for fish in the Lima estuary may include changes to nursery carrying capacity and functioning of the fish community (Amorim *et al.*, 2017). Taking this in consideration, there is the need to fully understand key early life cycle strategies of flounder that sustain nursery carrying capacity. This provides a baseline to detect potential changes in nursery habitat use and quality and tackle main management challenges towards its conservation. In this light, this thesis aimed to understand key factors of flounder lifecycle in a nursery habitat, including general spatial-temporal use patterns, feeding ecology and nursery habitat quality. The main hypothesis considered was that feeding was a major driving factor for habitat use of flounder juveniles in an estuarine nursery.

The following main objectives were pursued:

- to investigate nursery use of 0-group European flounder (juveniles that have not completed one year old), integrating estuarine colonization, abundance, feeding and growth patterns towards understanding of nursery habitat quality;
- to investigate the feeding ecology of the European flounder juveniles, including main prey, ontogenetic shifts in the diet, prey selectivity, and influence of prey-predator interactions;
- to determine main feeding areas of the European flounder juveniles through the integration of dietary indices and stable isotopes and investigate the relationship between feeding strategies and condition;
- to integrate the condition indices Fulton K, RNA:DNA, otolith post-settlement (PSGR) and recent growth rates (RG) of 0-group European flounder in order to assess the juvenile condition as proxy of the Lima nursery habitat quality.

This thesis is structured in five chapters. Chapter 1 starts with a general introduction on estuaries, the estuarine ecosystem and nursery value for fish, as well as the lifecycle of European flounder focusing on early life stages and nursery use, and a description of the study area. The main aims and the outline of the thesis are also presented. The Chapter 2 investigates general early life patterns of the 0-group flounder to corroborate previous abundance data from the Lima nursery (e.g. Ramos *et al.*, 2010, Amorim *et al.*, 2016). Thus, key early life traits based on otolith microstructure, diet and otolith growth rates were investigated towards understanding of nursery habitat quality. Then, Chapter 3 focused on the feeding ecology of the juvenile flounder, namely 0-group and 1-group European flounder (juveniles that have completed one year old). Ontogenetic shifts, prey-selectivity, and relationship between prey-predator interactions and juveniles distribution were investigated through stomach contents and macroinvertebrate community analysis. This chapter provided a first assessment of the recent diet of flounder juveniles. Then, Chapter 4 further investigates the main feeding areas by integrating stomach contents and stable isotopes towards a greater insight into trophic relationships. It also explores the relationship between feeding use and individual condition. Finally, there was the need to understand how the early life patterns and feeding strategies investigated throughout the thesis were sustaining condition and growth of the 0-group flounder in the Lima nursery. Therefore, Chapter 5 integrates condition indices and otolith growth rates of 0-group flounder as indicators of fish condition and proxies of nursery habitat quality. The final conclusions, as well as perspectives on future research are presented on Chapter 6.

CHAPTER 2

Early life of European flounder

Platichthys flesus in an estuarine
nursery

Abstract

Nursery successful occupation depends on the exploitation of habitats providing optimal trade-offs between physiological conditions and biotic variables such as prey availability for different life stages. This study investigated early life patterns of the 0-group European flounder *Platichthys flesus* in terms of estuarine colonization, spatial and temporal distribution of abundances, feeding and growth rates. The 0-group flounder hatched from February to June, followed by pelagic (33 ± 3 days) and metamorphic (16 ± 4 days) phases with migration into the estuary, and settlement peaked in May and June. These juveniles were almost restricted to the upper estuary and the diet reflected this spatial distribution as their main prey was typical of the low salinity upper estuary. An ontogenetic diet shift from Chironomidae to *Corophium* was evident at the end of post-settlement stage (< 50 mm TL). Recent (RG) and post-settlement (PSGR) growth rates based on otolith microstructure showed good individual condition of 0-group flounder. Early life strategies supported high growth rates throughout the post-settlement stage and showed the nursery value of the Lima estuary.

2.1. Introduction

The lifecycle of many fishes includes ocean spawning and migration of the early life stages to coastal and estuarine nurseries (Tanaka *et al.*, 1989; Hovenkamp, 1991; Bailey *et al.*, 2003), where they develop until recruiting to adult populations. Recruitment is mainly determined by species-specific biological factors and also environmental factors operating at the larval pelagic phase (Leggett and Deblois, 1994; Rijnsdorp *et al.*, 1995; Mikaela *et al.*, 2002). Early life traits (e.g. size at hatch, pelagic larval duration) are key aspects to understand temporal dynamics of nursery habitat use (Able and Fahay, 2010; Amorim *et al.*, 2018) as they affect connectivity to nursery habitats (Trembl *et al.*, 2012), growth (Searcy and Sponaugle, 2001) and survival (Raventos and Macpherson, 2005) at the subsequent juvenile stages.

In nursery habitats, trade-offs between physiological tolerances (e.g. salinity and temperature) (Power *et al.*, 2000; Ramos *et al.*, 2009; Post *et al.*, 2017), prey availability (Kopp *et al.*, 2013; Tableau *et al.*, 2016) and predation risk (Sogard, 1992; Harter and Heck, 2006; Camp *et al.*, 2011) are critical drivers of spatial (Rogers, 1992; Wennhage and Gibson, 1998) and temporal (Gibson *et al.*, 1998; Attrill and Power, 2004) distribution of the early life stages, including ontogenetic shifts (McBride *et al.*, 2001; Kimirei *et al.*, 2013; Amorim *et al.*, 2018). Successful nursery occupation is achieved by exploitation of habitats maximizing growth of the early life stages and recruitment to adult populations (Beck *et al.*, 2001; Nagelkerken *et al.*, 2015; Sheaves *et al.*, 2015). Faster growth promotes survival (Sogard, 1997), by increasing chances of escaping predators and capture food (Houde, 1987; Witting and Able, 1993; Leggett and Deblois, 1994). Thus, growth is considered a good premise for nursery habitat quality (Able, 1999). In this context, otoliths are widely used to set a record of past (Sogard, 2011; Watai *et al.*, 2017; Barrow *et al.*, 2018) and recent (Hovenkamp and Witte, 1991; Gilliers *et al.*, 2004; Peck *et al.*, 2015) growth rates of the early life stages of fishes. Analyses of the microstructure of otoliths provide age estimates based on daily increments (Pannella, 1971), whereas daily growth rates may be determined based on daily increment widths (Campana and Neilson, 1985; Karakiri *et al.*, 1991).

The Lima estuary is a nursery habitat to the European flounder *Platichthys flesus* (Ramos *et al.*, 2010; Mendes *et al.*, 2014; Amorim *et al.*, 2016). Previous studies showed that late larvae colonized the estuary during spring and newly settled fish

concentrated in the upper estuary (Ramos *et al.*, 2010; Amorim *et al.*, 2016). Differences in habitat suitability (Amorim *et al.*, 2018) between flounder juvenile stages have been associated to ontogenetic shifts in the diet (Mendes *et al.*, 2014). However, habitat loss (Amorim *et al.*, 2017) may compromise carrying capacity and nursery value of the estuary. Therefore, it is fundamental to fully understand the nursery use by integrating different ecological components for effective management and conservation purposes.

This study focused on the 0-group flounder and the main hypothesis was that early life strategies supported growth thereby maximizing nursery value. Therefore, main aims were to investigate the early life patterns of 0-group flounder in Lima nursery, namely: 1) to analyze spatial-temporal abundance patterns; 2) to determine key early life traits (age, hatch and settlement dates, and duration of pelagic and metamorphic phases) and growth rates (post-settlement and recent growth rates) based on otolith microstructure analysis; and 3) to determine diet including main prey and diet shifts towards assessment of nursery habitat quality.

2.2. Material and Methods

2.2.1. Study area

The Lima estuary, located at the NW Atlantic coast of Portugal, is a small open estuary with a semidiurnal and mesotidal regime with a range of 3.7 m. Salt intrusion can extend up to 20 km upstream, with an average flushing rate of 0.4 m s^{-1} , and a residence time of 9 days (Ramos *et al.*, 2006). The river mouth is partially obstructed by a 2 km long jetty, causing a deflection of the river flow to the south. For this study, ten stations were sampled covering the lower, middle and upper estuary (Figure 2.1.). The lower estuary (stations 1-3, average depth= 3.9), located in the initial 2.5 km, is a narrow, 9-m deep navigational channel, industrialized, with walled banks. It includes a shipyard, a commercial seaport, and a fishing harbour. The middle estuary (stations 4-7, average depth= 4.8) comprises a broad shallow intertidal saltmarsh zone, mainly colonized by the common rush (*Juncus* spp.). The upper estuary (Stations 8-10, average depth= 3.2) is a narrow shallow channel (< 3 m), less disturbed, with natural banks and small sand islands (Ramos *et al.*, 2010).

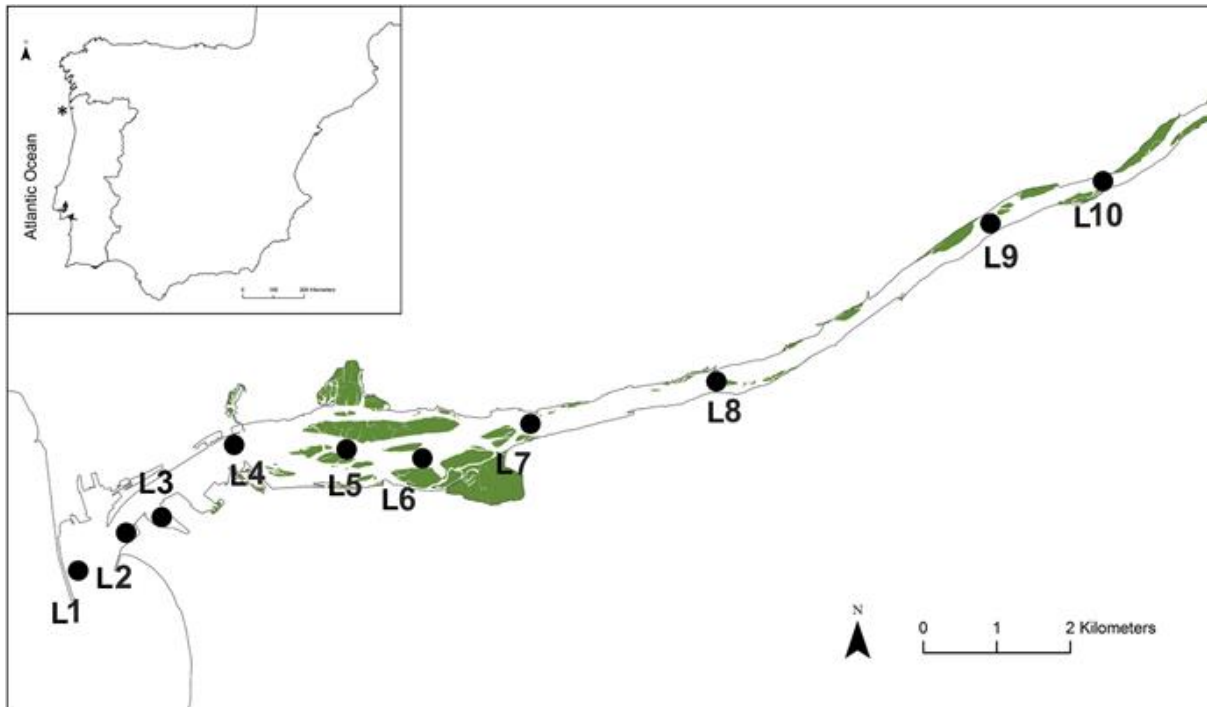


Figure 2.1. The Lima estuary with the location of the sampling points (adapted from Amorim *et al.*, 2018).

2.2.2. Data Collection

Flounder juveniles were collected monthly during nightly ebb tides, between June and October 2013, with a 2 m beam trawl, with a cod-end 5mm mesh, and a tickler chain. Trawls were made at a constant speed ($3 \text{ m} \cdot \text{s}^{-2}$) and lasted 10 min. All samples were kept on ice until further processing in the laboratory. Sampling geographic location was recorded with a Magellan 315 GPS. At each site, vertical profiles of temperature and salinity were obtained by means of a multi-parameter water quality probe YSI 6820. Flounder were measured for total length (TL, $\pm 1 \text{ mm}$) and weight (WW, wet weight, $\pm 0.01 \text{ g}$). The 0-group flounder with a maximum total length of 100 mm were sorted for otolith microstructure and stomach content analysis, as daily otolith rings were only countable for flounder within this size range.

2.2.3. Otolith preparation and analysis

Left sagittal otoliths were removed, cleaned and mounted with the sulcus side up on a slide embedded with crystalbond. Otoliths were polished using abrasive paper ($0.1 \mu\text{m}$ grain size silicon carbide abrasive papers; Buehler) until the nucleus daily rings were

visible. Whenever this procedure was not sufficient to expose these nucleus daily rings, otoliths were turned with the sulcus side down, and the polishing procedure was repeated on the unground side. Daily increments were analysed under a light microscope coupled to a digital camera at 100x and 400x, except for the nucleus that was analysed at 1000x.

Early life traits

Otolith daily increments were used to determine the duration (days) of the pelagic, metamorphic and post-settlement stages. The daily increments were counted according to the methods described in Martinho *et al.* (2013). The duration of the larval pelagic phase was determined as the number of rings between the first increment after the hatch check to the last complete increment before the first accessory primordium. The metamorphic phase corresponded to the increments between the first and last accessory primordia. It was assumed that the presence of secondary growth centres represented the settlement to the benthic habitat (Geffen *et al.*, 2007; Cardoso *et al.*, 2016). Thus, the post-settlement stage was counted between the first complete increment after the last accessory primordia to the edge of otolith. The age of 0-group flounder was estimated by the total increment counts of all three stages. Hatch dates were back-calculated based on age estimations and date of capture, while settlement date was determined by subtracting the number of increments since metamorphosis from the date of capture (Cardoso *et al.*, 2016).

Otolith growth rates

Daily otolith growth is an indicator of fish growth when there is a linear relationship between fish total length and otolith length (Campana and Neilson, 1985). A linear relationship between 0-group flounder total length (mm) and otolith length (mm) was fitted by a linear regression ($y = 0.0255 + 0.1844x$, $R^2 = 0.98$). Mean increment width between the first complete increment after the accessory primordia to the edge of the otolith was used to determine growth rates at the post-settlement stage (PSGR). The recent growth index (RG) was considered as indicator of recent condition (Suthers *et al.*, 1989; Hovenkamp and Witte, 1991) and was determined as the mean daily width of the last 10 daily increments (Amara *et al.*, 2009). The maximum diameter through the nucleus and daily increment widths were measured respectively at 10x and 100x

magnification with a coupled video system. All measurements were performed with ImageJ software.

2.2.4. Stomach content analysis

The stomachs of the 0-group flounder were excised and the contents were preserved in 70% alcohol. The prey items were identified to the highest taxonomic separation possible and counted under a binocular microscope (Leica MZ12-5). The prey were weighed (wet weight, ± 0.001) after blotting on a tissue paper (Mendes *et al.*, 2014).

2.2.5. Data analysis

Physical parameters of the bottom water column (1 m above bottom depth) were averaged for each location. The sampled area was determined for each location based on trawl opening (2 m) and distance travelled obtained by the Global Positioning System (GPS). Densities of 0-group flounder were standardized as the number of individuals per 1, 000 m² swept area. The 0-group flounder were further divided into 10 mm TL classes (from 10 to 100 mm TL). For each sampling date, densities were averaged across estuarine sectors for each size class.

Diet analysis was performed for all the sampled 0-group flounder and for each size class. The Relative Importance Index (IRI) (Pinkas, 1971) was determined based on the numerical (NI), occurrence (OI) and weight (WI) percentages of each prey taxa (Hyslop, 1980):

$$IRI (\%) = \frac{\%NI + \%WI}{\%OI}.$$

Diet diversity was determined by the Shannon-Wiener diversity index H' for all sampled juveniles and for each size class (Shannon and Weaver, 1949). Vacuity was determined as the percentage of empty stomachs.

The number of settling cohorts was identified through back-calculated hatching frequency distributions by grouping individuals into two-week age classes and visually identifying the number of modes (Cardoso *et al.*, 2016).

The PSGR and RG were determined for all the 0-group flounder and for each size class. Possible length effects were investigated through Pearson correlations between

0-group flounder total length and PSGR and RG. Pearson correlations between the 0-group flounder PSGR and RG were also investigated. A linear regression model was fitted to the relationship between total length (mm) and age (days). Differences between sampling months and between size classes were tested with one-way ANOVA for post-settlement and recent growth rates. Tukey post-hoc tests were applied to identify significant differences between levels within the month and size class factors. A significance level of 0.05 was used for all the statistic procedures. All statistical analyses were performed with R software (R Development Core Team, 2007).

2.3. Results

2.3.1. Physical-chemical parameters

The water column temperature ranged between 13.5 and 24.9 °C, with an average of 17.7 ± 2.2 °C. The temperature increased from the lower (16.7 ± 1.7 °C) to the middle (17.2 ± 1.7 °C) and upper (20.0 ± 2.1 °C) estuarine sections. The horizontal salinity gradient was evident, with salinity decreasing from the euhaline lower estuary (31.7 ± 3.3) to the polyhaline middle (27.9 ± 6.6) estuary, and finally to the mesohaline upper (5.7 ± 7.9) estuary.

2.3.2. Spatial and temporal distribution of 0-group European flounder

A total of 122 0-group European flounder were collected with total length ranging from 14 to 94 mm, and total weight from 1.0 to 370.0 g (Table 2.1.). The average density of 0-group flounder was 4.4 ± 13.5 fish 1,000 m⁻². Most 0-group flounder concentrated in the upper estuary (Figure 2.2.a) with an average abundance of 13.3 ± 22.1 fish 1,000 m⁻², compared to 0.2 ± 0.7 in the lower and 0.7 ± 1.7 individuals 1,000 m⁻² in the middle estuaries. Flounder below 50 mm TL were restricted to the upper estuary and were the most abundant classes in the Lima estuary (Table 2.1.). The European flounder below 20 mm TL were only observed in June. The 20-29 mm class was highly abundant in June, while larger 30-49 mm classes were more abundant in July (Figure 2.2.b). The post-settlement flounder were not observed in September and October (Figure 2.2.b).

In general, 0-group flounder abundances peaked in June (11.3 ± 29.9 fish.m⁻²) and decreased from there on to October (0.8 ± 2.4 fish.m⁻²) (Figure 2.2.a).

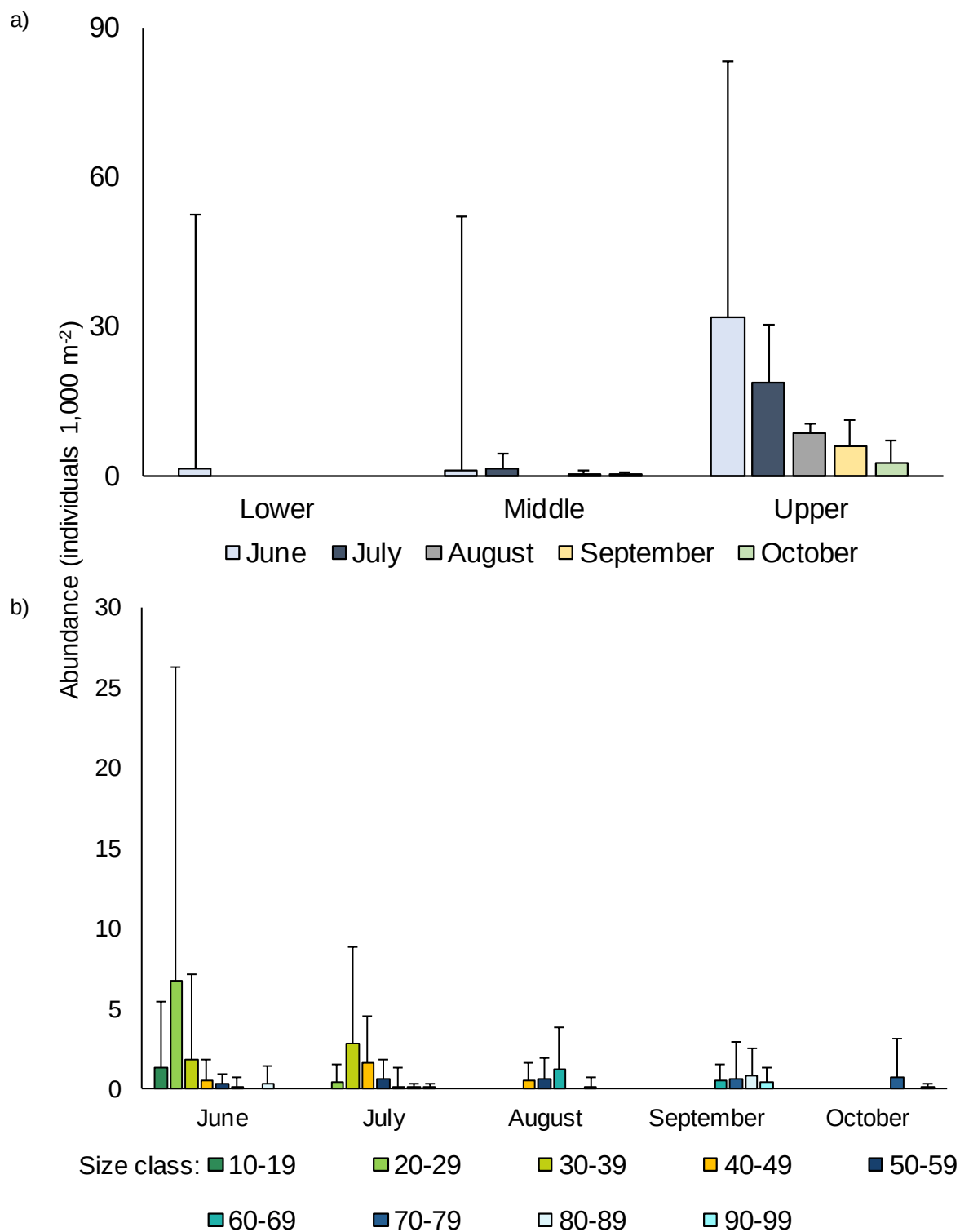


Figure 2.2. a) Spatial covering the lower, middle and upper sections, and b) temporal abundances of 0-group European flounder (individuals 1,000 m⁻²) per size class (10 mm TL) in the Lima estuary.

Table 2.1. Number of 0-group European flounder sampled (n), abundance (individuals 1,000 m⁻²), mean age (days), vacuity (%), Shannon-Wiener diet diversity (H') and mean post-settlement (PSGR) and recent (RG) growth rates per size class (10 mm TL). In brackets: number of 0-group flounder selected for otolith analysis.

Size class	n	Abundance (individuals 1,000 m ⁻²)	Age (days)	PSGR (µm.day ⁻¹)	RG (µm.day ⁻¹)	Vacuity (%)	H'
10-19	6 (5)	0.2 ± 1.7	52 ± 8	8.85 ± 2.32	6.15*	0	0.1
20-29	33 (17)	1.3 ± 8.1	64 ± 6	9.67 ± 1.43	9.40 ± 1.77	21	0.7
30-39	28 (20)	0.9 ± 3.6	74 ± 6	9.45 ± 1.44	9.10 ± 1.53	0	0.2
40-49	17 (12)	0.5 ± 1.6	83 ± 7	11.31 ± 1.52	11.18 ± 2.03	18	0.4
50-59	10 (7)	0.3 ± 0.9	94 ± 7	11.56 ± 2.23	11.66 ± 2.89	40	0.9
60-69	11 (11)	0.4 ± 1.3	100 ± 6	10.87 ± 2.70	11.34 ± 3.27	36	0.8
70-79	6 (5)	0.3 ± 1.5	113 ± 15	11.65 ± 2.95	10.98 ± 2.82	33	0.0
80-89	7 (7)	0.3 ± 1.0	120 ± 8	10.88 ± 1.71	10.38 ± 3.42	43	0.0
90-99	4 (3)	0.1 ± 0.4	119 ± 10	13.23 ± 2.76	15.27 ± 4.40	50	0.5

*Six 0-group European flounder belonging to the 10-19 mm class were sampled, but only one presented 10 increments for which the recent growth rate was determined.

2.3.3. Early life traits and growth rates of 0-group European flounder

A subsample of 88 0-group flounder proportionally covering all sampled size classes were selected for otolith analysis. The age varied between 44 and 131 days, with an average of 84 ± 21 days. The 0-group flounder hatched between February and June with a peak observed from March to mid-April (Figure 2.3.a). The pelagic larval and metamorphosis duration was 33 ± 3 and 16 ± 4 days, respectively. The settlement started in March and occurred until July, although most of the 0-group flounder settled between May and June (Figure 2.3.b). In agreement, age followed the progression of the cohort increasing from June (68 ± 16 days) to July (83 ± 14 days), and August (95 ± 12 days). Only older juveniles (≥ 70 mm TL, age= 118 ± 11 days) were observed in September and October with 111 ± 12 and 123 ± 4 days, respectively. In fact, age presented a linear relationship to flounder total length ($y = 0.9269x + 41.024$, $R^2 = 0.90$, Figure 2.4.a).

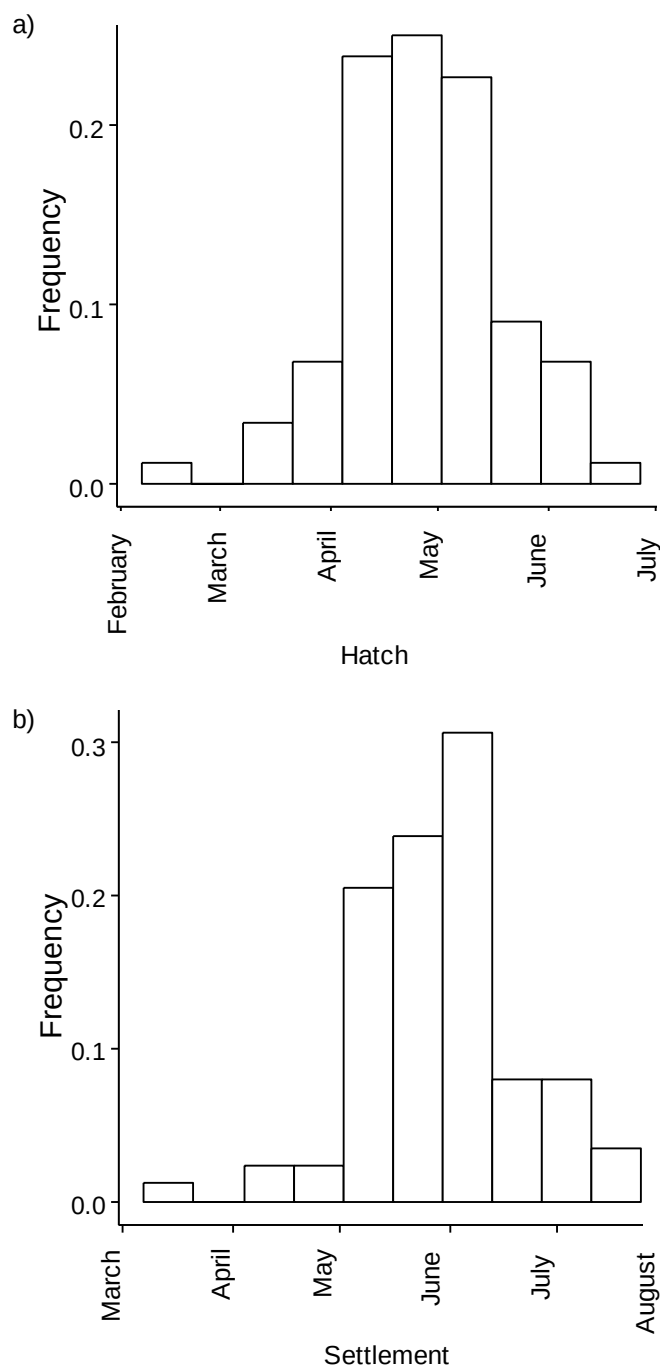


Figure 2.3. Back-calculated a) hatch and b) settlement dates of 0-group European flounder in the Lima estuary based on otolith daily ring counts.

The PSGR ranged between 6.71 and $16.39 \mu\text{m}\cdot\text{day}^{-1}$, with an average of $10.48 \pm 2.00 \mu\text{m}\cdot\text{day}^{-1}$. A total of 6 individuals, with less than 25 mm TL and collected in June at the upper estuary presented less than ten complete increment after the accessory primordia, indicating recent settlement before capture. Therefore, recent growth rates (RG) were not determined for these juveniles. The RG varied between 6.14 and 19.94

$\mu\text{m}\cdot\text{day}^{-1}$, with an average of $10.42 \pm 2.75 \mu\text{m}\cdot\text{day}^{-1}$. There was a significant correlation between total length and mean PSGR ($p < 0.05$, $r = 0.39$) and RG ($p < 0.05$, $r = 0.38$) growth rates (Figure 2.4.b, Table 2.1.). Moreover, the PSGR (F-statistic= 3.5, $p < 0.05$) and RG (F-statistic= 3.8, $p < 0.05$) varied significantly between months (Figure 2.5.a). Indeed, 0-group flounder caught in September presented higher PSGR (Tukey post-hoc, $p < 0.05$) and RG (Tukey post-hoc, $p < 0.05$) growth rates than flounder captured in June; as well as higher RG than flounder captured in July (Tukey post-hoc, $p < 0.05$). The RG were significantly correlated to the post-settlement growth rates ($p < 0.05$, $R^2 = 0.77$, Figure 2.5.b).

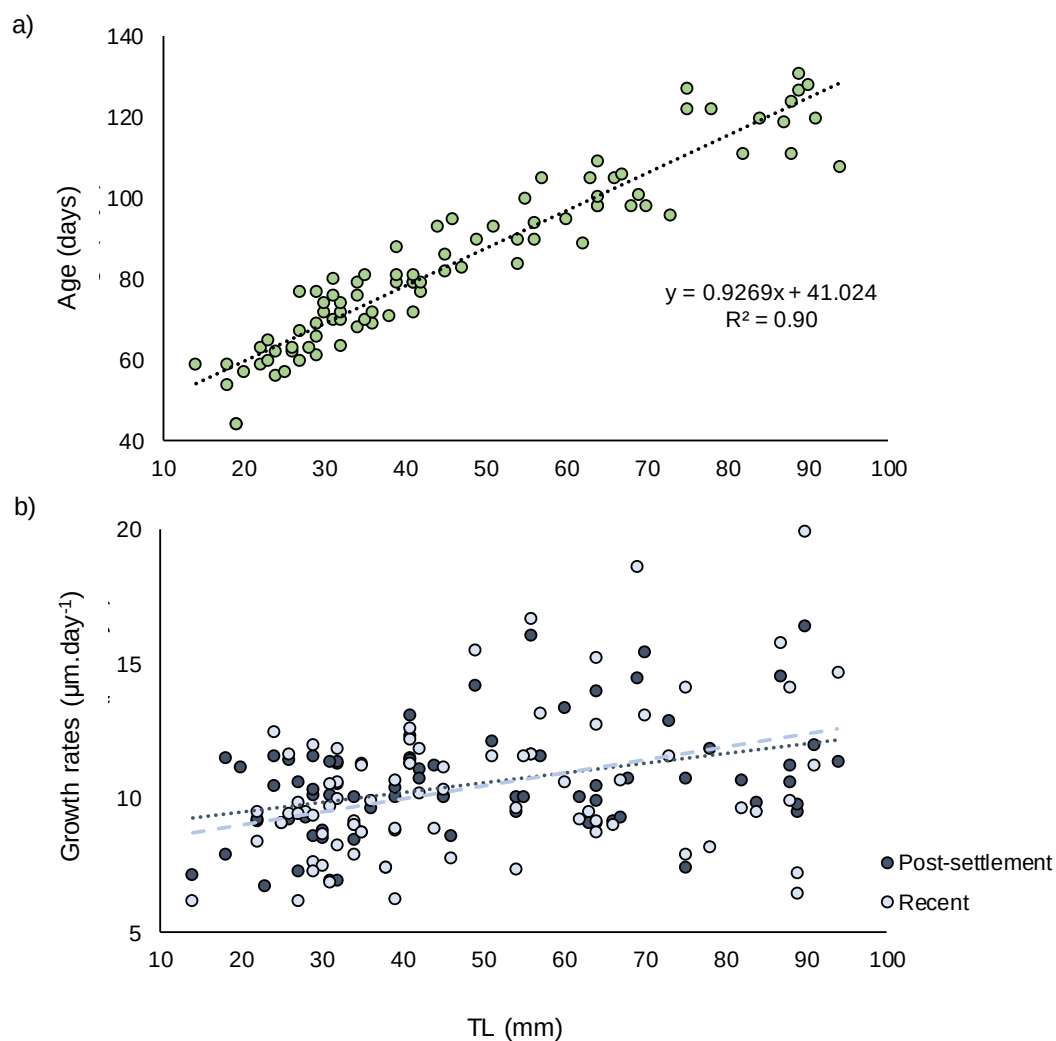


Figure 2.4. Relationship between total length and a) age (days); b) post-settlement and recent growth rates ($\mu\text{m}\cdot\text{day}^{-1}$) of 0-group European flounder based on otolith daily increments.

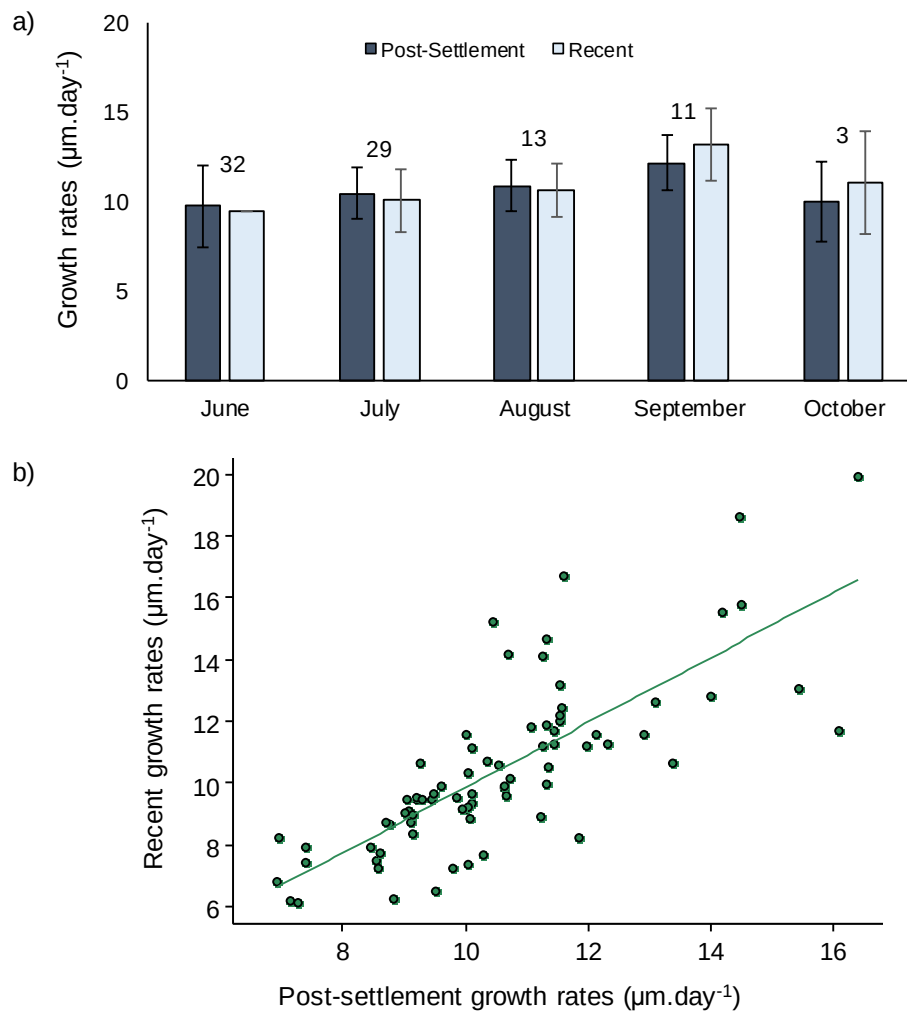


Figure 2.5. Growth rates of 0-group European flounder in the Lima estuary: a) temporal variation, and b) relationship between post-settlement and recent growth rates ($\mu\text{m}\cdot\text{day}^{-1}$) based on otolith daily increment widths. Number of otolith samples (n) represented above bars.

2.3.4. Diet patterns of 0-group European flounder

From the total flounder studied ($n=122$), 20% presented empty stomachs. Vacuity varied between 0% for the smaller juveniles (10-19 mm and 30-39 mm size class) to 50% for the largest juveniles (90-99 mm size class, Table 2.1.). The minimum vacuity was observed in September (8%), while the maximum vacuity (67%) occurred in October. Vacuity percentages of 24%, 17% and 21% were observed in June, July and August, respectively. A total number of 2,459 of prey were identified in the stomach contents of the 0-group flounder juveniles. The diet of 0-group flounder was dominated by Chironomidae insect larvae (IRI = 69%) and the amphipod *Corophium* (IRI = 29%). Other items (diet indices <1%) comprised Polychaeta, planktonic prey, such as

Copepoda and Cladocera, insect larvae such as Ephemeroptera and Odonata, and small crustaceans, including isopods and Sphaeromatidae.

The diet of the 0-group flounder varied temporarily and between size classes. Chironomidae larvae were the main prey of flounder below 50 mm TL (Figure 2.6.a). Moreover, other small insect larvae, including Coleoptera, and planktonic prey Cladocera, Copepoda and Clupeidae larvae were only present in the stomachs of the smaller flounder (< 30 mm TL) (Figure 2.6.a) collected in June and July (Figure 2.6.b). The diet of larger juveniles (≥ 50 mm TL) was dominated by *Corophium* (Figure 2.6.a) which was also the main prey from August to October (Figure 2.6.b). The polychaetes also represented important prey of the 60-69 mm and 90-99 mm size classes (Figure 2.6.a). Some prey were restricted to specific size classes and sampling months, such as Sphaeromatidae that occurred exclusively in the 50-59 mm individuals captured in July, or Gastropoda belonging to the 60-69 mm class and captured in August. In fact, these classes presented the highest diet diversity among the sampled 0-group flounder (Table 2.1.). The subsequent 70-79 and 80-89 classes presented the lowest diet diversity ($H'=0$). Temporally, 0-group flounder captured in June and July presented an H' of 0.49 and 0.40, respectively. The diet diversity was highest for 0-group flounder collected in August ($H'=0.81$) and reached a minimum in September ($H'=0.12$) and October ($H'=0$). The overall H' value for the sampled 0-group flounder was 0.70.

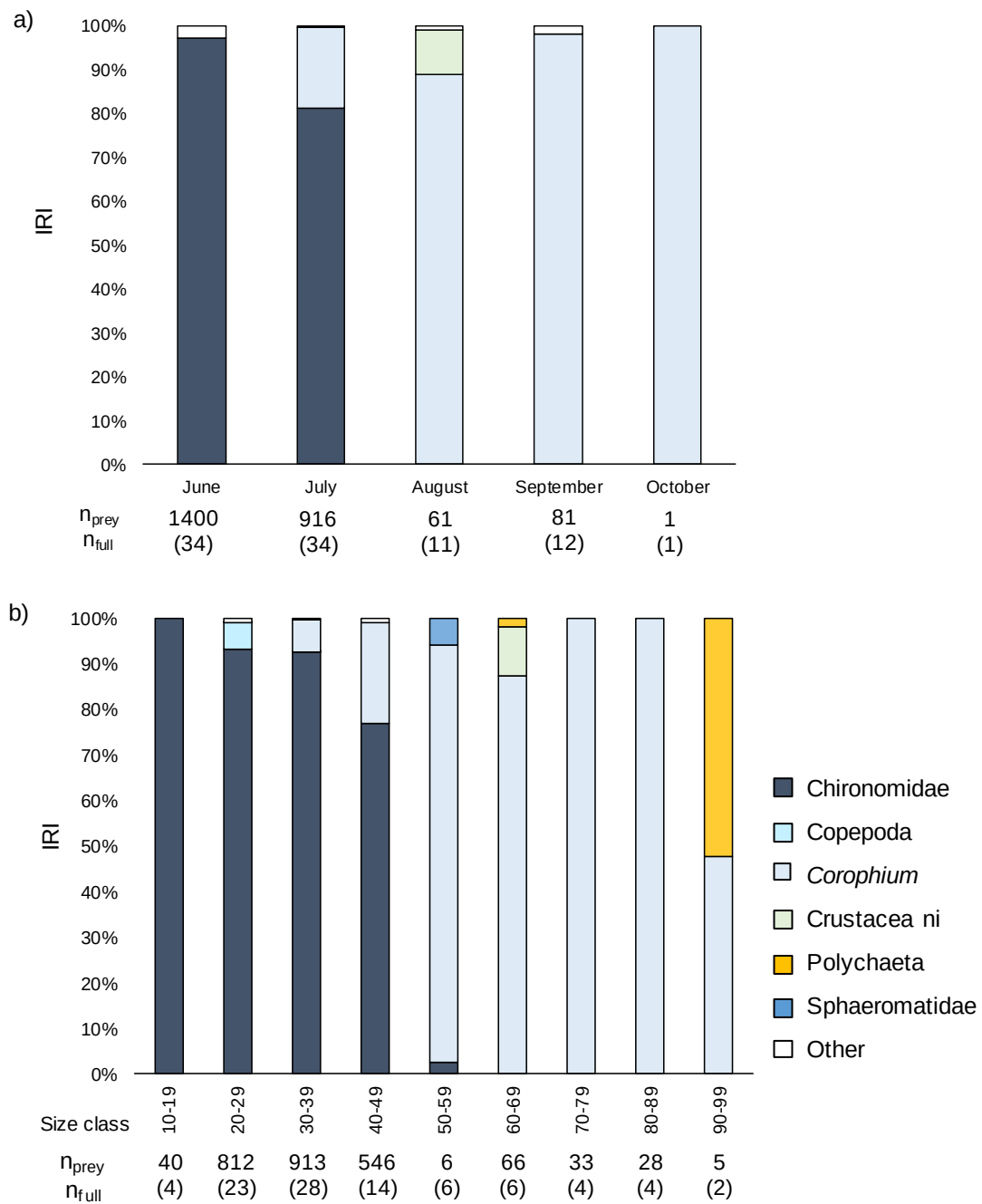


Figure 2.6. Index of Relative Importance (IRI) for the stomach contents of 0-group European flounder: a) temporal variation, and b) ontogenetic variation between size classes (10 mm total length). Number of counted prey (n_{prey}), and number of full stomachs analyzed (n) are presented below the graphs.

2.4. Discussion

2.4.1. The early life traits of European flounder

The flounder lifecycle generally includes spawning from winter to early spring (Summers, 1979; Muus and Nielsen, 1999; Primo *et al.*, 2013). Then, late phase larvae colonize the estuary in late spring (Summers, 1979; Amorim *et al.*, 2016) followed by direct settlement to benthic habitats (Jager, 1998; Bos, 1999; Ramos *et al.*, 2010). Otolith microstructure analysis together with spatial temporal abundances from this and previous studies in the Lima nursery (Ramos *et al.*, 2010; Amorim *et al.*, 2016) corroborated this lifecycle pattern. A single cohort of 0-group flounder was identified with more than 70% of 0-group flounder hatching in April and May and settling in May and June. Newly-settled (< 20 mm TL, Amorim *et al.*, 2016) flounder occurred in June, as well as peak abundances of post-settlement (< 50 mm TL, Amorim *et al.*, 2016) flounder, in agreement with previous studies of the Lima nursery (Amorim *et al.*, 2016). Post-settlement flounder were restricted to the upper estuary where most 0-group flounder concentrated in agreement with Ramos *et al.* (2010) and Amorim *et al.* (2016) as these juveniles tend to concentrate at shallow (Ryer *et al.*, 2010; Amorim *et al.*, 2018), low salinity areas (van der Veer *et al.*, 1991; Jager *et al.*, 1993; Selleslagh and Amara, 2008). Peak abundances in June corresponded mainly to post-settlement flounder and decreased thereafter as dispersal throughout the estuary tends to increase as the flounder juveniles grow (Kerstan, 1991; Ramos *et al.*, 2010; Primo *et al.*, 2013). Accordingly, lower abundances in the middle estuary corresponded mostly to flounder > 50 mm TL. The average abundance of 0-group flounder (4.4 ± 13.5 individuals $1,000\text{ m}^{-2}$) was within the same range previously reported for the Lima estuary (Ramos *et al.*, 2010; Mendes *et al.*, 2014; Amorim *et al.*, 2016) and other populations along the Portuguese coast (Vinagre *et al.*, 2005; Martinho *et al.*, 2007) and Wadden Sea (Kerstan, 1991; Jager *et al.*, 1995).

Flounder pelagic (33 ± 3 days) (Kerstan, 1991; van der Veer *et al.*, 1991; Amorim *et al.*, 2016) and metamorphic (16 ± 4 days) phases were within the latitudinal cline proposed by Martinho *et al.* (2013). The flounder pelagic phase lasts between 30-60 days (Grioche *et al.*, 2000; Martinho *et al.*, 2013). Therefore, longer pelagic stages in the Lima and other Southern estuaries (Martinho *et al.*, 2013) may reflect the distance to the spawning grounds (Bailey *et al.*, 2008), whose location off the Portuguese Coast

is still unknown (Amorim *et al.*, 2016), and adaptations to local hydrological features promoting transport and retention in favourable habitats (e.g. Ekman and selective tidal stream transport) (Jager, 1998; Griocche *et al.*, 2000). At lower latitudes, warmer temperatures may support faster development (Hovenkamp and Witte, 1991; Laurel *et al.*, 2014) hence shorter metamorphosis (Martinho *et al.*, 2013) which is temperature-dependent (Hutchinson and Hawkins, 2004).

2.4.2. Growth rates

The Lima nursery was able to sustain high growth rates of 0-group flounder throughout the growing season showing good nursery habitat quality. Indeed, the 0-group flounder presenting higher growth rates throughout the post-settlement stage also performed better in terms of recent growth rates. It is possible that growth rates reflected cumulative effects from previous life stages, including genetic and maternal effects, size at hatching or larval growth rates (Chambers *et al.*, 1988; Vigliola and Meekan, 2002; Hurst *et al.*, 2009). However, the low mobility of 0-group flounder (Raffaelli *et al.*, 1990; Dando, 2011; Le Pape and Cognez, 2016) may have contributed to these growth rates reflecting stable habitat conditions (Campana and Neilson, 1985; Vinagre *et al.*, 2009a; De Raedemaeker *et al.*, 2011).

The 0-group flounder RG were higher in the Lima nursery than in other nurseries, including the Canche (France) (mean=5.62), Authie (France) (mean=5.98) and Seine (France) (mean =4.40) (Amara *et al.*, 2009) estuaries. The Lima estuary is located at the southern geographical distribution limit of European flounder where warmer temperatures could potentially induce physiological stress. However, the 0-group flounder concentrated in the upper estuary where temperature (mean= 20 ± 2 °C) was within the optimal range (18-22 °C) for flounder growth (Fonds *et al.*, 1992). In fact, the growth rates increased with total length and achieved a maximum at late summer. Thus, this study did not provide evidence for sub-optimal growth due to food limitation at late summer in Southern European nurseries, as described for plaice *Pleuronectes platessa* (Amara, 2004; Nash *et al.*, 2007; Freitas *et al.*, 2012), common sole *Solea solea* (Amara, 2004) and European flounder (Freitas *et al.*, 2012). Actually, higher temperatures and longer photoperiod typical of lower latitudes may have promoted the early onset of spawning (Martinho *et al.*, 2013) and colonization of nursery habitats (Amara *et al.*, 2000; Martinho *et al.*, 2008; Amorim *et al.*, 2016), hence a longer growing

season, as well as faster growth (Henderson and Seaby, 2005; Vinagre *et al.*, 2009a). In the end, 0-group flounder have achieved similar body sizes to other European flounder populations including the Dollard (Netherlands) (Jager *et al.*, 1995), Mondego estuary (Portugal), Wadden Sea and Sorfjord (Norway) (Martinho *et al.*, 2013).

Successful nursery occupation implies exploitation of habitats within nurseries providing physical conditions and food resources (Sheaves *et al.*, 2015) that maximize early life stages growth and survival (Boesch and Turner, 1984; Gibson, 1994; Pihl *et al.*, 2007). Salinity is one of the main predictors of flounder early life stages distribution in estuarine habitats (Ramos *et al.*, 2009; Vasconcelos *et al.*, 2010; Amorim *et al.*, 2018) with young-of-year showing a preference for low salinity habitats (< 5, Bos and Thiel 2006) as the Lima upper estuary. Surprisingly, metamorphosing flounder presented faster growth at high constant salinities (30) (O'Neill *et al.*, 2011), while newly settled grew faster at intermediate salinities (5-15) (Gutt, 1985). Growth rates were higher at stable rather than fluctuating salinity habitats (Andersen *et al.*, 2005a) due to lower osmoregulation costs (Bœuf and Payan, 2001) at stable salinities (Hutchinson and Hawkins, 2004) and isosmotic conditions (Jobling, 1994). In the Lima upper estuary, the 0-group flounder presented high recent growth rates, independently of the salinity variability.

The 0-group flounder preference for upstream areas may represent a habitat trade-off (Beaumont and Mann, 1984; Bos, 1999; Ryer *et al.*, 2007; Grol *et al.*, 2014) where high prey availability may be favoured in contrast to optimal physiological conditions. Indeed, prey availability (Karakiri *et al.*, 1989; Berghahn *et al.*, 1995; van der Veer *et al.*, 2001), prey quality (van der Veer and Witte, 1993; Andersen *et al.*, 2005b), and ontogenetic diet shifts (Sheaves *et al.*, 2015) are important drivers of flatfish growth during the nursery stage. Moreover, flounder juveniles may use different feeding strategies reflecting opportunistic behaviour (De Groot, 1971; Hampel *et al.*, 2005; Martinho *et al.*, 2008) to achieve fast growth (Andersen *et al.*, 2005b) and good condition (Mendes *et al.*, 2014). In the Lima nursery, the diet of 0-group flounder showed the upper estuary as the main feeding area. Mendes *et al.* (2014) showed that 0-group flounder distribution in the Lima estuary correlated to prey availability. The main prey Chironomidae (Weatherley, 1989; Nissling *et al.*, 2007; Florin and Lavados, 2010) and the amphipod *Corophium* (Summers, 1980; Jager *et al.*, 1993; Selleslagh

and Amara, 2015) are common prey among flounder juveniles and highly abundant in the Lima upper estuary (Sousa *et al.*, 2006, Mendes *et al.*, 2014). The highly specialized diet on abundant and available prey (Grønkjær *et al.*, 2007) may optimize food intake (Andersen *et al.*, 2005b) and favour condition (Selleslagh and Amara, 2015).

The diet of newly-settled flounder focused on Chironomidae, similarly to other nurseries (Aarnio *et al.*, 1996) and included planktonic prey such as cladocerans and copepods. Planktonic prey may be present in the diet as newly-settled have recently gone through transition from pelagic larvae to benthic juvenile phase (Aarnio *et al.*, 1996; Andersen *et al.*, 2005b; Nissling *et al.*, 2007). Noteworthy, the RG was higher than in French estuaries where copepods were the main prey of 0-group flounder (Amara *et al.*, 2009). Thus, it is possible that a diet based on macrobenthic prey is more profitable and supported higher growth rates than planktonic prey.

Post-settlement juveniles fed entirely on macrobenthic prey and mainly on Chironomidae larvae. A diet shift was evident at the size of 50 mm TL, with *Corophium* replacing Chironomidae as the dominant prey. Ontogenetic diet shifts are common among flatfish juveniles (Toole, 1980; Scharf *et al.*, 2000; Tomiyama *et al.*, 2013) including flounder (Uzars *et al.*, 2003; Andersen *et al.*, 2005b; Florin and Lavados, 2010) and may be driven by changes in prey availability (Beaumont and Mann, 1984; Besyst *et al.*, 1999; Mendes *et al.*, 2014) or fish size (Keast and Webb, 1966; Dörner and Wagner, 2003; Selleslagh and Amara, 2015). In fact, diet seasonal patterns followed the progression of the flounder cohort: Chironomidae was the main prey in June and July when newly-settled and post-settlement were predominant, while *Corophium* was the main prey in the following months, when most 0-group flounder have reached ≥ 50 mm TL. Furthermore, foraging efficiency and energetic gains are also main drivers of diet shifts (Schoener, 1970; Pyke, 1984; Graeb *et al.*, 2006). Energetic gains depend on the balance between prey calorific content, and search and handling times (Graeb *et al.*, 2006) resulting from fish morphology and prey behaviour (Juanes *et al.*, 2001; Beukers-Stewart and Jones, 2004). Moreover, energetic value tends to increase with prey size (Aarnio *et al.*, 1996; Gning *et al.*, 2008; Selleslagh and Amara, 2015). Although *Corophium* and Chironomidae may have similar calorific value (Magnhagen and Wiederholm, 1982), the former is larger and highly active on

sediment surface (Magnhagen and Wiederholm, 1982; Hughes, 1988) increasing the encounter rate (Andersen *et al.*, 2005b). Therefore, it may represent higher energetic gains as flounder is a visual predator (De Groot, 1971). These diet shifts may minimize niche overlap and intraspecific competition (Florin and Lavados, 2010), while maintaining individual condition (Andersen *et al.*, 2005b; Mendes *et al.*, 2014). Overall, the 0-group flounder diet, including a focus on highly available macroinvertebrates and the diet shift at the end of post-settlement stage supported fast growth, including high RG which indicates recent condition and therefore it is sensitive to food availability (Suthers *et al.*, 1992; Selleslagh and Amara, 2013).

A potential additional advantage of the upper estuary is the exclusion of the flounder predators (Gibson *et al.*, 2002) shore crab *Carcinus maenas* and brown shrimp *Crangon crangon* (van der Veer and Bergman, 1987; van der Veer *et al.*, 1991; Ansell *et al.*, 1999). However, predatory pressure from bird predators such as the great cormorant *Phalacrocorax carbo* (Leopold *et al.*, 1998; Dias *et al.*, 2012) may still be high in these shallow areas. Potential competitors are also excluded from the upper estuary, including the common sole (Vinagre *et al.*, 2005; Ramos *et al.*, 2010) occurring at salinities > 20 (Marchand, 1991; Marshall and Elliott, 1998; Vasconcelos *et al.*, 2010), or 1-group flounder concentrating in downstream areas (Kerstan, 1991; Primo *et al.*, 2013; Amorim *et al.*, 2018).

In general, 0-group flounder nursery use, including estuarine colonization by newly settled in early summer, concentration in the upper estuary and feeding on highly available macroinvertebrate prey from the upper estuary supported the observed high growth rates and showed the high quality of the Lima nursery habitat. Moreover, this study reinforced the importance of integrating different ecological components of nursery use for a more comprehensive understanding of nursery value for an estuarine nursery dependent fish species.

2.5. Conclusions

The present study investigated several aspects of young of the year European flounder life in the Lima nursery habitat. Integrating all results, the otolith microstructure supported abundance data showing that 0-group flounder 1) hatched from February to June, had a pelagic (33 ± 3 days) and metamorphosis (16 ± 4 days) phases, and finally settled in May and June in the upper estuary 2) abundances peaked in June; 3) fed on upper estuary prey with a diet shift at 50 mm TL from Chironomidae to *Corophium*, and 4) presented high growth rates throughout the post-settlement stage and in terms of recent growth. Overall, early life patterns of 0-group flounder supported high growth rates quantifying nursery value of the Lima estuary.

CHAPTER 3

Feeding ecology of juvenile flounder *Platichthys flesus* in an estuarine nursery habitat: influence of prey- predator interactions

Mendes, C., Ramos, S. & Bordalo, A.A. (2014). Feeding ecology of juvenile flounder *Platichthys flesus* in an estuarine nursery habitat: influence of prey-predator interactions. *Journal of Experimental Biology and Ecology* 461, 458-468. DOI: 10.1016/j.jembe.2014.09.016.

Abstract

The present study aimed to investigate the feeding ecology and influence of prey-predator interactions on juvenile European flounder *Platichthys flesus* in an Atlantic estuarine nursery area (Lima estuary, NW Portugal), focusing on prey selection and ontogenetic shifts in the diet. The relationship between prey availability and flounder distribution was also investigated. Juvenile flounder diet included 21 taxa of macroinvertebrates and fishes, sand and plant debris. According to numerical, occurrence, and weight dietary indices, macroinvertebrates, namely Chironomidae and *Corophium* spp. were the main prey items. The diet diversity (i.e. niche breadth) tended to increase as juveniles grew, although some dietary overlap occurred between the early juveniles (50-149 mm total length TL). The diet diversity of the newly settled juveniles (< 50 mm TL) was particularly low, showing the importance of Chironomidae. Moreover, an ontogenetic shift was evident, since older juveniles (1-group) presented a distinct diet, including new items absent from the diet of the 0-group juveniles, namely Teleostei, *Carcinus maenas*, and Nemertea. The juvenile flounder presented an overall generalist behaviour, feeding on the most abundant macroinvertebrates namely Chironomidae and *Corophium* spp, as shown by the Strauss linear index. The spatial distribution of the 0-group flounder in the Lima estuary was associated with salinity and prey (Chironomidae and *Corophium* spp). These prey were characteristic of the upper estuary where most of the juveniles, especially the newly settled ones, were found. Hence, this study reinforces the importance of both abiotic and biotic factors as environmentally driven controls of habitat use during the early phases of the demersal life of European flounder.

3.1. Introduction

One of the most important roles that estuaries provide to fishes is the nursery function (Kerstan, 1991; McLusky and Elliott, 2004; Woodland *et al.*, 2012). A nursery habitat may be described as a restricted area where early development stages of a species spend a limited period of their life cycle, during which they are spatially and temporally separated from the adults (Beck *et al.*, 2001), even when spatial overlap occurs. In these areas, the survival of early development stages is enhanced through superior conditions for feeding, growth, and/or predation refuge (Beck *et al.*, 2001; Pihl *et al.*, 2007).

Habitat selection in the nursery areas results from a compromise between different environmental factors, both biotic and abiotic (Burrows *et al.*, 1994; Hugie and Dill, 1994). The influence of each factor varies throughout the ontogenetic development (Phelan *et al.*, 2001), and also at a variety of temporal and spatial scales (Gibson *et al.*, 1996). For example, as diet and main predators change throughout ontogeny, juveniles may reorganize their distribution around the nursery habitat (Burke, 1995; Modin and Pihl, 1996; Castillo-Rivera *et al.*, 2000). Furthermore, both differences in ontogenetic state and seasonal fluctuations in the abiotic and biotic factors act together to produce characteristic distribution patterns and differential habitat use at different spatial and temporal scales (Gibson *et al.*, 2002).

Flatfish, including the European flounder *Platichthys flesus*, are among the species that use estuaries as nursery areas. The distribution of *P. flesus* is commonly described as ranging from the coasts of northern Europe to the Mediterranean, constituting an important component of demersal fish assemblages economically exploited (Maes *et al.*, 1998; Thiel and Potter, 2001). However, it has been recently suggested that the present southern distribution limit of this species is at the coast of Portugal (Cabral *et al.*, 2001). This euryhaline species tolerate salinities from 0 to 35, although the juveniles preferentially use low salinity areas as nursery grounds (Hemmer-Hansen *et al.*, 2007; Zucchetta *et al.*, 2010). This broad distribution and tolerance to a wide range of environments, makes *P. flesus* particularly interesting to study the mechanisms underlying habitat use. Abiotic factors affecting *P. flesus* distribution include salinity (Ramos *et al.*, 2009), temperature (Power *et al.*, 2000), depth (Cabral *et al.*, 2007; Vasconcelos *et al.*, 2010), dissolved oxygen (Maes *et al.*, 1998; Power *et al.*, 2000),

turbidity, and sediment composition (Kerstan, 1991; Zucchetta *et al.*, 2010). The correlation between abiotic factors and the abundance of juveniles does not always imply that the former has a direct effect on the distribution patterns of the latter. Instead, abiotic factors may be used by fishes to locate habitats with favorable biotic conditions, such as reduced risk of predation or high food availability (Gibson *et al.*, 2005).

Prey availability has been shown to affect the distribution of juvenile flatfish in nursery habitats (Vinagre *et al.*, 2005; Le Pape *et al.*, 2007; Nicolas *et al.*, 2007). As the diet changes throughout the ontogenetic development (Aarnio *et al.*, 1996; Andersen *et al.*, 2005b; Florin and Lavados, 2010), prey distribution may be responsible for differential ontogenetic distribution of the juveniles (Burke, 1995). The ontogenetic shifts may also contribute to reduce the trophic overlap between the different development stages (Aarnio *et al.*, 1996). Thus, this resource partitioning strategy, either through spatial segregation or variations in the type of prey consumed, may be important to minimize niche overlap and hence competition for food in nurseries (Cabral *et al.*, 2002), where high densities of juveniles are typically observed. Consequently, survival may be enhanced, possibly increasing recruitment to adult populations (Russo *et al.*, 2008). However, few studies have investigated prey-predator interactions influence on flatfish (Burke, 1995; Le Pape *et al.*, 2007), and in particular on flounder (Vinagre *et al.*, 2008) nursery habitats, compared to the many studies regarding abiotic factors (e.g. Power *et al.*, 2000; Cabral *et al.*, 2007; Zuccheta *et al.*, 2010).

The Lima estuary (NW Portugal) has been previously identified as an important nursery for the flounder *P. flesus* (Ramos *et al.*, 2010), and juvenile flounder spatial distribution has been related with abiotic features, namely the salinity regime and sediment type (Ramos *et al.*, 2009). Thus, the present study aimed to investigate the feeding ecology and influence of prey-predator interactions on the juvenile flounder in this nursery habitat, answering the following questions: (1) Do juveniles exhibit prey selectivity? (2) Are there ontogenetic shifts in the diet? (3) Is there a relationship between prey availability and juveniles distribution? The study represents a step forward towards the understanding of the early life of the European flounder at its southern distribution limit. This information will help the detection of eventual changes in the habitat use of the species in this area, and consequently meet the management challenges regarding their conservation.

3.2. Material and Methods

3.2.1. Study area

The Lima estuary, located in the NW Atlantic coast of Portugal, is a small open estuary with a semidiurnal and mesotidal regime (3.7 m). Salt intrusion can extend up to 20 km upstream, with an average flushing rate of 0.4 m s^{-1} , and a residence time of 9 days (Ramos *et al.*, 2006b). The river mouth is partially obstructed by a 2 km long jetty, causing a deflection of the river flow to the south, against the Coriolis effect. For this study, nine sampling stations covering the lower, middle and upper estuary were established (Figure 3.1.a). The lower estuary (stations L1-L3, average depth= 3.4 m), located in the initial 2.5 km, is a narrow, 9 m deep navigational channel, industrialized, with walled banks. It includes a shipyard, a commercial seaport, and a fishing harbor. The middle estuary (stations L4-L6, average depth= 4.2 m) comprises a broad shallow intertidal saltmarsh zone, mainly colonized by the common rush (*Juncus* spp.). The upper estuary (Stations L7-L9) is a narrow shallow channel (< 3 m), less disturbed, with natural banks and small sand islands. The average depth of the sampling sites in this estuarine section was 2.4 m.

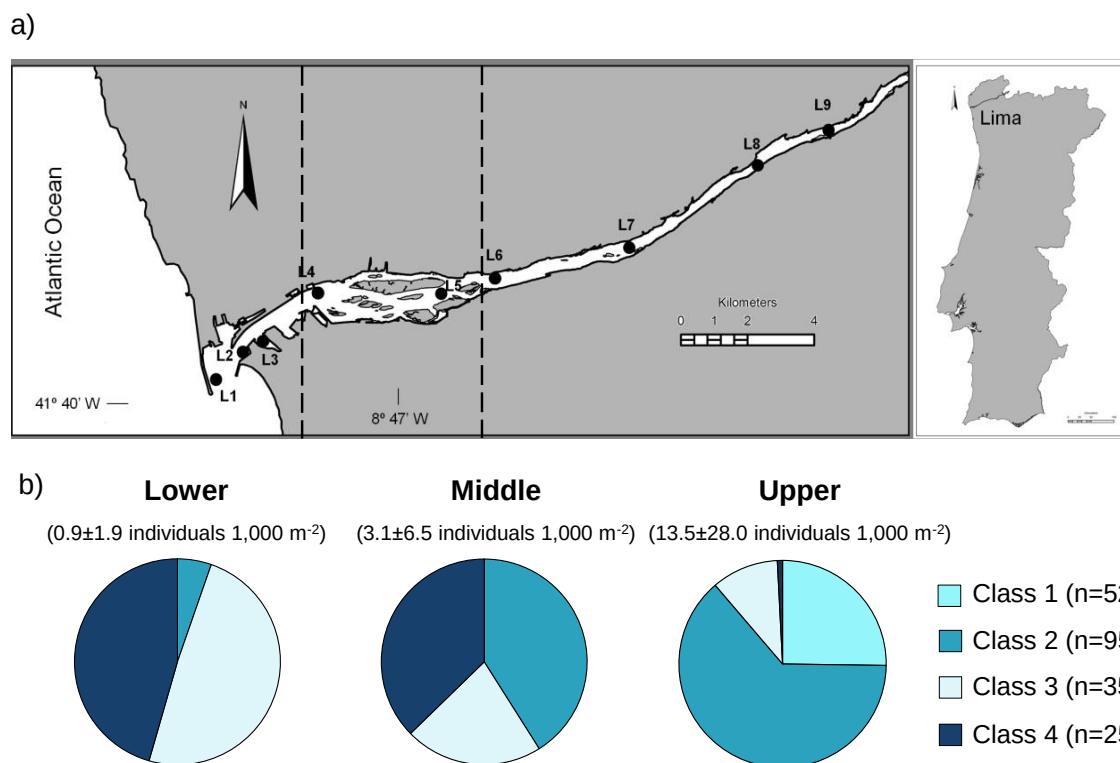


Figure 3.1. a) Lima estuary with the location of sampling stations, (L1, L2, L3- lower estuary; L4, L5, L6- middle estuary; L7, L8, L9- upper estuary); and b) average (individuals 1, 000 m⁻²) and relative abundances (%) of *P. flesus* juveniles of the lower, middle and upper sections of the Lima estuary; (in brackets: total number of fishes sampled per size class).

3.2.2. Data Collection

Macroinvertebrates

Macroinvertebrates are one of the main prey items of flounder juveniles as shown by diet studies (e.g. (Chapter 2, Link *et al.*, 2002; Martinho *et al.*, 2008). Thus, sediment grain characterization and the macroinvertebrate community were surveyed seasonally, in February, April, July and October of 2010, representing winter, spring, summer and autumn, respectively. For each survey, triplicate sediment samples were retrieved at each sampling site by means of a Petite Ponar grab with an area of 0.023 m² both for sediment and macroinvertebrate community characterization. Sediment samples for macroinvertebrate analysis were immediately fixed in 5% buffered formalin stained with Rose Bengal, and stored for further laboratory analysis (Mucha *et al.*, 2005).

Fishes

Flounder juveniles were collected monthly during nightly ebb tides, between September 2009 and October 2010, with a 2 m beam trawl, with a mesh size of 5 mm in the cod end and a tickler chain. Trawls were made at a constant speed (3 m s^{-2}) and lasted 10 min. Samples were refrigerated in boxes with ice and transported to the laboratory where they were frozen (-18°C) until sorting. The geographic location of the sampling stations and the distance travelled during each tow was measured with a Magellan 315 GPS. At each site, vertical profiles of temperature and salinity were obtained by means of a multi-parameter water quality probe YSI 6820.

3.2.3. Laboratory Procedures

Sediment characterization

Unfixed sediments were treated in order to determine the percentage of organic matter, by drying the samples at 105°C (24 h), and then by loss on ignition at 500°C (4 h; (APHA, 1992). Sediments were dried at 100°C , and grain size analysis was performed by wet (fraction $< 0.063 \text{ mm}$), and dry (other fractions) sieving (CISA Sieve Shaker Mod. RP.08). Sediments were divided into four fractions: silt and clay ($<0.063 \text{ mm}$), fine sand ($0.063\text{--}0.250 \text{ mm}$), sand ($0.250\text{--}1.000 \text{ mm}$), and gravel ($>1.000 \text{ mm}$). Each fraction was weighed and expressed as a percentage of the total weight.

Macroinvertebrates

Sediment samples were sieved on a 0.5 mm mesh size, and the macroinvertebrates were kept in 70% alcohol until sorting (Mucha *et al.*, 2005). Macroinvertebrates were then counted and identified to the species level whenever possible, using a binocular microscope (Leica MZ12-5). Whenever individuals were fragmented, only the heads were considered for counting purposes.

Fish

Fishes were measured for total length (TL, $\pm 1 \text{ mm}$ precision) and weight (WW, wet weight, $\pm 0.01 \text{ g}$ precision). Considering that the flounder length at first sexual maturity is 200 mm TL (Dinis, 1986), fishes measuring less than 200 mm TL were considered juveniles. Maximum mouth gape width was determined by carefully opening the mouth

of the juveniles with the aid of fine-tipped forceps and then measuring across the distance of the gape, using a caliper. Stomachs were excised, contents removed and preserved in alcohol 70%, for further prey identification. Each prey item was identified to the highest taxonomic separation possible, using a binocular microscope (Leica MZ12-5), counted, allowed to drip on a tissue paper and weighed (wet weight to 0.001 g). As mentioned above, whenever individuals were fragmented, only the heads were considered for counting purposes. Additionally, the length (mm) of each prey item was also determined whenever possible taking in consideration the preservation state of the prey.

3.2.4. Data Analysis

Macroinvertebrates community

Macroinvertebrates abundance data were standardized as the number of individuals per m² of sediment. The most abundant taxa were determined by the average of each taxon abundance per estuarine section. Diversity of macrobenthos was expressed by the Shannon-Wiener index (H') (Shannon and Weaver, 1949). Two-way ANOVA was performed to assess spatial and temporal differences on the macrofauna abundance and diversity (H'), with estuarine sections and seasons as fixed factors. Abundance data were log transformed ($\log(x + 1)$). Furthermore, in the event of significance, an *a posteriori* Fisher test was used to determine which means were significantly different at a 0.05 level of probability (Zar, 1996). These analyses were performed with Statistica software (version 10.0, Statsoft Inc., Tulsa, OK, USA). Two-way crossed analysis of similarity (ANOSIM) was used to investigate seasonal and spatial variations in the macrofauna structure. Similarity percentages analysis (SIMPER) was used to assess which species contributed more to the dissimilarities observed. Tests were based on a Bray-Curtis similarity matrix calculated based on $\log(x+1)$ transformed abundance data. These analyses were performed with the software package PRIMER v6 (Plymouth Routines Multivariate Ecological Research) (Clarke and Warwick, 2001).

Flounder diet

Trawl opening (2 m) and distance travelled (determined by GPS) were used to estimate the sampled area. Therefore, densities were standardized as the number of individuals per 1,000 m² swept area. Fishes were divided into four size classes according to their

total length: class 1 (0-49 mm), class 2 (50-99 mm), class 3 (100-149 mm), and class 4 (150-199 mm). Classes 1, 2 and 3 correspond to 0-group juveniles and class 4 to 1-group juveniles, according to Teixeira *et al.* (2010). Fish condition was assessed by the Fulton's condition factor, K (Ricker, 1975). Feeding activity was evaluated by the vacuity index (I_v), defined as the percent of empty stomachs (Hyslop, 1980). The relative contribution of the different prey taxa was assessed by the percent of numerical abundance (NI), occurrence in the stomachs (OI) and weight (WI) (Hyslop, 1980). These dietary indices were also calculated for each size class. Hierarchical agglomerative clustering with group average sorting was used to investigate dietary variations throughout the flounder juvenile development. SIMPROF test was applied to assess the significance of the clusters produced (Clarke and Warwick, 2001). Analyses were based on Bray-Curtis similarity (Bray and Curtis, 1957) matrix calculated using log ($x+1$) transformed NI, OI and WI data. Multivariate analyses were performed with the software package PRIMER v6 (Plymouth Routines Multivariate Ecological Research) (Clarke and Warwick, 2001).

Prey-predator interactions

For prey selection analysis, only macroinvertebrates were considered since these were the main prey items of flounder juveniles. Samples were divided according to the estuarine section: lower, middle and upper, and season: autumn (September-November), winter (December-February), spring (March-May), and summer (June-August). Prey selection by flounder juveniles was quantified for each size class by the Strauss linear index L (Strauss, 1979):

$$L = r_i - p_i$$

where r_i is the relative frequency of the item i in the diet, and p_i is the relative frequency of the item i in the environment (Lima macroinvertebrate community). This index compares the proportion of a prey item in the diet with its proportion in the environment. It ranges from -1 to +1, with negative values indicating avoidance or inaccessibility and positive values indicating preference. Zero indicates random feeding. Extreme values occur when a prey is rare but consumed almost exclusively or is highly abundant but rarely consumed. In order to avoid false results, for example when a given prey type

and fish size class does not co-occur in the estuary, prey selection was only analyzed in the most abundant section of the estuary as following:

i) for 0-group flounder (classes 1, 2 and 3) that were concentrated in the upper section, only data from the upper Lima was considered for the Strauss index; ii) for 1-group juveniles, only the lower estuary was considered, where most of the stomach contents with macroinvertebrate prey were found. The Shannon-Wiener diversity index H' (Shannon and Weaver, 1949) was used to evaluate the diversity of each size class diet. Additionally, the potential diet overlap between the four size classes was measured by the Schoener index (SI) (Schoener, 1970):

$$SI = 1 - 0.5 \left(\sum_{i=1}^n |P_{iA} - P_{iB}| \right)$$

where p_{iA} and p_{iB} are the numerical or weight proportions of the item i in the size class A and B, respectively. Values of the diet overlap vary between 0, when no food was shared and 1, when there was the same proportional use of all food resources. Wallace and Ramsey (1983) suggested that values higher than 0.6 indicate biologically significant overlap.

The influence of prey size on the flounder diet was investigated through the relationship between the prey length and fish length. Data and residuals were plotted and inspected for trends in order to assess if the underlying assumptions of independence, linearity, homocedasticity, and normality were met. The relationship between fishes total length and minimum, maximum and mean prey length for the overall individuals, and for each size class were determined through linear regressions, using R software (R Development Core Team, 2007). Differences in prey size between different juvenile flounder size classes were assessed by non-parametric test Kruskal–Wallis and multiple comparison procedures. These analyses were performed with Statistica software (version 10.0, Statsoft Inc., Tulsa, OK, USA).

The influence of environmental variables in the juvenile flounder distribution in the Lima estuary was investigated. Biotic factors such as prey availability tend to act on a finer scale (Le Pape *et al.*, 2007), where abiotic factors such as salinity are favourable to fish occurrence. Thus, the environmental variables investigated comprised both water

parameters and prey availability. Generalized Linear Models (GLM) were applied to analyze juvenile flounder distribution patterns, using a Gamma regression with a log link (McCullagh and Nelder, 1989) of the positive abundance values, therefore excluding null values. Since the flounder juveniles in the Lima estuary were mostly 0-group, only those individuals were used in the model. The main prey *Corophium* spp. and Chironomidae, as well as the physical-chemical variables temperature, salinity and sediment composition were considered as explanatory variables. In order to evaluate potential variables, a stepwise procedure was applied where the significance of predictor variables was tested, and first order interactions were included. The significance of the difference in the deviance between two models resulting from the addition of a new variable was assessed by a chi-square test. The goodness of fit was evaluated by the percentage of total deviance explained and relative contribution of each variable. Statistical analyses were performed using R software (R Development Core Team, 2007). A significance level of 0.05 was considered for all the statistical procedures.

3.3. Results

3.3.1. Environmental parameters

During the study period, the water column temperature ranged between 9.1 °C and 24.9 °C, with a mean of 14.9 ± 2.4 °C. It followed the usual seasonal pattern with lower values during winter. The typical estuarine horizontal salinity gradient was always present, with salinity decreasing upstream. On average, the lower estuarine zone was in the euryhaline range (32.3 ± 5.4), while the middle estuary was in the polyhaline (29.3 ± 9.1), and the upper section was in the mesohaline range (10.3 ± 10.9).

Sediment composition varied across the estuary. The lower estuary was mainly composed of sand (47.1%) and fine sand (32%), while in the upper estuarine section gravel (65.6%) was the dominant fraction, as expected because of the higher current speeds. The middle estuary presented the most equal distribution of different types of sediment, with a prevalence of gravel (38.2%) and sand (32.7%). There was an upstream increase of the gravel and a decrease of the silt and clay fractions (lower

estuary: 14.8%; upper estuary: 0.2%). The organic matter content throughout the year showed a general trend to decrease from the lower (36.9 mg g⁻¹), to the middle (27.6 mg g⁻¹), and upper (7.8 mg g⁻¹) estuarine sections following the increase in grain size.

3.3.2. Macroinvertebrate community

A total of 3,601 individuals were identified in the macroinvertebrate community, belonging to 63 taxa. Oligochaeta, *Corophium* spp., and *Hediste diversicolor* were the most abundant taxa (Figure 3.2.), corresponding to 29.6%, 21.3% and 10.3% of the total macrofauna, respectively. The abundance of macrofauna averaged $1,788 \pm 2,597$ individuals m⁻². No significant seasonal ($F = 2.8$, $p = 0.06$), or spatial ($F = 2.1$, $p = 0.15$) variations of abundance were observed. In general, the lower estuary tended to comprise more species, with an average of 17 species, followed by the middle (average of 13), and upper estuarine sections (average of 8). Diversity (H') presented a significant spatial variation ($F = 3.9$, $p < 0.05$), but did not vary seasonally ($F = 2.5$, $p = 0.08$). Similarly to the number of species, diversity was generally higher in the lower ($H' = 1.9$) and middle estuarine sections ($H' = 1.5$), comparatively to the upper estuary ($H' = 1.3$). According to ANOSIM results, the structure of the macroinvertebrates community varied significantly ($R = 0.6$, $p < 0.05$) between the estuarine sections, but no significant ($R = 0.1$, $p = 0.24$) seasonal variation was observed. In fact, the macroinvertebrate community found in the upper estuary was significantly different from the one observed in the lower ($R = 0.7$, $p < 0.05$) or middle ($R = 0.7$, $p < 0.05$) sections (Table 3.1.). SIMPER results identified Oligochaeta, particularly abundant in the lower estuary (Figure 3.2.), and *Corophium* spp. and Chironomidae, more abundant in the upper estuary (Figure 3.2.), being responsible for 43% of the average dissimilarity observed between the macrobenthic community of the lower and the upper sections of the estuary (Table 3.1.). Regarding the differences between the middle and upper estuary, *Hediste diversicolor*, which was more abundant in the upper estuary (Figure 3.2.), and *Capitella* spp. and Oligochaeta, more abundant in the middle estuary (Figure 3.2.), contributed together to 43% of the total dissimilarity (Table 3.1.).

Table 3.1. Results of ANOSIM (R values and significance levels) and SIMPER analyses on abundance of macroinvertebrate taxa (SIMPER results for the three most important taxa contributing to dissimilarities are shown). ni = not identified.

Groups	ANOSIM		Average dissimilarity (%)	SIMPER Discriminating taxa	Cumulative contribution (%)
	<i>R</i>	<i>P</i>			
Lower vs. Middle	0.5	0.06	69.8	<i>Hediste diversicolor</i>	15.3
				Oligochaeta ni	30.4
				Nemertea ni	30.9
Lower vs. Upper	0.7	0.03*	83.4	Oligochaeta ni	15.1
				<i>Corophium</i> spp.	30.1
				Chironomidae ni	43.3
Middle vs. Upper	0.7	0.03*	78.4	<i>Hediste diversicolor</i>	14.9
				<i>Capitella</i> spp.	29.3
				Oligochaeta ni	43.4

* significant value

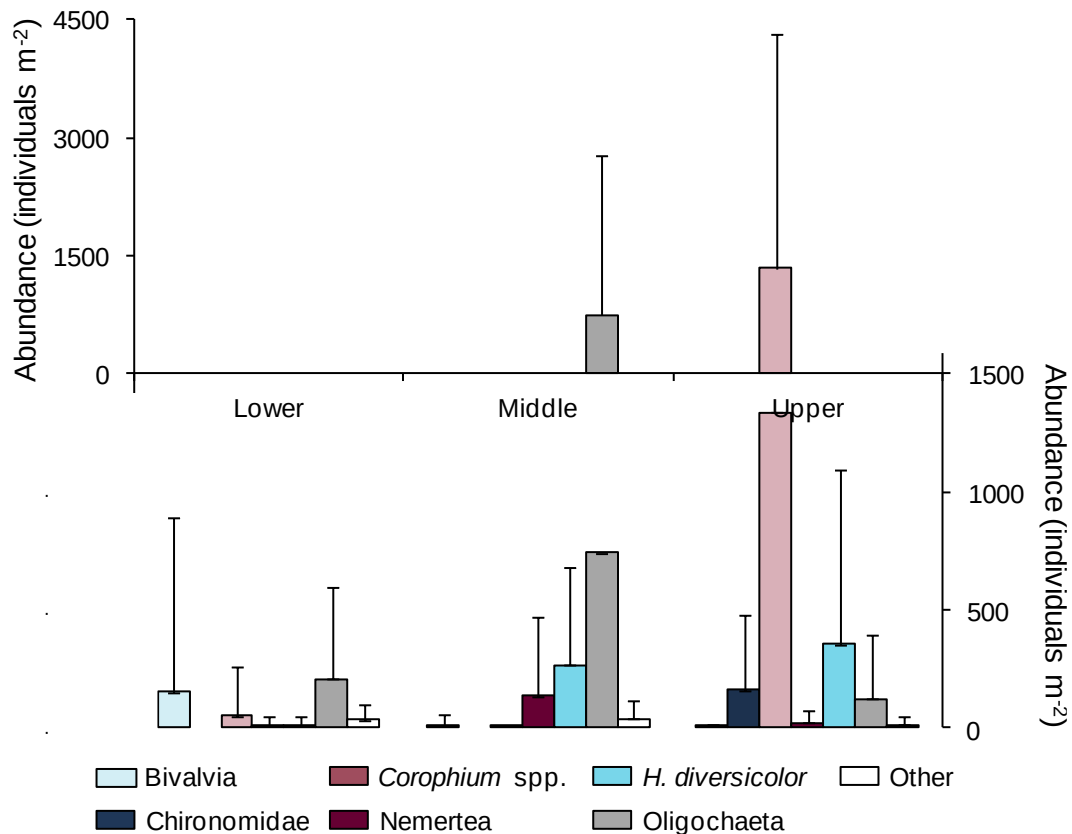


Figure 3.2. Average abundance (individuals m⁻²) per estuarine section (lower, middle and upper) of the main taxa of the Lima estuary macroinvertebrate community including main prey of juvenile flounder.

3.3.3. *P. flesus* juvenile distribution

A total of 207 flounder juveniles were collected (Table 3.2.), with the total length ranging 19 - 197 mm, and total weight varying between 0.1 and 84.6 g. The average density of flounder was 6.0 ± 17.4 individuals 1,000 m⁻². Class 1 juveniles, with an overall abundance of 1.7 ± 8.6 individuals 1,000 m⁻² were restricted to the upper estuary (Figure 3.1.b) and were only present in the Spring (4.53 ± 8.00 individuals 1,000 m⁻²), Summer (0.55 ± 1.38 individuals 1,000 m⁻²), and Autumn 2 (0.22 ± 0.67 individuals 1,000 m⁻²). Class 2 was the most abundant (3.0 ± 14.1 individuals 1,000 m⁻²), occurring throughout the year, with densities ranging from 0.48 ± 0.99 in the Summer and 10.45 ± 29.60 individuals 1,000 m⁻² in the Autumn 1. This class tended to occur mostly in the upper estuary although their presence was also noticed in other

estuarine sections (Figure 3.1.b). A similar spatial pattern was observed for Class 3 flounder, whose densities peaked in the Autumn 1 (1.53 ± 3.69 individuals $1,000 \text{ m}^{-2}$), while the lowest densities were observed in the Spring (0.50 ± 0.79 individuals $1,000 \text{ m}^{-2}$). The 1-group juveniles (Class 4) with an overall abundance 0.5 ± 1.5 individuals $1,000 \text{ m}^{-2}$ were more frequent in the middle section of the Lima estuary (Figure 3.1.b) and were present in all seasons except Spring. Abundances of this class ranged from 0.56 ± 0.88 to 0.87 ± 1.20 individuals $1,000 \text{ m}^{-2}$ in the Winter and Autumn, respectively.

Table 3.2. Number of *P. flesus* juveniles sampled per size class, mean total length (mm) and mean total weight (g).

Class	Class 1	Class 2	Class 3	Class 4
Number	52	95	35	25
Mean total length (mm)	29.1 ± 8.1	71.9 ± 12.6	121.3 ± 16.1	172.8 ± 13.3
Mean total weight (g)	0.3 ± 0.3	4.3 ± 2.1	19.7 ± 8.9	56.7 ± 14.6

3.3.4. Diet of *P. flesus* juveniles

The overall percentage of empty stomachs reached 33.3%, and tended to increase with fish size, with Class 1 presenting the lowest value (11.5%), followed by classes 2 (30.5 %), 3 (51.4%), and 4 (64.0%). The condition of the flounder juveniles, expressed by Fulton's *k* factor varied between 0.3 (Class 2) and 1.6 (Class 2), and presented an average value of 1.0 ± 0.2 , not varying between size classes.

The diet composition of the overall *P. flesus* juveniles analyzed included 21 different taxa, containing macroinvertebrates, fishes, plant debris and sand. In terms of number and occurrence, the diet of flounder juveniles was mainly composed by macroinvertebrate prey, namely Chironomidae, *Corophium* spp., and Elmidae. Gravimetrically, *Corophium* spp., and the brown shrimp *Crangon crangon* were the most important items.

The diet of the newly settled flounder (Class 1) included 1,453 items. Chironomidae were the dominant item, in terms of number (NI= 92.4%), occurrence (OI= 68.2%), and weight (WI= 64.0%) (Figure 3.3.). *Corophium* spp. was important in terms of occurrence (OI= 12.7%), and weight (WI= 21.8%). Elmidae appeared as a minor item

(NI= 5.3%, OI= 3.2%, WI= 9.7%), as well as Bivalvia, Ephemeroptera, and Caenidae. The diet of Class 2 juveniles was more diversified, including Amphipoda, Polychaeta, Bivalvia, Mysidacea, and Insecta larvae, with a total of 550 items. *Corophium* spp. was the main prey item in any of the three applied dietary indices (NI= 55.5%, OI= 47.2%, WI= 75.1%) (Figure 3.3.). The diet also comprised Chironomidae and Elmidae, which were important in terms of number (NI= 22.5%; 16.0%, respectively), and occurrence (OI= 10.1%; 22.5%). Moreover, *C. crangon* was gravimetrically important (WI= 8.5%). The diet of Class 3 comprised 63 items. Similarly to Class 2, *Corophium* spp. was the main item of Class 3, according to all dietary indices (NI= 50.8%; OI= 31.3%; WI= 63.1%) (Figure 3.3.). Chironomidae and the gastropod *Ecrobia truncata* were numerically important (NI= 19.0%; 17.5%), as well as in terms of occurrence (OI= 12.5%; 12.5%), while *C. crangon* assumed a great importance in terms of weight (WI= 34.3%), and occurrence (OI= 18.8%). Other items were also present in the diet of this size class, namely Bivalvia, Polychaeta, the gastropod *Potamopyrgus jenkinsi*, and Simuliidae, but with low values. The diet of older juveniles (Class 4) consisted of only 5 taxa, and a total of 20 items. Nemertea (NI= 40.0%) and *Corophium* spp. (NI= 30.0%) were the main items according to the numerical index, while Teleostea (WI= 5.6%) and Crustacea (WI= 68.4%), including *C. maenas* (WI= 29.5%) and *Corophium* spp. (WI= 9.25%), were the most important items in terms of weight (Figure 3.3.). All items presented similar values of occurrence (OI= 14.3%).

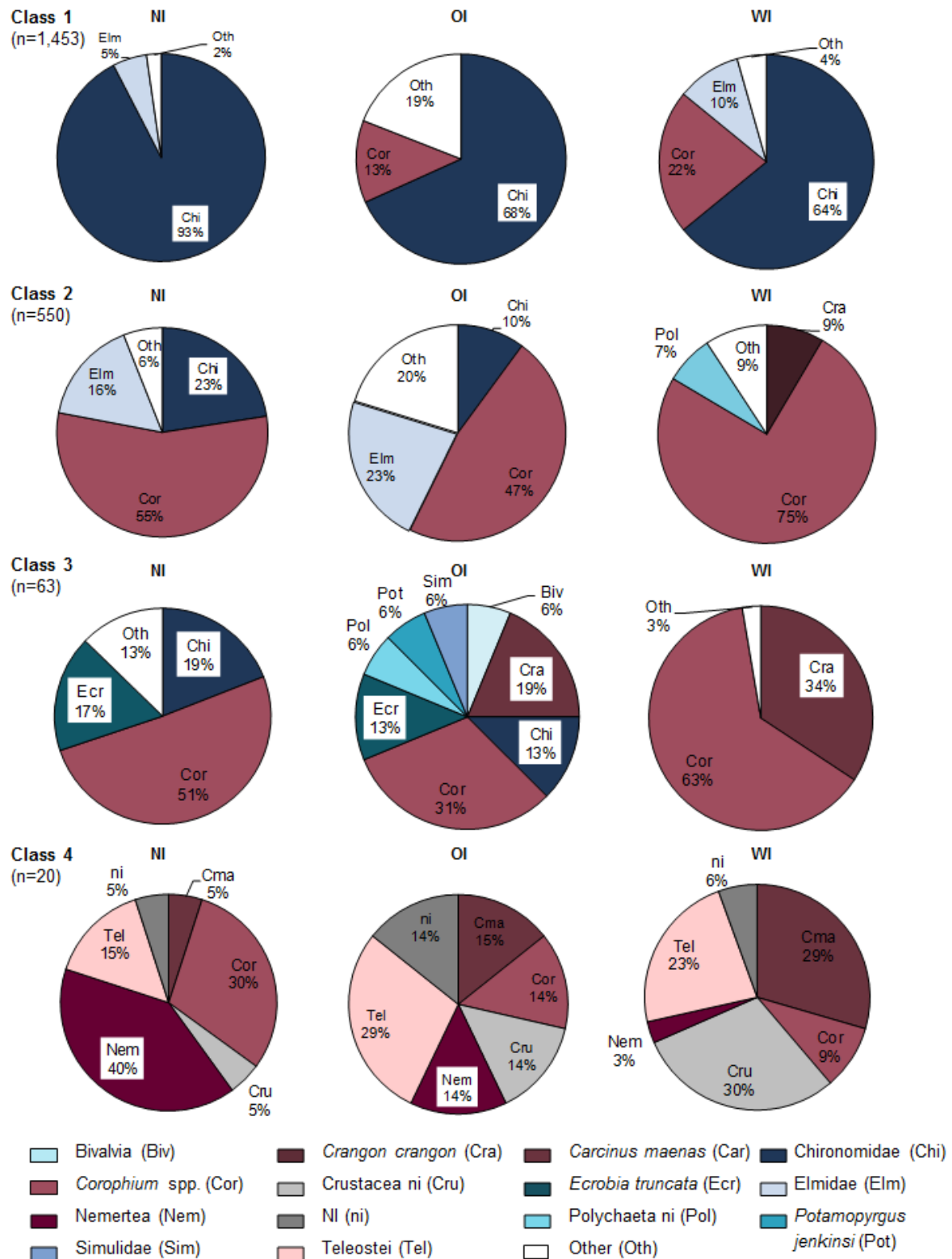


Figure 3.3. Numerical (NI), occurrence (OI) and weight (WI) indices for stomach contents of *P. flesus* juveniles for each size class; (in brackets: number of prey items per size class).

Macroinvertebrates were the only prey items preserved sufficiently to allow the measurement of the respective total length. Thus, macroinvertebrate prey total length varied significantly ($H = 29.42$, $p < 0.05$) between different juvenile flounder size classes. Class 1 prey size (mean = 2.23 ± 0.87 mm) was significantly lower than prey size of classes 2 (mean = 5.31 ± 1.77 mm), 3 (mean = 7.92 ± 3.88 mm), but not from class 4 (mean = 4.71 ± 3.12 , $p = 0.31$). The minimum prey size observed for classes 1 and 2 was 1 mm, while the maximum prey length was 7 mm for class 1, and 13 mm for class 2. The prey length of class 3 ranged between 3 and 28 mm, while for class 4 values ranged between 2 and 9 mm. The minimum ($R^2 = 0.9$), mean ($R^2 = 0.9$), and maximum ($R^2 = 0.5$) prey length (Table 3.3.) significantly ($p < 0.05$) increased with total length of class 3 individuals. However, such trends were not always observed when considering the remaining classes (Table 3.3.). When considering NI and WI, there was a diet overlap between the 0-group juveniles of classes 2 and 3 ($SI = 0.7$). In fact, cluster analysis showed that the diet of classes 2 and 3 clustered at a level between 50% and 60% of similarity, based on NI, OI and WI (Figure 3.4.). In contrast, the diet of the 1-group juvenile flounder (Class 4) differed significantly from the diet of the 0-group juveniles (Class 1-3), as shown by the SIMPROF analysis ($p < 0.05$).

Table 3.3. Statistics for linear regression analysis (R^2 and p value) on minimum, mean and maximum prey length and juvenile flounder total length, according to fish size class.

Size class	Prey length					
	Minimum		Mean		Maximum	
	R^2	p	R^2	p	R^2	p
All fishes	0.26	$p < 0.05$	0.43	$p < 0.05$	0.17	$p < 0.05$
Class 1	0.12	$p < 0.05$	0.04	$p = 0.07$	0.03	$p = 0.27$
Class 2	0.02	$p = 0.46$	0.20	$p < 0.05$	0.06	$p = 0.13$
Class 3	0.94	$p < 0.05$	0.87	$p < 0.05$	0.51	$p < 0.05$
Class 4	0.42	$p = 0.40$	0.85	$p = 0.36$	0.26	$p = 0.49$

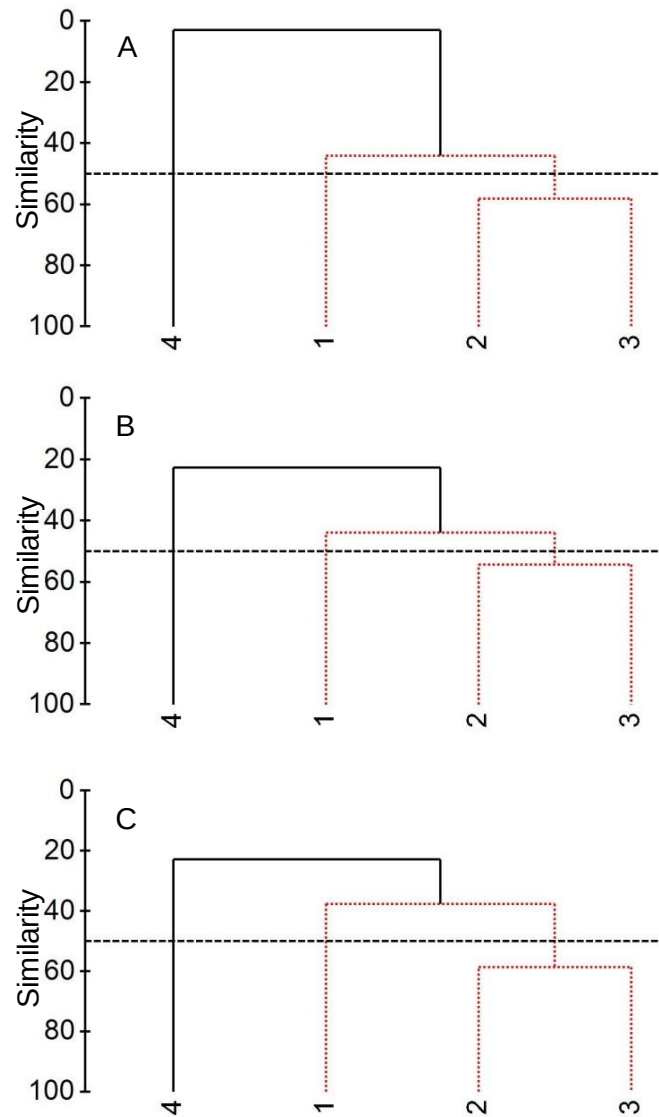


Figure 3.4. Cluster analysis of the four *P. flesus* size classes, based on numerical index (NI)(A), occurrence index (OI)(B) and weight index (WI)(C). Significant clusters according to SIMPROF are shown in red.

3.3.5. Prey selection

The diet diversity increased with an increase in the fish size classes, according to the Shannon-Wiener index, from $H' = 0.3$ for Class 1, $H' = 1.3$ for Class 2, $H' = 1.4$ for Class 3 and $H' = 1.5$ for Class 4. Diet diversity index revealed a non-specialized character of the diet, with an overall value of $H' = 0.97$, i.e. with a high niche breadth.

According to the Strauss linear index (L), each size class exhibited a different prey selection pattern (Figure 3.5.). For example, Class 1 individuals presented high L values for Chironomidae during spring and summer (Figure 3.5.b), and negative values during autumn that coincided with the absence of this taxon among the macroinvertebrate community of the upper estuary (Figures 3.5.a). However, near zero and negative values were observed for *Corophium* spp. during summer and autumn (Figure 3.5.b) respectively, although its abundance in the upper estuary was high during these periods (Figure 3.5.a). L values for Bivalvia and Isopoda were near zero, indicating random feeding on these items (Figure 3.5.b). Class 2 L values were high for Chironomidae during winter and spring (Figure 3.5.c). A negative L value occurred in summer, concomitant with a decrease in the respective abundance (Figure 3.5.a), and an increase in the proportion of *Corophium* spp. in the diet. Concerning class 3, positive L values for Chironomidae were only observed during winter, while for *Corophium* spp., positive values occurred in spring and autumn (Figure 3.5.d), the later corresponding to its peak abundance in the macroinvertebrate community (Figure 3.5.a). Class 4 individuals consumed *Corophium* spp. and Nemertea during winter, presenting positive L values for these items (Figure 3.5.e). The juveniles from this class caught during other seasons always presented empty stomachs. Polychaetes, although dominant in the macrobenthic community, were almost absent from the diet of all the fish size classes. Thus, L values for polychaetes were always negative. Some important prey items, namely Elmidae and *E. truncata* were not considered due to their absence in the macrobenthos samples.

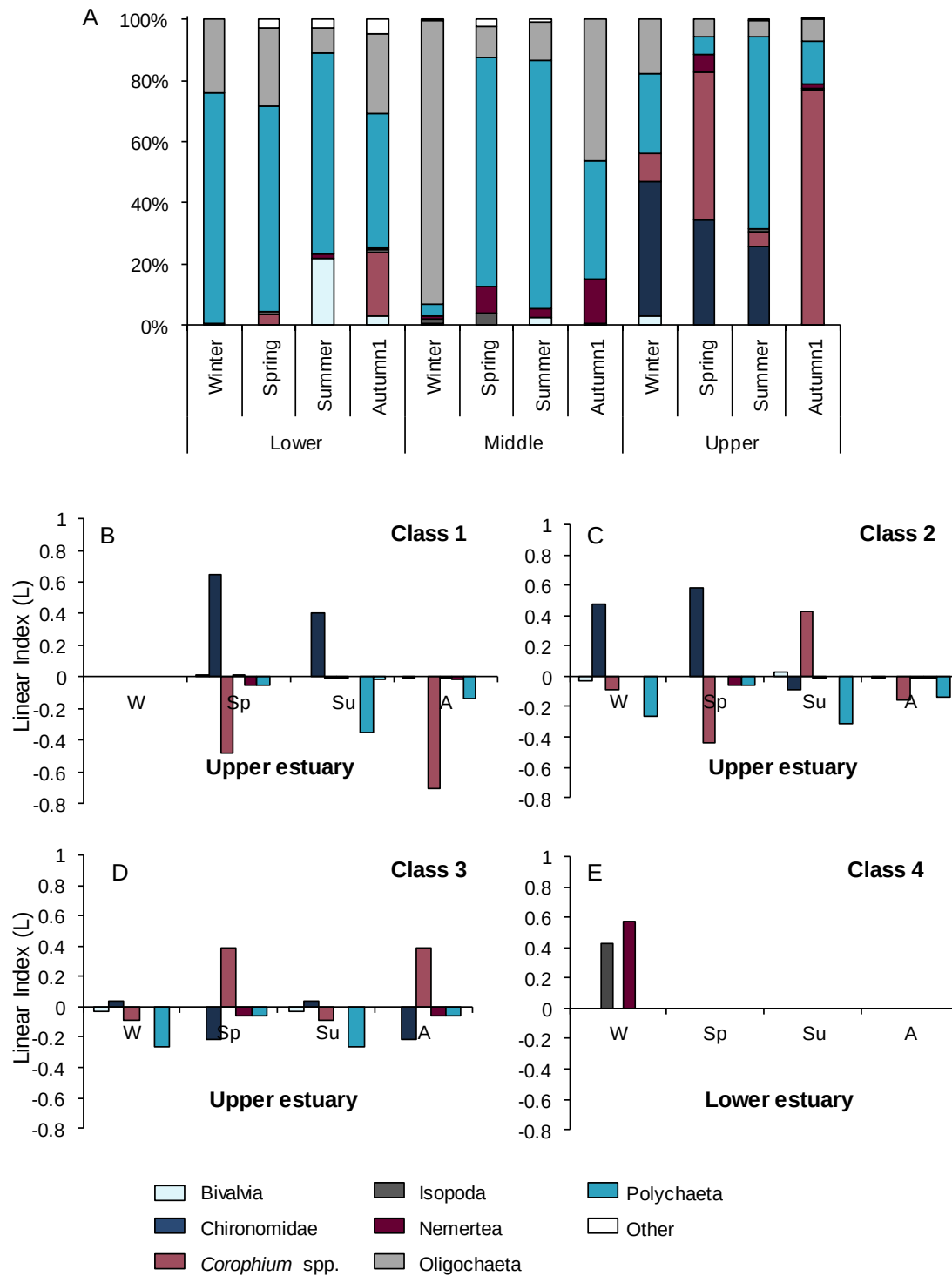


Figure 3.5. Seasonal abundance of macrobenthos prey in the Lima estuary (A); Strauss linear index values for the main prey items of *P. fesus* size classes: 1 (B), 2 (C), 3 (D) and 4 (E)(W- winter; Sp- spring; Su- summer; A- autumn).

Generalized Linear models showed that salinity ($p < 0.05$) together with the interaction between Chironomidae and *Corophium* spp. ($p < 0.05$, Table 3.4.) were the main factors associated with the 0-group flounder abundance in the Lima estuary. Accordingly, higher densities of 0-group flounder were observed in the upper estuary where both Chironomidae and *Corophium* spp. were more abundant than in the other areas where only *Corophium* spp. or none of these main prey were present. Chironomidae were also significantly ($p < 0.05$) associated with these flounder, although with a minor contribution to percentage of deviance explained by the model (Table 3.4.). The model presented an explanatory value of 62% of variability.

Table 3.4. Statistics for the Gamma regression models fitted to 0-group *P. flesus* densities in the Lima estuary (residual deviance, deviance, percentage of the total deviance explained by each factor and p value).

	Residual deviance	Deviance	% explained	p value
NULL	26.31			$p < 0.05$
Salinity	15.99	10.32	39.23	$p < 0.05$
<i>Corophium</i> spp.	15.97	0.02	0.06	$p = 0.84$
Chironomidae	15.85	0.12	0.46	$p < 0.05$
<i>Corophium</i> spp. : Chironomidae	9.95	5.90	22.42	$p < 0.05$
TOTAL			62.17	

3.4. Discussion

The main prey items of flounder juveniles in the Lima estuary included the macroinvertebrates Chironomidae and *Corophium* spp.. Chironomidae commonly occurs in the flounder diet (Nissling *et al.*, 2007), particularly of smaller juveniles (Weatherley, 1989; Florin and Lavados, 2010). Indeed, Chironomidae dominated the diet of the newly settled juveniles (Class 1) in the Lima estuary and was also a major item of classes 2 and 3. *Corophium* spp. was an important item across all size classes of juveniles, similarly to several studies (Summers, 1980; Hampel *et al.*, 2005), including in Portuguese estuaries (Costa and Bruxelas, 1989; Vinagre *et al.*, 2005). Moreover, the inclusion of Elmidae in the diet of classes 1 and 2 emerged as a characteristic behaviour of juvenile flounder of the Lima estuary, since no reports of Elmidae integrating the diet of flounder were found in other studies. Paradoxically, this taxon was absent from the macroinvertebrate community. Since these organisms are typical of freshwater environments, juveniles could have been feeding further upstream of the sampling area.

Although polychaetes dominated the macroinvertebrate community in the Lima estuary, they were only present as minor prey items of all flounder size classes. The large size and burrowing ability of polychaetes represents a high handling time and energy cost that may limit their capture by the small flounder (Vinagre *et al.*, 2008). However, polychaetes were not an important item of older 0-group juveniles (Class 3) as reported elsewhere (Vinagre *et al.*, 2008; Selleslagh and Amara, 2015). Specifically, the polychaete *Hediste diversicolor* and oligochaetes were totally absent in the diet of flounder juveniles, despite being dominant taxa in the macroinvertebrate community and common prey of juvenile flounder (Costa and Bruxelas, 1989; Weatherley, 1989; Hampel *et al.*, 2005). While the burrowing behaviour of oligochaetes may prevent their capture by the flounder juveniles (Andersen *et al.*, 2005b), *H. diversicolor* is active on the sediment surface (Muus, 1967; Fauchald and Jumars, 1979; Scaps, 2002). Moreover, *H. diversicolor* individuals found in the Lima macroinvertebrate community were within the range of the prey consumed by all the juvenile classes, including the newly settled. Thus, the present results seem to indicate that prey size was not the reason for its absence in the flounder diet. Gastropods *E. truncata* and *P. jenkinsi*, although common items in the diet, were not observed in the benthic samples during

this study. However, in the context of other studies, these species have been frequently observed in the Lima estuary (data not shown), explaining their occurrence in the flounder diet.

A general increase of prey length was observed along flounder juvenile development, a pattern commonly associated with the increase of the mouth gape width (Keast and Webb, 1966; Dörner and Wagner, 2003; Juanes *et al.*, 2008). Noteworthy, the prey consumed by 1-group juveniles did not present a significantly ($p > 0.05$) higher total length in comparison to the prey consumed by the newly settled flounder. This feature might have been a consequence of the low number of prey analysed for this class and their advanced digestion state that prevented the measurement of larger prey such as Teleostei and unidentified crustaceans that were also fragmented. Moreover, despite the ability to ingest larger prey, larger flounder diet continued to include small food items (Vinagre *et al.*, 2008) such as Nemertea. The increase in vacuity also observed along the size classes suggests a higher feeding activity of the smaller flounder, as they tend to present higher consumption rates when compared to the older ones (Fonds *et al.*, 1992). Fish consumption rates depend on the energy content, particle size and conversion efficiency of their prey (Brett and Groves, 1979; Bowen *et al.*, 1995; Arrington *et al.*, 2002). Accordingly, the consumption of larger and energetically more profitable prey by the older juveniles may have enabled an increase of the feeding intervals. Furthermore, the ingestion of larger prey may also explain why older juveniles (Class 4) presented such reduced number of prey items and similar condition factor of the younger flounder. These results suggest that fishes maintained the same nutritional state throughout their juvenile phase. Moreover, the flounder condition in the Lima estuary ($k = 1.0$) was within the range of the results obtained for other European estuaries ($k_{\min} = 0.73$, $k_{\max} = 1.20$, Amara *et al.* 2009), but slightly higher than for other Iberian estuaries, namely Minho, Douro and Mondego ($k = 0.73$) (Vasconcelos *et al.*, 2009).

Flounder are capable of an adaptive feeding behaviour, optimizing the use of the food resources (Andersen *et al.*, 2005b). In fact, flounder feeding becomes more specialized when prey are highly available, as in sandy habitats, in contrast to vegetated habitats (Andersen *et al.*, 2005b). The upper section of the Lima estuary, where most of juveniles concentrated, was mainly composed of gravel, being the vegetation scarce.

Thus, the low diversity of the flounder juveniles diet may have resulted from the presence of *Corophium* spp., an active and abundant prey that has been indicated as main item in these types of habitats (Grønkjær *et al.*, 2007). Nevertheless, diet diversity remained within the range of values reported for flounder juveniles (Aarnio *et al.*, 1996; Andersen *et al.*, 2005b; Hampel *et al.*, 2005). The diet diversity was particularly low for the newly settled juveniles (Class 1), since their diet was mostly based on Chironomidae (NI= 92.4%), and increased along the size classes, in agreement with other studies (Aarnio *et al.*, 1996). This feature might reflect the ability of larger fish to ingest larger and consequently, a wider range of prey. However, the high diversity of the class 4 diet needs to be interpreted with caution, since the stomach contents analyzed comprised only 20 prey items belonging to 5 taxa. Thus, the high diet diversity of class 4 resulted from an equal contribution of the prey items in the diet and should not be interpreted as more diverse in terms of including more prey items.

The diet of 0-group juveniles of classes 2 and 3 denoted a relevant diet overlap. Indeed, *Corophium* spp. and Chironomidae in terms of NI, and *C. crangon* in terms of WI comprised the main prey items of these classes. The diet overlap may be the result of a common distribution pattern along the middle and upper estuaries where the same kind of prey items was available. The diet of 1-group juveniles (Class 4) differed from the young flounder diet, indicating an ontogenetic shift in the diet. In the nursery grounds, where high densities of flatfish juveniles of different species can occur, both inter- and intraspecific competition may arise (Martinsson and Nissling, 2011). Ontogenetic shifts in the diet have been reported (Florin and Lavados, 2010), enabling resource partitioning between different life stages, and minimizing niche overlap, hence reducing intraspecific competition. The diet shift observed for 1-group juveniles resulted from the incorporation of new items such as *C. maenas*, Nemertea and Teleostea. Moreover, this feature may be related to the ability of older juveniles to consume larger prey, as the diet shift was coincident with an important increase of the prey size of the larger 0-group (Class 3). Uzars *et al.* (2003) reported a similar diet shift to large amphipods, decapods, and fishes, but at a lower fish size (90-150 mm TL). Noteworthy, Nemertea which is a small food item was not observed in the diet of smaller classes. This item was included in the diet of flounder juveniles caught in the lower estuary, while the smaller juveniles were concentrated in the upper estuary where other small prey, namely Chironomidae and *Corophium* spp. were more

abundant. Thus, the ontogenetic shift in the diet may also have been driven by a partial spatial partitioning of 0-group and 1-group flounder in the Lima estuary.

A relationship between flounder juvenile distribution and salinity, sediment characteristics and prey availability was observed in several Portuguese estuaries, using GLM (Vasconcelos *et al.*, 2010). In the Lima estuary, the spatial distribution of flounder juveniles was also negatively correlated with salinity (Table 2.4). Indeed, younger juveniles (Class 1 and most of Class 2) were restricted to the upper estuary in agreement with Ramos *et al.* (2010), suggesting a preference for low salinity waters (Bos and Thiel, 2006). Furthermore, older juveniles (classes 3 and 4) assumed a broader distribution throughout the estuary as they developed as regularly observed in other estuarine habitats (Kerstan, 1991). However, salinity tolerance may not be sufficient to explain these distribution patterns, as older 0-group and 1-group flounder have also been commonly found in estuarine low salinity areas (Martinho *et al.*, 2007; Freitas *et al.*, 2009). Moreover, laboratory experiments showed that growth (Gutt, 1985) and condition (Gutt, 1985; O'Neill *et al.*, 2011) of newly settled were not enhanced in low salinity conditions, suggesting that other factors such as high food supply, low predation, and competition (Beaumont and Mann, 1984; Bos, 1999), may also be related to the newly settled preference for upstream areas. Accordingly, a relationship between prey abundance and the distribution of flatfish juveniles has been described (Le Pape *et al.*, 2007; Vinagre *et al.*, 2009b), including for flounder (Vasconcelos *et al.*, 2010). Furthermore, environmental variables such as sediment composition and salinity may only act indirectly (Gibson *et al.*, 2005), by influencing the distribution of the macroinvertebrate prey (Gibson, 1994; McConnaughey and Smith, 2000; Amezcua and Nash, 2001). In fact, Ramos *et al.* (2010) showed that the spatial distribution of *P. flesus* juveniles was related to the sediment composition in the Lima estuary possibly through its effect on prey abundance. Indeed, the 0-group flounder distribution in the Lima estuary was also related to the abundance of the main prey Chironomidae and *Corophium* spp. (Table 2.4), highly abundant and characteristic of the upper estuary. Chironomidae is typical of freshwater, often being the dominant group of insects in these environments (Armitage *et al.*, 1995). On the other hand, *Corophium* spp. is highly tolerant to a wide range of salinities, from 2 to 50 (McLusky, 1967), and in the Lima estuary, *Corophium multisetosum*, one of the recorded species, showed a preference for low salinities (Sousa *et al.*, 2007b). Thus,

although highly available prey such *Corophium* spp. were present throughout the estuary, the 0-group flounder concentrated in the upper estuary where abundances of this prey were higher and Chironomidae was also available. In conclusion, salinity was the main environmental driver of the juvenile flounder spatial distribution, which jointly with high food availability of the upper section of the Lima estuary, explained the concentration of the newly settled flounder in that section of the estuary. Our results here showed that both abiotic and biotic factors affect the suitability of fish habitats. However, the contribution of these prey to the 0-group flounder distribution model may be related to their co-occurrence in the upper estuary, especially when considering the feeding opportunist behaviour reported for flounder (De Groot, 1971; Martinho *et al.*, 2008), and the high densities of Chironomidae and *Corophium* spp. in the upper estuary. Further studies are required to test these hypotheses, by comparing areas with similar salinity and distinct *Corophium* spp. and Chironomidae abundances, a comparison that was not possible to find in our dataset of the Lima estuary.

Variations of the proportions of the main prey items in the diet of flounder juveniles, namely Chironomidae and *Corophium* spp. tended to reflect fluctuations in the Lima estuarine macroinvertebrate community. The diet of class 1 presented a high proportion of Chironomidae during Spring and Summer when this item was highly available in the macroinvertebrate community. Nevertheless, Chironomidae abundances may be higher than those reported, as these organisms are also present in the bottom water column (Walton, 1979) where newly settled may feed. As a result, an overestimation of Chironomidae selection may have occurred. On the other hand, the decrease of *Corophium* spp. in the diet of class 2 coincided with its peak abundance in the macroinvertebrate community during autumn. At this time of year, Chironomidae were not observed in the macroinvertebrate community, whereas Elmidæ emerged in the diet as a major item. As stated above, Elmidæ was not observed in the macroinvertebrate community, and therefore prey selection for this item was not evaluated. Hence, seasonal variations of the main prey items revealed that juvenile flounder adapted their diet according to the availability of prey (Aarnio *et al.*, 1996), feeding on abundant items.

3.5. Conclusions

The present study showed that:

- (1) Juvenile European flounder in the Lima estuary fed mainly on macroinvertebrates, and the diet became more diverse as the juveniles grew, including other groups and larger size prey;
- (2) 0-group European flounder fed on highly available items (Chironomidae and *Corophium* spp.), exhibiting a highly specialized diet;
- (3) The newly settled flounder were restricted to the Lima upper estuary, and fed almost exclusively on Chironomidae and *Corophium* spp, typical species of this estuarine section.
- (4) An ontogenetic shift in the diet occurred from 0-group to 1-group juveniles.
- (5) Salinity and prey Chironomidae and *Corophium* spp. were the main factors associated with 0-group flounder distribution in the Lima estuary.

CHAPTER 4

Feeding strategies and condition of
juvenile European flounder

Platichthys flesus in a nursery habitat

Abstract

Estuarine habitats are major nurseries for the European flounder *Platichthys flesus*, with different year classes sharing food and space resources. Hence, an understanding of feeding strategies that optimize resource use and sustain nursery carrying capacity is fundamental for sustained and successful ecosystem management. The main feeding areas of European flounder juveniles were investigated in the Lima estuary (northern Portugal) nursery ground by integrating stomach contents with stable isotopic values and fish condition. The 0-group flounder presented the lowest $\delta^{13}\text{C}$ value ($-25.58 \pm 1.86\text{‰}$) that was associated with the upper estuary, while the 1-group flounder exhibited a higher $\delta^{13}\text{C}$ value $-22.59 \pm 2.51\text{‰}$ related with the lower and middle sections. The two European flounder groups did not differ in terms of $\delta^{15}\text{N}$, with the 0-group juveniles showing an average of $13.93 \pm 0.29\text{‰}$, and 1-group flounder with $13.50 \pm 0.96\text{‰}$. The upper estuary was the main feeding area of 0-group flounder (74%), while 1-group flounder fed along the estuary both upstream (52%) and downstream (48%). The flounder juveniles presented good individual condition based on Fulton K (0-group: 1.05 ± 0.08 ; 1-group: 1.07 ± 0.05) and RNA:DNA (0-group: 1.70 ± 0.70 ; 1-group: 1.41 ± 0.47). The relationship between stable isotopes and condition indices was investigated, based on the assumption that stable isotope ratios reflected food use with lower $\delta^{13}\text{C}$ and higher $\delta^{15}\text{N}$ associated to feeding on the upper estuary. Results showed that 0-group flounder with higher $\delta^{13}\text{C}$ and lower $\delta^{15}\text{N}$ presented higher RNA:DNA indicating that the feeding strategy affected the recent condition of the individuals. The condition of 1-group flounder did not vary with stable isotopes, despite the variability in feeding locations. It is concluded that the different feeding strategies supported condition of the flounder juveniles in the nursery habitat.

4.1. Introduction

Estuaries are highly productive systems (McLusky and Elliott, 2004) that support the nursery function for many fish species (Kerstan, 1991; Beck *et al.*, 2001; Potter *et al.*, 2015). In theory, early life stages of fishes find suitable conditions in nursery habitats that enhance body condition, growth and survival, such as high food availability and shelter from predation (Boesch and Turner, 1984; Gibson, 1994; Pihl *et al.*, 2007). Ultimately, these conditions promote recruitment and therefore maintenance of the adult populations (Gibson, 1994; Rijnsdorp *et al.*, 1995; Beck *et al.*, 2001). However, there has been a high historical loss of estuarine areas and habitats (Wolanski and Elliott, 2015) which now, through management measures, is having to be remedied (Elliott *et al.*, 2016). Hence it is important to know whether food and space resources have diminished for fishes and whether this has in turn decreased the carrying capacity in terms of the maximum number of individuals or biomass that can be ecologically supported by an area (Elliott *et al.*, 2007a).

The dispersal of juveniles supports ecological interactions between the different units that comprise nursery habitats, ensuring connectivity (Sheaves, 2009; Palmer *et al.*, 2014). Losses in connectivity may decrease trophic interactions and lead to food web fragmentation, compromising the system resilience and nursery function (Vinagre *et al.*, 2011; Selleslagh *et al.*, 2015). Similarly, a loss of connectivity could increase competition for scarce resources within an area. Moreover, distribution patterns will reflect abiotic factors, such as temperature, salinity, depth, sediment type (Power *et al.*, 2000; Andersen *et al.*, 2005a; Vasconcelos *et al.*, 2010), and biotic factors such as prey availability, predation and competition (van der Veer *et al.*, 2000; Darnaude *et al.*, 2001; Amara *et al.*, 2009; Sheaves *et al.*, 2015). In particular, prey availability may affect the condition and growth of juveniles (Cabral *et al.*, 2002; Amara *et al.*, 2009; De Raedemaeker *et al.*, 2012b) and hence nursery value. Therefore, migrations to areas with high prey availability may either optimize resource use or prevent food limitation, hence minimizing competition (Tableau *et al.*, 2016), and this migration may shape habitat and resource partitioning between different life stages and species in nursery habitats (Darnaude *et al.*, 2001; Russo *et al.*, 2008; Vinagre *et al.*, 2009b). Apart from migration, other resource use strategies include feeding on highly abundant prey (Molinero and Flos, 1992; Vinagre *et al.*, 2005), ontogenetic shifts in the diet (Aarnio

et al., 1996; Kopp *et al.*, 2013) and temporal differences on habitat use (Cabral and Costa, 1999).

Feeding strategies based on stomach contents analysis offer a snapshot of the fish diet (Hyslop, 1980; Marshall and Elliott, 1997), which can be combined with stable isotope information on a wider temporal scale (DeNiro and Epstein, 1978; Minagawa and Wada, 1984; Peterson and Fry, 1987) to give greater insight into trophic relationships. Carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) stable isotope ratios provide information on the diet composition of consumers (DeNiro and Epstein, 1978; Fry and Sherr, 1989), while $\delta^{15}\text{N}$ can also give trophic position (Deniro and Epstein, 1981; Post, 2002). Recently, stable isotope mixing models (Parnell *et al.*, 2013; Phillips *et al.*, 2014) have been used to identify the main prey and organic matter sources (Le Pape *et al.*, 2013; Hoffman *et al.*, 2015), and to characterize trophic niche widths and niche overlap between species (Kostecki *et al.*, 2012; Vaslet *et al.*, 2015). This information may then be integrated to track movements in variable temporal and spatial scales, and identify key habitats regarding food use (Green *et al.*, 2012; Kopp *et al.*, 2013; Selleslagh *et al.*, 2015).

Fish condition indices provide a valuable tool to assess nursery habitat quality (Gilliers *et al.*, 2004; Fonseca *et al.*, 2006; De Raedemaeker *et al.*, 2012b) by measuring individual somatic nutritional status and hence the potential for growth and reproduction. These indices, including morphometric and biochemical ones, respond to environmental conditions in various temporal scales (Suthers *et al.*, 1992; Fonseca *et al.*, 2006). The Fulton condition factor, K (Ricker, 1975) is a morphometric index widely used to measure fish somatic condition (Amara *et al.*, 2009; Vasconcelos *et al.*, 2009; De Raedemaeker *et al.*, 2012b), considering that for a given length, the heavier fish are in better condition. Complementary to this, the biochemical RNA:DNA ratio indicates recent fish nutritional condition and growth (Clemmesen, 1994; Malloy and Targett, 1994; Chícharo and Chícharo, 2008). It assumes that the DNA content in a somatic cell remains constant, while the RNA concentration reflects the capacity for protein synthesis, responding to changing environmental conditions (Bulow, 1970; Buckley *et al.*, 1999), i.e. in a non-stressful situation, higher RNA:DNA ratio reflects higher somatic growth of the fish.

The flatfish European flounder *Platichthys flesus* (L. 1758) uses estuarine habitats along their geographical distribution as nurseries (Henderson and Holmes, 1991;

Kerstan, 1991; Martinho *et al.*, 2008), including the Lima estuary (northern Portugal) (Ramos *et al.*, 2010) where they are an important component of the estuarine fish community. It is of note that the NW Portuguese coast represents the southern limit distribution for flounder (Nielsen, 1986), with a recent decline in abundance associated with rising seawater temperatures (Cabral *et al.*, 2007). Moreover, food limitation has been linked to interspecific competition and higher metabolic demands in Southern European nurseries, resulting in reduced growth rates of flounder compared to higher latitudes (Freitas *et al.*, 2012).

As in other estuaries (Elliott and Hemingway, 2002), previous studies have showed that the spatial distribution of flounder in the Lima estuary varies with age (Ramos *et al.*, 2009; Mendes *et al.*, 2014). Young of the year flounder tend to aggregate in the upper estuary, while older flounder juveniles are more abundant in the middle estuary (Ramos *et al.*, 2009; 2010; Mendes *et al.*, 2014). The juveniles of this species typically feed on common prey such as polychaetes (Summers, 1980; Piet *et al.*, 1998; Martinho *et al.*, 2008) and amphipods (Aarnio *et al.*, 1996; Vinagre *et al.*, 2005; Mendes *et al.*, 2014). This work has led to the hypothesis that juvenile abundance and feeding are related within any nursery area in a manner to minimize competition through resource partitioning and ensure good growth and condition. In order to test this hypothesis, the present study aims to: 1) determine the main feeding areas using dietary indices and stable isotope mixing models, 2) assess flounder juveniles condition through morphometric and RNA:DNA indices, and 3) investigate the relationship between feeding strategies and condition of flounder juveniles in the Lima estuary. This information increases our understanding of the feeding use of flounder in a nursery system near its southern geographic distribution limit, information needed for better management decisions.

4.2. Material and Methods

4.2.1. Study area

The Lima estuary, NW Atlantic coast of Portugal, is a small open estuary with a semidiurnal and mesotidal regime (3.7 m). Salt intrusion can extend up to 20 km upstream, with an average flushing rate of 0.4 m s^{-1} , and a residence time of 9 days (Ramos *et al.*, 2006b). This study sampled at five stations covering the lower, middle, and upper estuary (Figure 4.1.). The lower estuary (station 1, average depth of 4.9 m), located in the first 2.5 km, is a narrow, 9-m deep navigational channel, industrialized, with walled banks, including a shipyard, a commercial seaport, and a fishing harbour; the middle estuary (stations 2-3, average depth 5.1 m) comprises a broad shallow intertidal saltmarsh zone, mainly colonized by the common rush (*Juncus* spp.); the upper estuary (Stations 4-5, average depth 2.1 m) is a narrow shallow channel, less disturbed, with natural banks and small exposed sandbanks (Ramos *et al.*, 2010).

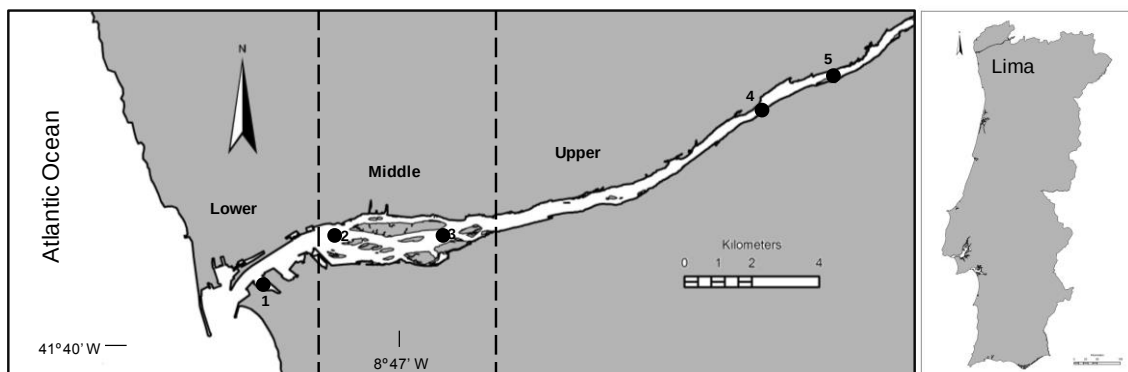


Figure 4.1. Lima estuary with the location of the sampling stations (1- lower estuary; 2 and 3 – middle estuary; 4 and 5 - upper estuary).

4.2.2. Data collection

European flounder juveniles, macroinvertebrates, sediment and water samples were collected in August 2013. This sampling date ensures that 0-group flounder have reached isotopic equilibrium to the estuarine habitats considering that this process could take weeks to months (Herzka, 2005) following the typical late spring estuarine colonization (Martinho *et al.*, 2008; Ramos *et al.*, 2009). Bottom water samples for

particulate organic matter (POM) analysis were collected at each sampling site with a Van Dorne bottle. Samples for sediment organic matter (SOM) and macroinvertebrate analysis were retrieved at each station with a Petit Ponar grab of area 0.023 m². European flounder juveniles, as well as the shore crab *Carcinus maenas* were collected during two nightly ebb tides with a 2 m beam trawl, with a cod-end 5 mm mesh, and a tickler chain, and sorted immediately. All samples were kept on ice until further processing in the laboratory. The geographic location of each sampling station was recorded with a Magellan 315 GPS, and vertical profiles of water temperature and salinity were obtained using a multi-parameter probe YSI 6820.

4.2.3. Stomach content analysis

Fishes were measured for total length (TL, ± 1 mm) and wet weight (WW, ± 0.01 g). European flounder juveniles were separated according to the total length at first sexual maturity 200 mm; Dinis, 1986). The stomachs were excised, and the contents removed and preserved in 70% alcohol. The prey items were identified to the highest taxonomic level possible using a binocular microscope (Leica MZ12-5), counted, and weighed (WW, ± 0.001 g) after blotting on a tissue paper (Mendes *et al.*, 2014).

4.2.4. Stable isotope analysis

POM samples were obtained by pre-filtering 1 L of water sample through a 200 μ m nylon mesh to remove zooplankton and debris and then through pre-combusted GF/F glass fibre filters (Harmelin-Vivien *et al.*, 2008; Suzuki *et al.*, 2008). Filters were acidified with 10% HCl for carbonate removal (Vizzini *et al.*, 2002; Kennedy *et al.*, 2005) which is $\delta^{13}\text{C}$ enriched compared to organic carbon (DeNiro and Epstein, 1978), dried at 60 °C for 48h, and stored at -80 °C. Sediment samples for SOM analysis were dried at 60 °C and ground to a fine powder. In addition, SOM samples for $\delta^{13}\text{C}$ analysis were acidified with 10% HCl to remove carbonates, and dried at 60 °C. Samples were stored in a desiccator, until further analysis. Bivalvia, *Carcinus maenas*, Chironomidae, *Corophium* spp., Gastropoda, Isopoda and Polychaeta were considered as main prey groups (Vinagre *et al.*, 2005; Martinho *et al.*, 2008; Mendes *et al.*, 2014) and sorted from the sediment samples. Whole individuals were used for prey stable isotope analysis, except for larger prey as *C. maenas* for which muscle tissue was collected from the claws. Dorsal white muscle was removed from the European flounder juveniles. Prey and fish samples were kept frozen (-80 °C) until analysis. Prior to stable

isotope analysis, animal tissue samples were dried and ground to a fine powder using a mortar and pestle. *Corophium* spp. and gastropods were acidified (10% HCl) to remove carbonates (Ng *et al.*, 2007; Selleslagh *et al.*, 2015) from the samples for $\delta^{13}\text{C}$ analysis. Carbon and nitrogen stable isotope analysis was performed on individual samples of fish and crabs. For other prey groups, several individuals were pooled in order to have sufficient material for analysis. Ratios of $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ in each sample were determined by continuous flow isotope mass spectrometry (CF-IRMS) (Preston and Owens, 1983) using a Thermo Scientific Delta V Advantage IRMS via a ConFlo IV interface (Interdisciplinary Centre of Marine and Environmental Research—CIIMAR- University of Porto). The delta (δ) notation was used to express the stable isotope ratios as ppt differences from a standard reference material:

$$\delta X (\text{‰}) = (R_{\text{sample}} \times R_{\text{standard}}^{-1} - 1) \times 10^3$$

where X is ^{13}C or ^{15}N and R is the ratio of $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$. Isotope ratios were measured relative to the international standards of PeeDee Belemnite for carbon and atmospheric N_2 for nitrogen. Analytical precision (standard deviation) was $\pm 0.2 \text{ ‰}$ of reference material for carbon and nitrogen.

4.2.5. Condition analysis

The individual condition of the European flounder juveniles was assessed through the Fulton index and RNA:DNA ratio indices. The Fulton condition factor, K (Ricker, 1975) was determined following the formula:

$$K = W \times (TL^3)^{-1} \times 100 ,$$

where W is the wet weight (mg) and TL is the total length (mm). For the RNA:DNA, dorsal white muscle samples from European flounder juveniles were preserved in liquid nitrogen upon collection and kept at -80 °C until analysis. Prior to analysis, muscle samples were homogenised in 500 μl TEN-SDS buffer (0.05 M Tris, 0.01 M EDTA, 0.1 M NaCl, 0.01% SDS, pH 8). RNA:DNA was determined for three replicate samples of dorsal white muscle (10 mg) of each juvenile flounder, by the fluorometric method described in Caldarone *et al.* (2001) and modified by De Raedemaeker *et al.* (2012b). Fluorescence was measured on a Fluoroscan Ascent FL microplate reader with 530 nm excitation wavelength and 590 nm emission wavelength. RNA and DNA concentrations were determined based on standard curves prepared with baker's

yeast RNA (Sigma) and pure calf-thymus DNA (Sigma). The RNA:DNA was determined as the ratio between average RNA and DNA concentrations of each sample. The ratio between the slopes of RNA and DNA standard curve was of 2.5 which can be used as standardization factor for inter-calibration with other studies (Caldarone *et al.*, 2006).

4.2.6. Data analysis

European flounder juveniles were separated according to age class based on minimum and maximum total length for each class: <150 mm for 0-group flounder and <200 mm for 1-group flounder (Dinis, 1986). For each group, feeding activity was evaluated by the vacuity index (Iv), defined as the percent of empty stomachs (Hyslop, 1980). Key prey were identified based on the numerical (NI), and weight (WI) percentages of each prey item in the diet (Hyslop, 1980). Differences of $\delta^{13}\text{C}$ between POM and SOM sources, and across estuarine sectors (lower, middle and upper) were tested with a two-way ANOVA coupled with a Tukey post-hoc test. Data were log-transformed in order to meet parametric assumptions. The high $\delta^{15}\text{N}$ variability between replicates for POM and SOM sources, possibly linked to the low nitrogen content of the samples, did not allow the use of this isotope to assess POM and SOM sources. Principal Coordinates Analysis (PCO) was carried out on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ prey data to investigate patterns among estuarine sectors and prey groups. Differences of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ between prey were tested with a permutational multivariate analysis of variance (PERMANOVA), using prey groups as a nested factor within estuarine sector. Pair-wise tests between levels of the estuarine sector and prey groups within sectors were performed in a separate PERMANOVA routine. Multivariate dispersion was tested with the PERMDISP routine. The PCO, PERMANOVA, and PERMDISP analyses were based on the Euclidean distance dissimilarity matrix. For each European flounder group, correlations were determined between total length and isotopic values of carbon and nitrogen. Differences of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ between flounder groups were tested with a PERMANOVA. PCO and PERMANOVA were performed with PRIMER v6.1 (Primer-E Ltd, UK), and PERMANOVA+1.0.1 add-on software (Clarke and Gorley, 2006; Anderson, 2008).

The main feeding locations of the flounder juveniles were investigated with stable isotope mixing models, using the SIAR package in R software (Parnell *et al.*, 2010).

Prey $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ data (mean $\pm$ standard error) were sorted according to estuarine area classification based on POM and SOM isotopic results, namely *upstream* (comprising the upper estuarine sector) and *downstream* (including the lower and middle sections), see below section 3.2.1. *Organic matter sources*). These data were used to assess prey relative contribution to the diet of each flounder group. One general set of trophic enrichment factors (TEF), 1‰ for $\delta^{13}\text{C}$ and 3.4‰ for $\delta^{15}\text{N}$ per trophic level, was used based on a meta-analysis from the scientific literature, and with an associated standard error of ± 0.5 (Kostecki *et al.*, 2012).

For each flounder group, Pearson correlations between total length, Fulton K and RNA:DNA were determined in order to investigate a potential length effect on condition and for links between the condition indices. Differences in condition indices between flounder age groups were investigated with a t-test. Based on the assumption that stable isotopes reflect food use, linear regressions between each condition index and $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were applied to each flounder group in order to investigate how food use affected juveniles condition. Data and residuals were plotted and inspected for trends in order to assess if the underlying assumptions of independence, linearity, homocedasticity, and normality were met. A significance level of 0.05 was used for all the statistical analyses that were performed using R software (R Development Core Team, 2007).

4.3. Results

4.3.1. Stomach contents analysis

A total of 42 juveniles were collected in the Lima estuary, comprising two age groups: 0-group flounder (n= 22) and 1-group flounder (n= 20) (Table 4.1). All the 22 0-group flounder were caught in the upper estuary (Table 4.1.), except two juveniles that were collected in the middle estuary. The 20 1-group flounder were collected from the middle estuary (Table 4.1.). The 0-group flounder presented a lower vacuity index (8%) compared to the 70% of the 1-group flounder.

Table 4.1. Number (n) of 0-group and 1-group European flounder sampled in the Lima estuary, mean total length (mm), total weight (g), Fulton's K, RNA:DNA and muscle carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) stable isotope signatures (‰).

Flounder	n	TL (mm)	WW (g)	K	RNA:DNA	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)
0-group	22	75±20	5.43	1.05±0.08	1.70±0.70	-25.58±1.86	13.93±0.29
1-group	20	163±10	46.96	1.07±0.05	1.41±0.47	-22.59±2.51	13.50±0.96

The diet of the 0-group flounder was dominated by the amphipod *Corophium* spp. (NI = 94%, WI= 88%), followed by the polychaete *Hediste diversicolor* that reached 12% of the WI (Figure 4.2.a). Other prey such as *Crangon crangon*, Chironomidae, Mysidae, and Oligochaeta were also identified as minor items (less than 1%). The diet of the 1-group flounder comprised mainly polychaetes (NI= 48%; WI= 69%). Isopoda (NI= 20%, WI= 3%) and *C. crangon* (NI= 16%, WI= 17%) were also important prey items, in terms of number and weight, respectively (Figure 4.2.b). Other items of the 1-group flounder diet included Bivalvia, *Carcinus maenas*, and *Corophium* spp. Both flounder groups also had plant debris and sand in their stomach contents.

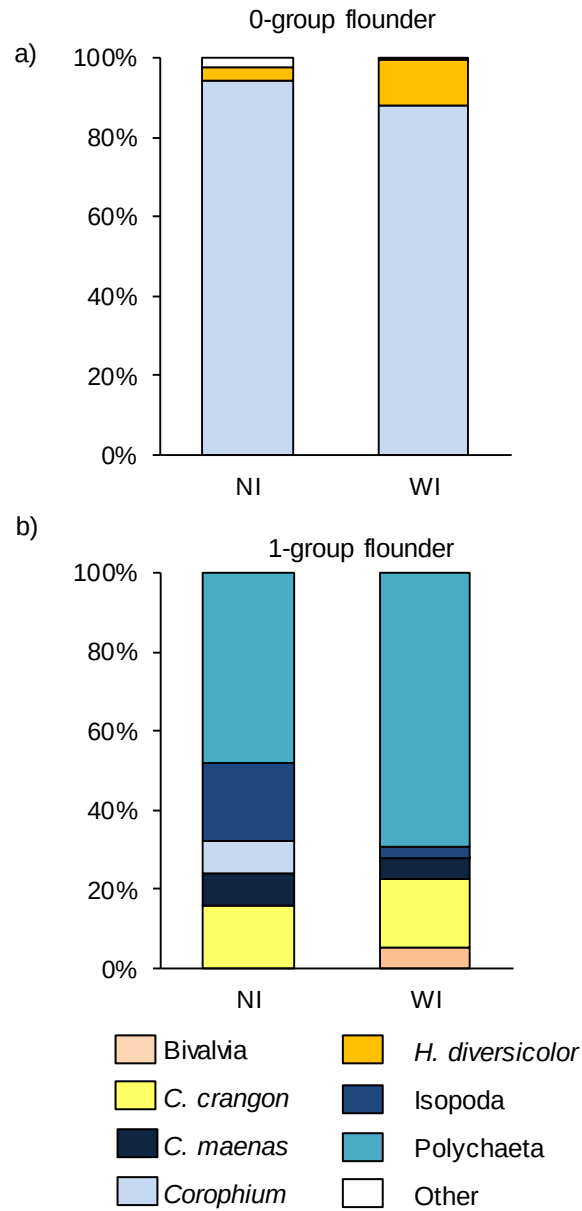


Figure 4.2. Numerical (NI) and weight (WI) indices for stomach contents of a) 0-group and b) 1-group European flounder in the Lima estuary.

4.3.2. Stable isotope analysis

Organic matter sources

The POM and SOM $\delta^{13}\text{C}$ signature varied significantly between the three estuarine sectors (two-way ANOVA; $F = 11.1$, $p < 0.05$). The upper estuary presented a depleted carbon signature ranging from -32.66 to -25.55‰ , compared to the lower and middle estuaries with a $\delta^{13}\text{C}$ varying from -24.57 to -19.45‰ . This variation was consistent with the salinity gradient, as the oligohaline upper estuary showed an average salinity of 7.2 ± 5.4 , while the lower and middle estuaries had average salinities of 29.9 ± 0.1 and 29.5 ± 0.3 , respectively. Thus, samples were grouped as *upstream* (upper estuarine samples) and *downstream* (lower and middle estuarine samples) (Table 4.2.). The POM and SOM carbon signatures of *upstream* and *downstream* sections did not vary significantly (two-way ANOVA; $F = 1.3$, $p \geq 0.05$) (Table 4.2.), across all samples.

Table 4.2. Mean carbon stable isotope $\delta^{13}\text{C}$ (‰) of particulate organic matter (POM) and sediment organic matter (SOM) sources in the upstream and downstream areas of the Lima estuary.

Source	$\delta^{13}\text{C}$ (‰)	
	Downstream	Upstream
POM	-23.62 ± 0.32	-28.88 ± 3.34
SOM	-23.15 ± 2.47	-26.11 ± 0.39

Prey

A total of 5 groups of macroinvertebrates were included in the stable isotope analysis: Chironomidae, *Corophium* spp., *C. maenas*, Gastropoda and Polychaeta. Samples from the upper estuary comprised Chironomidae, *Corophium* spp., and Polychaeta. The taxa *C. maenas* and Polychaeta from the lower and middle estuaries were analyzed, as well as Gastropoda from the lower estuary. Rare prey groups such as Bivalvia and Isopoda were not included due to insufficient material available for the stable isotope analysis. Stable isotope signatures varied across estuarine sectors (PERMANOVA, Pseudo-F= 12.08, $p < 0.05$) and between prey groups within each estuarine sector (PERMANOVA, Pseudo-F= 7.93, $p < 0.05$). Moreover, the PCO showed the upper estuary prey to have a distinct stable isotope signature (Figure 4.3.). This separation occurred mostly along the first ordination axis that explained 94.7% of the total variance of prey isotopic signatures, and was mainly associated to $\delta^{13}\text{C}$ ratios ($|r| = 0.99$). In contrast, differences within each sector were associated to $\delta^{15}\text{N}$ values ($|r| = 0.97$).

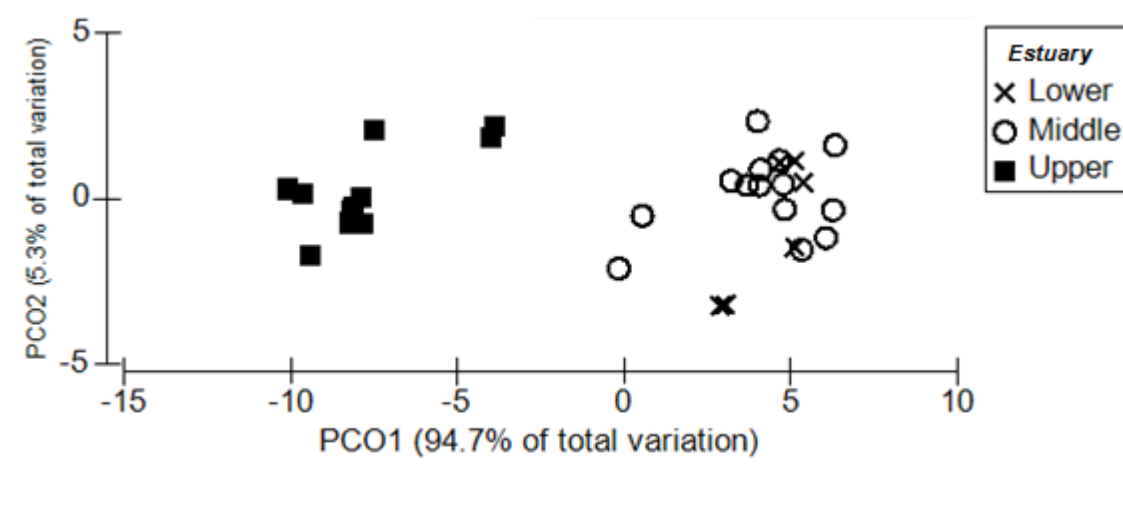


Figure 4.3. Ordination diagrams for the Principal Coordinate Analysis (PCO) performed on carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) stable isotope signatures of European flounder prey in the Lima estuary.

Based on these results, prey groups were further divided according to *upstream* (upper estuary), and *downstream* (lower and middle estuaries). Indeed, prey from the

upstream areas (*Corophium* spp., Polychaeta, and Chironomidae) presented generally lower $\delta^{13}\text{C}$ compared to other prey (Table 4.3.). In the downstream areas, *C. maenas* and gastropods presented the most enriched $\delta^{13}\text{C}$, while polychaetes presented the lowest $\delta^{15}\text{N}$ values (Table 4.3.). Further analysis followed this new sample classification to identify the main feeding areas of the European flounder juveniles.

Table 4.3. Mean carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) stable isotope signatures of the main prey groups of European flounder juveniles in the upstream and downstream areas of Lima estuary.

Group	Estuarine Zone	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$
Chironomidae	Upstream	-28.47 ± 0.05	13.05 ± 0.02
<i>C. maenas</i>	Downstream	-16.20 ± 0.93	12.32 ± 0.96
<i>Corophium</i> spp.	Upstream	-29.50 ± 0.90	10.50 ± 0.65
Gastropoda	Downstream	-15.58 ± 0.11	11.26 ± 1.38
Polychaeta	Downstream	-18.26 ± 2.46	9.54 ± 1.21
	Upstream	-24.92 ± 0.08	13.20 ± 0.22

European flounder juveniles

European flounder juveniles carbon ($p \geq 0.05$; 0-group flounder: $R = 0.16$; 1-group flounder: $R = -0.16$), and nitrogen ($p \geq 0.05$; 0-group flounder: $R = 0.04$; 1-group flounder: $R = 0.14$) did not vary significantly with total length. Therefore, it was not necessary to correct the data for length effects. The stable isotopes varied significantly between flounder groups (PERMANOVA, Pseudo- $F = 13.62$, $p < 0.05$). The 1-group flounder presented higher $\delta^{13}\text{C}$ and lower $\delta^{15}\text{N}$ values than the 0-group flounder (Table 4.1.).

4.3.3. SIAR outputs

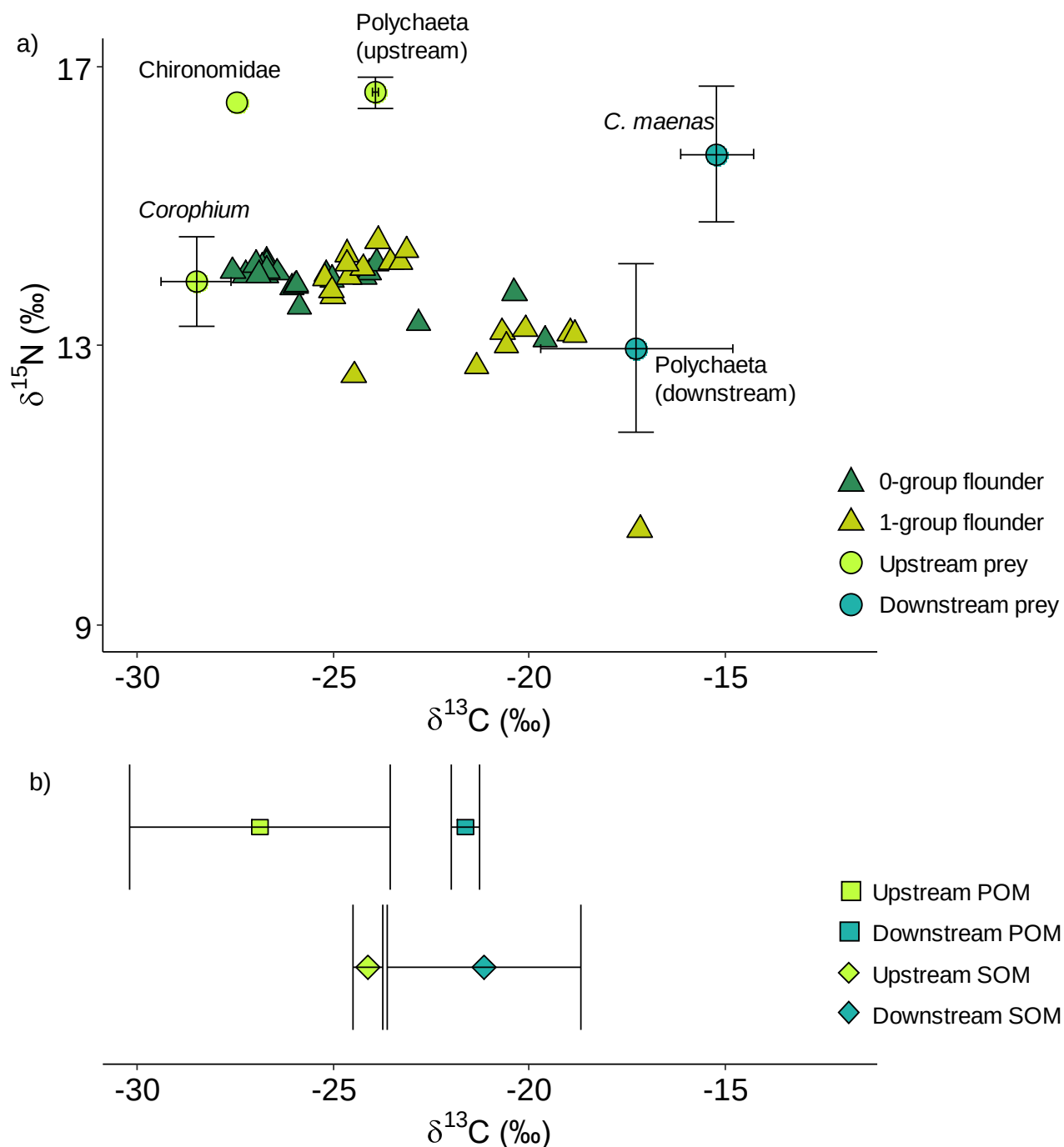


Figure 4.4. Carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) stable isotopes (‰) of a) 0-group and 1-group European flounder, and respective upstream and downstream prey; b) sediment (SOM, upstream and downstream) and water particulate (POM, upstream and downstream) organic matter sources. Trophic enrichment factors were applied to sources ($\delta^{13}\text{C}$: $\pm 2\text{‰}$), and to prey ($\delta^{13}\text{C}$: $\pm 1\text{‰}$, $\delta^{15}\text{N}$: $\pm 3.4\text{‰}$).

The diet of 0-group flounder depended primarily on *Corophium* spp. and other prey from the upper estuary (Figure 4.4.a), with a total of 74% contribution according to SIAR (Figure 4.5.a). However, some 0-group flounder presented higher $\delta^{13}\text{C}$ values (Figure 4.4.a), between -24 and -18 ‰, than expected if the diet depended only on the upper estuary resources; polychaetes from the downstream areas constituted 26% of the diet. The dual isotope plots displayed one cluster of 1-group flounder (Figure 4.4.b) with isotopic signatures similar to *Corophium* spp., and sources from upstream, while the remaining individuals presented more scattered values consistent with the sources and prey from downstream areas (Figure 4.4.b). Indeed, the SIAR model applied to 1-group flounder data showed that their diet relied equally on prey from the upstream (48%) and downstream areas (52%) (Figure 4.5.b). Specifically, polychaetes from downstream areas (46%) and *Corophium* spp. (42%), from upstream represented the main prey of 1-group flounder.

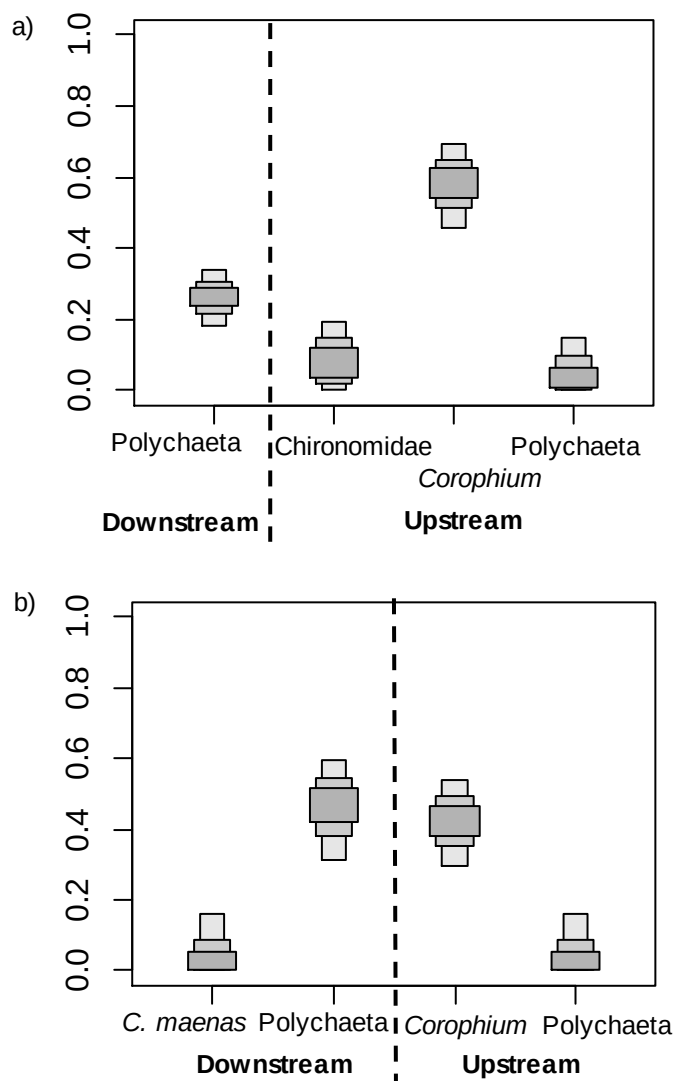


Figure 4.5. Boxplots of the mixing models estimates of prey contribution to the diet of a) 0-group and b) 1-group European flounder. Prey groups divided according to upstream and downstream areas.

4.3.4. Condition analysis

There was no correlation between flounder juveniles total length and Fulton K ($p \geq 0.05$, 0-group flounder: $R = 0.1$; 1-group flounder: $R = -0.25$) and RNA:DNA ($p \geq 0.05$, 0-group flounder: $R = 0.25$; 1-group flounder: $R = 0.21$). Therefore, no corrections for length effect were applied to the indices. There were no significant differences in condition between 0-group flounder and 1-group flounder (Table 4.1.), in terms of Fulton K (t-test, $t = -0.89$, d.f. = 40, $p \geq 0.05$) and also in terms of RNA:DNA (t-test, $t = 1.58$, d.f. = 40, $p \geq 0.05$) and the two condition indices Fulton K and RNA:DNA were

also not correlated ($p \geq 0.05$, 0-group flounder: $R = 0.06$, 1-group flounder: $R = -0.44$). The linear regression models (Figure 4.6.) showed no significant relationship between Fulton K and $\delta^{13}\text{C}$ ($p \geq 0.05$, 0-group flounder: $R^2 = -0.1$; 1-group flounder: $R^2 = -0.05$, Figure 4.6.a) and Fulton K and $\delta^{15}\text{N}$ ($p \geq 0.05$, 0-group flounder: $R^2 = -0.1$; 1-group flounder: $R = -0.1$, Figure 4.6.b). On the other hand, there was a significant relation between RNA:DNA of 0-group flounder and the signatures of $\delta^{13}\text{C}$ ($p < 0.05$, $R^2 = 0.30$) and $\delta^{15}\text{N}$ ($p < 0.05$, $R^2 = -0.28$). In particular, flounder juveniles with higher $\delta^{13}\text{C}$ (Figure 4.6.c) and lower $\delta^{15}\text{N}$ (Figure 4.6.d) presented higher RNA:DNA values. Regarding 1-group flounder, there were no significant relationships between RNA:DNA and $\delta^{13}\text{C}$ ($p \geq 0.05$, $R^2 = 0.12$) and $\delta^{15}\text{N}$ ($p \geq 0.05$, $R^2 = -0.05$).

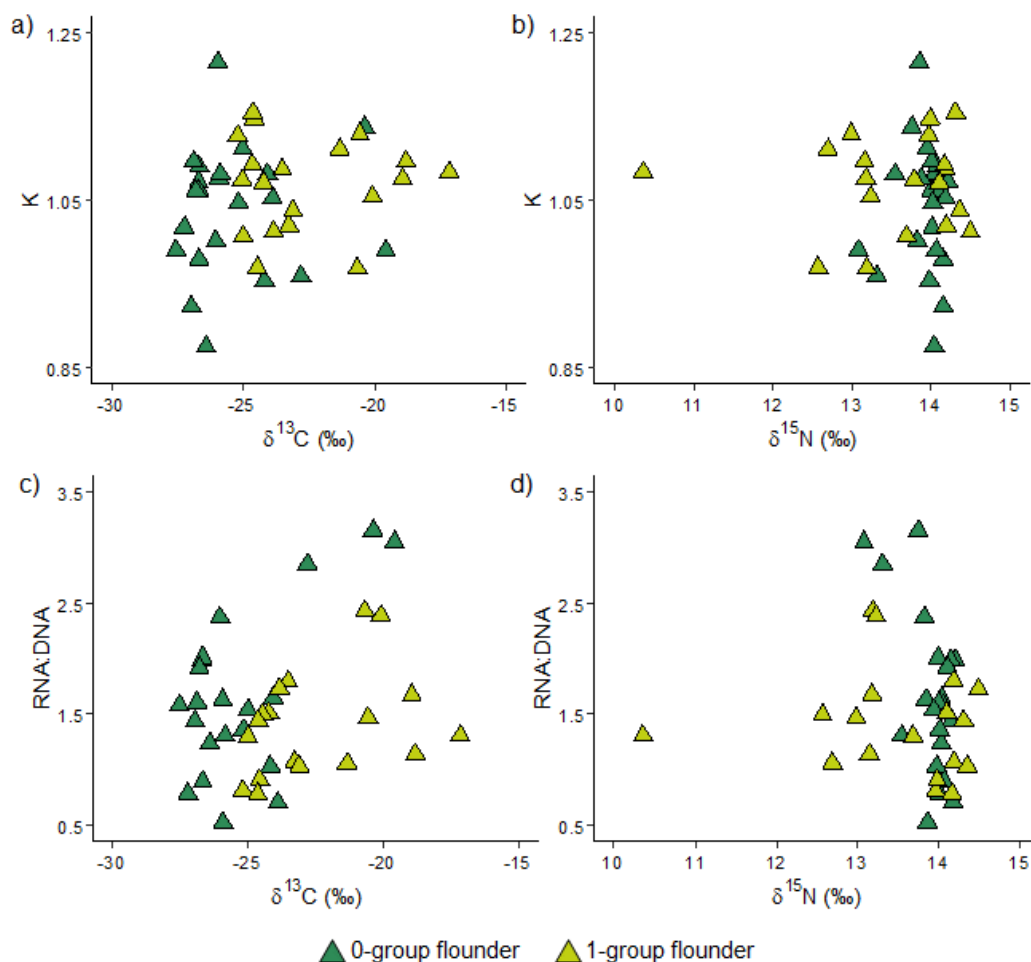


Figure 4.6. Relationship between Fulton K and a) carbon stable isotopes ($\delta^{13}\text{C}$) and b) nitrogen stable isotopes ($\delta^{15}\text{N}$); RNA:DNA and c) carbon ($\delta^{13}\text{C}$) and d) nitrogen ($\delta^{15}\text{N}$) stable isotopes for 0-group and 1-group European flounder.

4.4. Discussion

4.4.1. Integrating stomach contents and stable isotope analysis

Stomach content analysis provided a first indication of the flatfish juvenile recent diet, enabling identification of main prey such as polychaetes, *Corophium* spp. and *C. maenas*, to be included in the stable isotope analysis. A discrimination between sources and prey signatures, was necessary to reconstruct the consumer diet based on stable isotope analysis (Vander Zanden *et al.*, 1997; Post, 2002). The $\delta^{13}\text{C}$ depletion usually increases along the horizontal salinity gradient from marine to estuarine and terrestrial sources (Fry, 2002). In the Lima, the $\delta^{13}\text{C}$ similarity between lower and middle estuaries reflected the euryhaline regime of these downstream areas. The depleted $\delta^{13}\text{C}$ signatures in the upper estuary were related to the increasing terrestrial input in the upstream areas (Darnaude *et al.*, 2004; França *et al.*, 2011). The POM and SOM presented similar $\delta^{13}\text{C}$ values, providing good indicators of local $\delta^{13}\text{C}$ signatures.

In general, prey from upstream areas consistently presented higher $\delta^{15}\text{N}$ values than the downstream prey. The $\delta^{15}\text{N}$ variability between prey groups within each estuarine area may have resulted from differences in trophic position (Minagawa and Wada, 1984; Peterson and Fry, 1987; Post, 2002). Hence, the epibenthic predator *C. maenas* (Raffaelli *et al.*, 1989) presented the highest $\delta^{15}\text{N}$ from the downstream prey, while the omnivorous polychaetes from upstream areas presented higher $\delta^{15}\text{N}$ than Chironomidae and *Corophium*, respectively opportunistic omnivores (Armitage *et al.*, 1995) and suspension and deposit feeders (Gerdol and Hughes, 1994). Prey from the upstream areas showed markedly depleted $\delta^{13}\text{C}$ compared to the more saline downstream areas thus reflecting the use of local OM sources. This discrimination between upstream and downstream prey enabled the use of stable isotope mixed models to identify the main feeding locations, and trace movements of European flounder juveniles in the Lima estuary.

The stomach content results complemented the insights provided by the stable isotope analysis. Discrepancies between these two methods may be related to the fact that stomach contents reflect recent feeding (Hyslop, 1980), while stable isotope analysis indicates and integrates long term patterns (Vander Zanden *et al.*, 1997). Hence, easily

digested prey, such as polychaetes, were probably underestimated in 0-group flounder dietary indices even though these were important prey, together with *Corophium*, according to SIAR results. Also, the near absence of the upper estuary prey *Corophium* spp. in 1-group flounder dietary indices contrary to SIAR, reflected the recent feeding on polychaetes in the middle estuary, where juveniles were caught.

4.4.2. European flounder juvenile movements and main feeding areas within the Lima estuary

Results showed that the upper estuary was the main feeding area of 0-group flounder, supporting the typical preference of early European flounder juveniles for upstream areas (see also Kerstan, 1991; van der Veer *et al.*, 1991; Freitas *et al.*, 2009), and a dependence on freshwater-derived sources (Pasquaud *et al.*, 2008; Selleslagh *et al.*, 2015). Prey availability (Bos, 1999; Florin and Lavados, 2010; Vasconcelos *et al.*, 2010), and reduced competition for space and food (Beaumont and Mann, 1984; Złoch and Sapota, 2010; Souza *et al.*, 2013), may explain this preference for upstream areas especially as prey availability and salinity have been previously correlated with the concentration of 0-group flounder in the Lima upper estuary (Ramos *et al.*, 2009; Mendes *et al.*, 2014). Despite this, 0-group flounder feeding was not restricted to the upper estuary since polychaetes from downstream areas were also identified as prey items.

Limited mobility, and consequent patchy segregation along upstream-downstream estuarine gradients, have been reported for 0-group flatfish (Raffaelli *et al.*, 1990; Le Pape and Cognez, 2016). However, these movements between feeding habitats (Summers, 1980; Wirjoatmodjo and Pitcher, 1984; Dando, 2011; Souza *et al.*, 2013) may be the result of the 0-group flounder increasing mobility given that the individuals sampled were approaching 1-group age class (≥ 150 mm). The risk of feeding in downstream areas was reduced as individuals attained size-based refuge from predation by the crustaceans *C. maenas* (> 50 mm TL) and *C. crangon* (> 30 mm TL) (van der Veer and Bergman, 1987; Burrows *et al.*, 2001).

Feeding use affected the condition of the 0-group flounder and higher RNA:DNA were associated with $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ indicating downstream feeding. Accordingly, laboratory experiments showed that low salinity typical of upstream areas may limit condition and growth (Gutt, 1985; O'Neill *et al.*, 2011) of flounder juveniles. The predator-prey trade-

offs mentioned above may explain why the juveniles remain upstream despite these limitations. In parallel, the downstream areas may have promoted higher condition as the juveniles approached 1-group age size and become less vulnerable to predation (van der Veer and Bergman, 1987; Burrows *et al.*, 2001), while consuming larger and more diverse prey (Mendes *et al.*, 2014).

The 1-group fed throughout the estuary, relying equally on prey from downstream where they were more abundant (Ramos *et al.*, 2009), and upstream areas. It is of note that a limited home range and high site fidelity has been reported for flounder juveniles (Raffaelli *et al.*, 1990; Dando, 2011). However, diel and tidal migrations (Edwards and Steele, 1968; Edwards *et al.*, 1970; Gibson, 1973) to areas with high prey availability (Modin and Pihl, 1996), reflected movements between estuarine habitats. Moreover, a gradual use of the downstream areas throughout development (Kerstan, 1991; Ramos *et al.*, 2010; Primo *et al.*, 2013; Souza *et al.*, 2013) is typical of the flounder life cycle. Thus, the wide range of 1-group flounder $\delta^{13}\text{C}$ may indicate recent migration from upstream to downstream areas, where most 1-group flounder were caught, considering that muscle tissue takes many weeks to reach isotopic equilibrium (Vander Zanden *et al.*, 1997; Herzka, 2005). Overall, connectivity between upstream and downstream estuarine habitats has also been observed for 1-group flounder in other estuarine habitats (Vinagre *et al.*, 2011; Selleslagh *et al.*, 2015). This connectivity did not affect condition of the 1-group flounder that did not vary with stable isotope signatures. Such connectivity may also allow the use of alternative resources if one of the habitats is compromised, hence increasing the ability of flounder to tolerate environmental change (Selleslagh *et al.*, 2015).

4.4.3. Feeding strategies promoting European flounder condition in the Lima estuary and management implications

The issue of food limitation in nursery habitats is still debated (Le Pape and Bonhommeau, 2015), as several authors suggest that feeding on abundant prey (van der Veer *et al.*, 2000; Amara *et al.*, 2009; Selleslagh and Amara, 2013) and resource partitioning strategies (Evans, 1983; Besyst *et al.*, 1999; Hampel *et al.*, 2005; Martinho *et al.*, 2008; Haynes *et al.*, 2011) may reduce the effects of niche overlap and prevent competition. Therefore, resource use is optimized, preventing carrying capacity being exceeded (Le Pape and Bonhommeau, 2015). Accordingly, the distinct isotopic

signatures 0-group and 1-group European flounder and the feeding on prey such as *Corophium* and polychaetes, highly abundant in the Lima estuary (Sousa *et al.*, 2006b) did not support evidence for competition. Moreover, the morphometric condition of European flounder juveniles in the Lima estuary was within the same range observed for this species in estuaries such as Canche (mean=0.89), Authie (mean=1.0) (Amara *et al.*, 2009), Minho, Mondego and Douro (mean=0.70) (Vasconcelos *et al.*, 2009).

The RNA:DNA of European flounder in the Lima estuary was lower than observed in the laboratory (O'Neill *et al.*, 2011) and in the Minho and Douro nurseries (Vasconcelos *et al.*, 2009), while in the same range as Canche, Authie (Amara *et al.*, 2009) and Mondego (Vasconcelos *et al.*, 2009). However, differences in methodology may limit comparisons of RNA:DNA between studies (Caldarone *et al.*, 2006). It is of note that European flounder juveniles were sampled in the late summer when sub-optimal growth has been observed for juveniles of this species (Jager *et al.*, 1995; Freitas *et al.*, 2012) and similarly to other flatfish such as plaice *Pleuronectes platessa* L. 1758 (Freitas *et al.*, 2012; Ciotti *et al.*, 2013a) and common sole *Solea solea* Quensel 1806 (Fonseca *et al.*, 2006; Laffargue *et al.*, 2007; Teal *et al.*, 2008). These reduced growth rates have been linked to food limitation, which may be caused by changes in prey quality and availability (Teal *et al.*, 2008), and intraspecific (Edwards *et al.*, 1970; Laffargue *et al.*, 2007) and interspecific competition (Jager *et al.*, 1995; van der Veer *et al.*, 2010; Freitas *et al.*, 2012; Ciotti *et al.*, 2013c). Hence, the Lima estuary promoted good condition of the juveniles in a wide temporal frame integrated by the Fulton K and stable isotope analysis, while short term changes in environmental conditions or prey availability and competition may justify the low RNA:DNA as this index is sensitive to fish recent feeding (Clemmesen, 1994; Malloy and Targett, 1994) and condition (Buckley, 1984; Gwak and Tanaka, 2001). Moreover, the different lag response to environmental conditions of the Fulton K and RNA:DNA may explain the lack of correlation between these indices (Suthers *et al.*, 1992; Ferron and Leggett, 1994; De Raedemaeker *et al.*, 2012b). Further studies are required to estimate carrying capacity and the relative amounts of prey available to predators.

Historical habitat loss is a major environmental problem in estuaries (Wolanski and Elliott, 2015) and there are increasing measures to remedy that loss (Elliott *et al.*, 2016). Coordinated management initiatives (e.g. Lonsdale *et al.* 2015) will aim to balance the effects of the various estuarine users and their demands on the systems.

Hence, the present study is important in determining which species and stages use habitats within and adjacent to the estuary (Amorim *et al.*, 2017). In particular there is the continued need for information on the food and space resources required by fish species to ensure a maximal carrying capacity of an estuarine fish habitat.

4.5. Conclusions

In short, the present study showed that: 1) feeding of 0-group European flounder concentrated in the upstream area to explain the abundance of these juveniles; 2) feeding of 1-group European flounder in upstream and downstream areas showed dispersal throughout the estuary; 3) feeding use affected the recent condition of 0-group European flounder, with higher $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ associated with higher RNA:DNA, while long term condition assessed by Fulton did not vary with stable isotopes; 4) the condition of 1-group European flounder did not vary with stable isotopes, despite the variability in feeding locations; 5) the combined use of stomach content and stable isotope analyses provided an integrated insight on trophic relationships. This study reinforced the importance of understanding feeding strategies at an intraspecies level for future management decisions.

CHAPTER 5

Condition and growth of 0-group

European flounder *Platichthys flesus*
in a nursery habitat

Abstract

Condition and growth patterns of early life stages of fishes can reflect the nursery habitat quality and affect recruitment to adult populations. Fishes individual condition can be assessed at the morphometric, tissue, and biochemical level. Otolith microstructure can be used to assess age given by the total number of daily increments, and growth rates by measuring daily increment widths of larvae and juveniles. This study combined different fitness indicators namely morphometric Fulton K and biochemical RNA:DNA condition indices, as well as the otolith post-settlement growth rates (PSGR) and the Recent Growth index (RG) to assess physiological status of the 0-group European flounder *Platichthys flesus* and thus investigate the nursery habitat of the Lima estuary. The 0-group European flounder presented an average Fulton K and RNA:DNA of 1.05 ± 0.08 and 1.70 ± 0.70 , respectively. The RG ($26.23 \pm 4.16 \mu\text{m}.\text{day}^{-1}$) was correlated to the PSGR ($25.38 \pm 3.86 \mu\text{m}.\text{day}^{-1}$) growth rates suggesting that 0-group European flounder presented stable growth patterns. The other indices were not correlated possibly due to the different lag time responses to environmental conditions. The integration of these indices provided a measure of nursery habitat quality at the short (RNA:DNA and RG) and long (Fulton K and PSGR) temporal frame. The condition and growth rates showed good individual condition of the 0-group European flounder suggesting good nursery habitat quality of the Lima estuary.

5.1. Introduction

The life cycle of marine fish often includes offshore spawning and migration of the early life stages to estuarine and coastal nurseries until they reach maturity. Nursery habitats promote survival of the early life stages and recruitment to adult populations (Rijnsdorp *et al.*, 1995; Able, 1999; Potter *et al.*, 2015) by providing high food availability, low predation and enhanced growth (Gibson, 1994; Beck *et al.*, 2001; Pihl *et al.*, 2007). In fact, high food availability prevents intra- and interspecific competition and supports maximum growth according to the maximum growth/optimal food hypothesis (Karakiri *et al.*, 1991; Amara *et al.*, 2001; Reichert, 2003). Juvenile fishes with higher growth rates attain size-refuge from predation, consume a wider range of prey (Ellis and Gibson, 1995; Sogard, 1997; Wennhage, 2000) and have higher chances of survival until the end of the nursery period (Houde, 1987; Pepin, 1991; Suthers, 1998). However, fishes individual condition and growth in nursery areas vary across temporal (Glass *et al.*, 2008; Ciotti *et al.*, 2013c) and spatial scales (Gibson *et al.*, 1996; De Raedemaeker *et al.*, 2012b). This variability is primarily driven by physiological responses to environmental conditions, including temperature, salinity and dissolved oxygen (Stierhoff *et al.*, 2009; Ciotti *et al.*, 2010), and biotic factors, such as prey availability (Paperno *et al.*, 2000; De Raedemaeker *et al.*, 2012a), prey composition (van der Veer and Witte, 1993; Gibson, 1994), and predation (Boesch and Turner, 1984; Pihl *et al.*, 2007; De Raedemaeker *et al.*, 2012a). Sub-optimal growth has been observed for flatfish species including the common sole *Solea solea* (Amara, 2004) and plaice *Pleuronectes platessa* (Amara, 2004; Nash *et al.*, 2007; Freitas *et al.*, 2012) contradicting the maximum growth/optimal food hypothesis. Moreover, the extra stress imposed by anthropogenic pressures may compromise the carrying capacity of estuarine nurseries (Wolanski and Elliott, 2016), and ultimately affect the recruitment of adult marine stocks. Therefore, it is fundamental to investigate the link between condition, growth and survival of the early life stages in nursery habitats to better support and inform management decisions.

Fish individual condition can be assessed at the morphometric, tissue, and biochemical level (Ferron and Leggett, 1994). The Fulton condition factor, K (Ricker, 1975) is a morphometric index widely used in fisheries studies (Amara *et al.*, 2009; De Raedemaeker *et al.*, 2012b; Parsons *et al.*, 2013). It provides an overall measure of

fish well-being (Suthers, 1992) considering that heavier fish for a given length are in better condition. The RNA:DNA ratio (Buckley, 1984) assumes that DNA content is constant in somatic cells (Clemmesen, 1988), while RNA content is a measure of protein synthesis (Clemmesen, 1994), so higher contents of RNA indicate increase of protein synthesis and ultimately increase in somatic growth. This index responds rapidly to environmental conditions and is a proxy for recent growth (Bulow, 1970; Buckley *et al.*, 1999) and recent nutritional condition (Richard *et al.*, 1991; Meyer *et al.*, 2012; Selleslagh and Amara, 2013). Otolith microstructure provides insights into fish life history, as fish age, growth rates and time of important life events as metamorphosis, based on the assumption that there is daily deposition of material in the otolith (Pannella, 1971; Campana, 1990). In larvae and juveniles, the number of daily increments provides fish age, and growth rates are determined based on daily increment widths (Campana and Neilson, 1985). The recent growth index (RG) considers the width of the last ten daily otolith increments to assess recent fish condition (Suthers *et al.*, 1989; Hovenkamp and Witte, 1991; Gilliers *et al.*, 2004) and is sensitive to food availability (Suthers *et al.*, 1992; Selleslagh and Amara, 2013). Each index has different lag responses to environmental conditions (Suthers, 1992) providing complementary information on condition of juvenile fishes. Thus, the integration of different condition and growth indices provides a robust assessment of juveniles condition and ultimately nursery habitat quality (Fonseca *et al.*, 2006; De Raedemaeker *et al.*, 2012b; Selleslagh and Amara, 2013).

The flatfish European flounder *Platichthys flesus* uses estuarine nurseries (Summers, 1980; Kerstan, 1991; Morais *et al.*, 2011) along its geographic range, including the Portuguese coast that is the species southern distribution limit (Nielsen, 1986). According to previous studies, in the Lima estuary (northern Portugal), the late stage larvae arrive in late spring, and after settlement the 0-group European flounder tend to aggregate in the upper estuary (Ramos *et al.*, 2009; Amorim *et al.*, 2016). Reduced growth rates of European flounder in Southern European estuaries have been associated with food limitation resulting from interspecific competition and higher metabolic demands (Freitas *et al.*, 2012). Thus, this study aimed to: 1) analyse 0-group flounder condition in the Lima nursery habitat; 2) investigate correlations between the condition indices including Fulton K, RNA:DNA, RG and otolith post-settlement growth rates (PSGR), and 3) evaluate nursery habitat quality by integrating condition and

growth indices at the short (RNA:DNA and RG) and long (Fulton K and PSGR) temporal frame, based on 0-group European flounder data in the Lima estuary. The understanding of condition and growth patterns of early life stages of fishes provides further understanding of the nursery habitat quality and ultimately of species recruitment, fundamental to promote sustainable adult populations.

5.2. Material and Methods

5.2.1. Study area

The Lima estuary, NW Atlantic coast of Portugal, is a small open estuary with a semidiurnal and mesotidal regime (3.7 m). The average flushing rate is 0.4 m.s^{-1} with a residence time of 9 days, and salt intrusion can extend up to 20 km upstream (Ramos *et al.*, 2006b). Five stations were sampled covering the lower, middle and upper estuary (Figure 5.1.) as described in Chapter 4. The lower estuary (station 1, average depth of 4.9 m) is a narrow, 9-m deep navigational channel with walled banks and industrialized that includes a shipyard, a commercial seaport, and a fishing harbour; the middle estuary (stations 2-3, average depth 5.1 m) is a broad shallow intertidal saltmarsh zone, mainly colonized by the common rush (*Juncus* spp.); the upper estuary (stations 4-5, average depth 2.1 m) comprises a narrow shallow channel with natural banks and small exposed sandbanks and less disturbed (Ramos *et al.*, 2010).

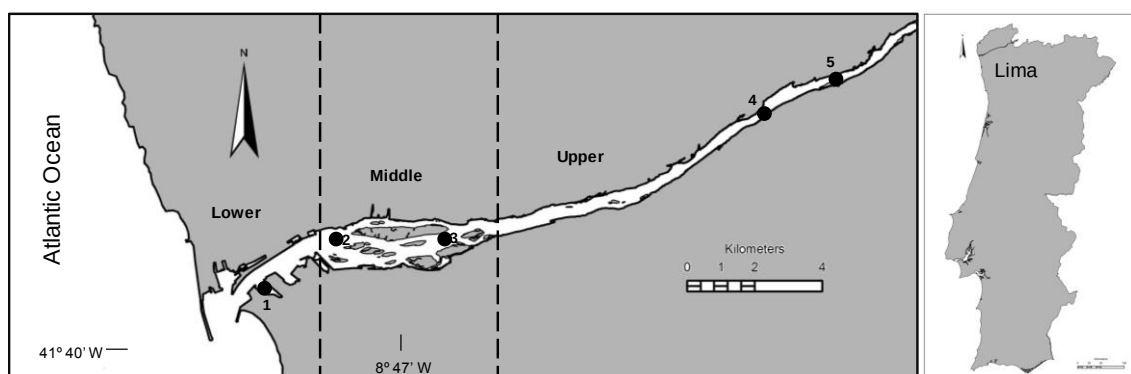


Figure 5.1. The Lima estuary and sampling locations along the lower (station 1), middle (stations 2 and 3) and upper (stations 4 and 5) estuaries.

5.2.2. Collection and sorting of the 0-group European flounder

The European flounder juveniles were collected during two nightly ebb tides in August 2014 with a 2-m beam trawl, with a cod-end 5 mm mesh, and a tickler chain, in the upper estuary where the 0-group European flounder concentrate (Ramos *et al.*, 2009; Amorim *et al.*, 2016). All the juveniles were sorted immediately and kept on ice until further processing. The geographic location of the sampling stations was recorded with a Magellan 315 GPS.

The flounder were measured for total length (TL, ± 1 mm) and wet weight (WW, ± 0.01 g). The 0-group flounder were sorted considering a maximum total length of 150 mm (Dinis, 1986). Dorsal white muscle samples of 0-group flounder were preserved in liquid nitrogen upon collection and then kept at -80°C for further RNA:DNA analysis.

5.2.3. Condition and growth analysis

The individual condition of the 0-group European flounder in the Lima nursery was assessed through the morphometric index Fulton K, the biochemical index RNA:DNA and otolith growth rates, including the mean otolith growth rates at the post-settlement stage (PSGR) and the recent growth index (RG). The Fulton K and PSGR were used to assess long-term physiological status, while RNA:DNA and RG represented measures of recent condition and growth of 0-group European flounder. The Fulton condition factor, K (Ricker, 1975) was determined through the formula:

$$K = W \times (TL^3)^{-1} \times 100,$$

where W is the wet weight (mg) and TL is the total length (mm). For the RNA:DNA, dorsal white muscle samples of 0-group flounder were homogenised in 500 μl TEN-SDS buffer (0.05 M Tris, 0.01 M EDTA, 0.1 M NaCl, 0.01% SDS, pH 8). RNA:DNA was determined for three replicate samples of dorsal white muscle (10 mg) of each 0-group flounder, by the fluorometric method described by Caldarone *et al.* (2001) and modified by De Raedemaeker *et al.* (2012b). Fluorescence was measured by means of a Fluoroscan Ascent FL microplate reader, 530 nm excitation and 590 nm emission wave length. RNA and DNA concentrations were determined based on standard curves prepared with baker's yeast RNA (Sigma) and pure calf-thymus DNA (Sigma). The RNA:DNA was determined as the ratio between average RNA and DNA concentrations of each sample. The ratio between the slopes of RNA and DNA standard curve was of

2.5 which can be used as standardization factor for inter-calibration with other studies (Caldarone *et al.*, 2006).

The age (days) of 0-group European flounder was determined as the total number of otolith daily increments and according to the methods described in Martinho *et al.* (2013). Daily otolith growth ($\mu\text{m}\cdot\text{day}^{-1}$) can be used as an indicator of fish growth when there is a linear relationship between fish total length and otolith length (Campana and Neilson, 1985). A linear relationship between 0-group European flounder total length and otolith length was fitted by a linear regression ($y=0.0195x + 0.321$). Mean daily increment width between the first complete increment after the accessory primordia to the edge of the otolith was used to determine PSGR. The mean width of the last 10 daily increments were used to determine the RG (Amara *et al.*, 2009). Otoliths were analysed under a light microscope at 100x with a coupled video system. The daily increments were measured with ImageJ software.

The condition indices were investigated for possible length effects through Pearson correlations between total length and Fulton K, RNA:DNA, the RG and PSGR. Whenever a significant correlation was observed, indices were corrected for the length effect by subtracting the slope of the linear regression and multiplying by the total length. Pearson correlations between the different condition and growth indices were investigated. A significance level of 0.05 was used for all the statistic procedures. All statistical analyses were performed with R software (R Development Core Team, 2007).

5.3. Results

5.3.1. Condition and growth of 0-group European flounder

A total of 22 0-group European flounder were collected in the Lima upper estuary (Table 5.1.) with a total length that ranged between 40 and 136 mm. The age varied between 50 and 104 days with an average of 76 ± 15 days (Figure 5.3.a., Table 5.1.). The Fulton K varied between 0.88 and 1.23, while RNA:DNA varied between 0.52 and 3.15 (Table 5.1.). There was no correlation between the 0-group European flounder total length and K ($p \geq 0.05$, $R = 0.03$, Figure 5.2.a) and RNA:DNA ($p \geq 0.05$, $R = 0.25$,

Figure 5.2.b). The otoliths of two 0-group European flounder were excluded from the microstructure analysis as the increments were not countable. Significant correlations were found between the 0-group flounder total length and otolith growth rates, both for the PSGR ($p < 0.05$, $R = -0.56$, Figure 5.2.c) and RG ($p < 0.05$, $R = -0.55$, Figure 5.2.d). The PSGR presented an average $14.37 \pm 3.86 \mu\text{m}\cdot\text{day}^{-1}$, while RG presented an average value of $14.52 \pm 4.17 \mu\text{m}\cdot\text{day}^{-1}$. After length corrections, PSGR varied between 20.07 and $32.32 \mu\text{m}\cdot\text{day}^{-1}$, while the RG varied between 19.48 and $33.50 \mu\text{m}\cdot\text{day}^{-1}$ (Table 5.1.).

Table 5.1. Number (n) of 0-group European flounder *Platichthys flesus* sampled in the Lima estuary, mean total length (mm), total weight (g), age (days) and condition indices Fulton K, RNA:DNA, otolith post-settlement growth rates (PSGR, $\mu\text{m}\cdot\text{day}^{-1}$) and recent growth index (RG, $\mu\text{m}\cdot\text{day}^{-1}$).

0-group European flounder	
n	22
TL (mm)	75 ± 20
WW (g)	5.43 ± 5.65
Age (days)	76 ± 15
K	1.05 ± 0.08
RNA:DNA	1.70 ± 0.70
PSGR	25.38 ± 3.86
RG	26.23 ± 4.16

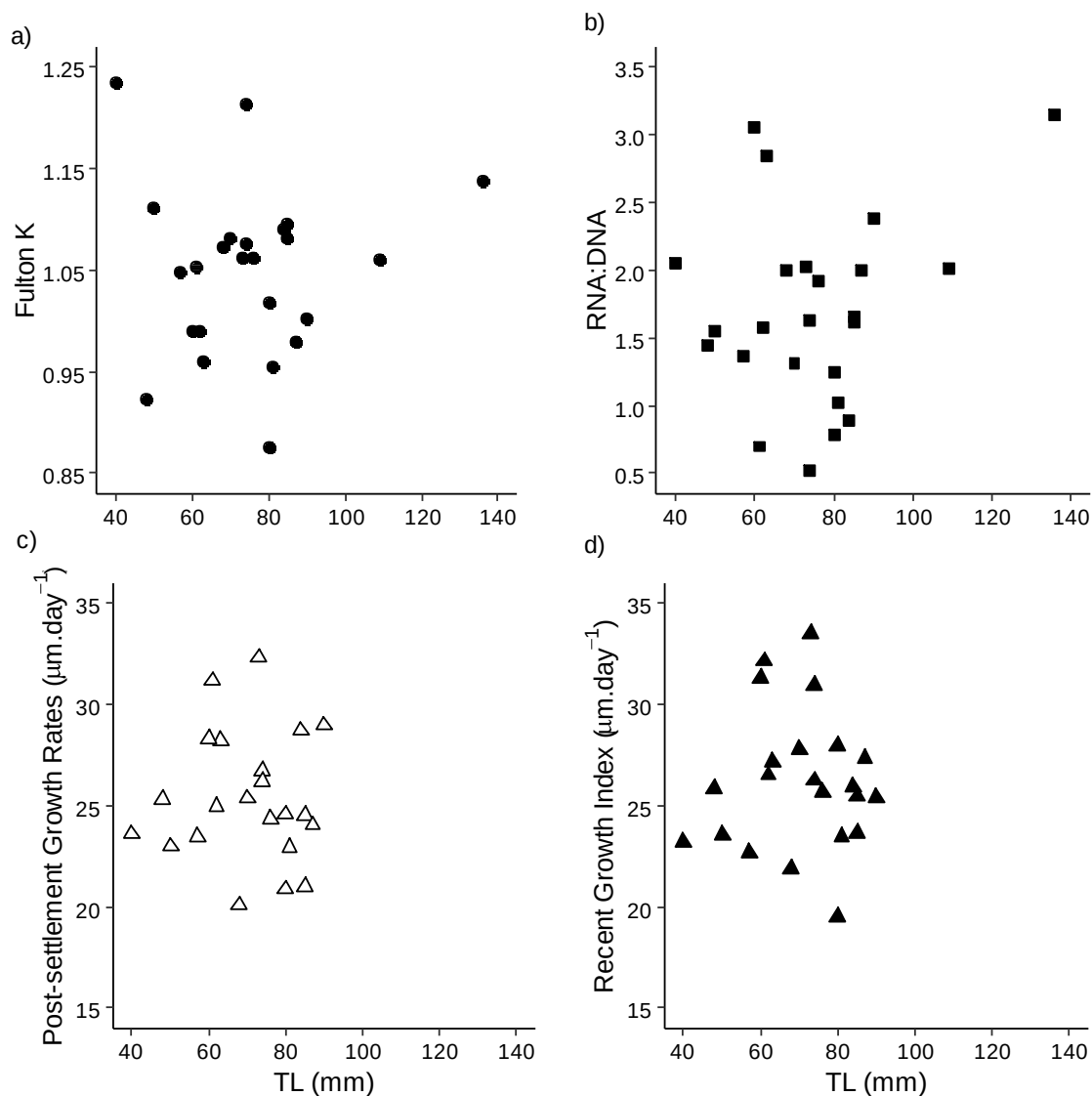


Figure 5.2. Relationship between total length and a) Fulton K, b) RNA:DNA, c) Post-settlement growth rates ($\mu\text{m}\cdot\text{day}^{-1}$), and d) Recent Growth index ($\mu\text{m}\cdot\text{day}^{-1}$) of 0-group European flounder *Platichthys flesus*.

The Fulton K was not correlated to any of the indices used, namely the RNA:DNA ($p \geq 0.05$, $R = 0.06$), PSGR ($p \geq 0.05$, $R = -0.05$) and RG ($p \geq 0.05$, $R = -0.15$). The RNA:DNA was also not correlated to the PSGR ($p \geq 0.05$, $R = 0.17$) and RG ($p \geq 0.05$, $R = 0.11$). The PSGR were significantly correlated to the RG ($p < 0.05$, $R = 0.82$, Figure 5.3.b.).

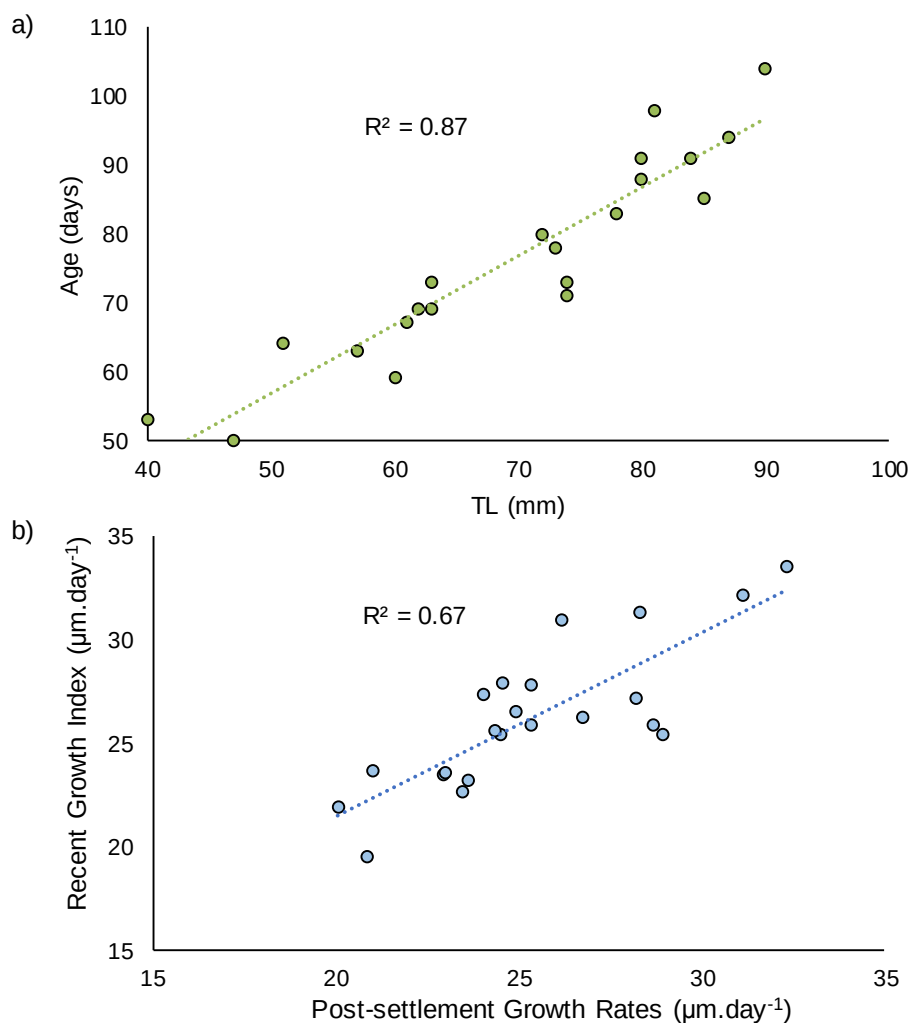


Figure 5.3. Relationship between a) total length (mm) and age (days), and b) mean otolith growth rates at the post-settlement stage ($\mu\text{m}\cdot\text{day}^{-1}$) and the Recent Growth index ($\mu\text{m}\cdot\text{day}^{-1}$) of the 0-group European flounder *Platichthys flesus*.

5.4. Discussion

Condition indices respond to environmental conditions within different lag times (Suthers, 1998) providing complementary information in the assessment of nursery habitat quality. The Fulton K was not correlated to the RNA:DNA and to the RG of 0-group European flounder, similarly to other flatfish (Suthers *et al.*, 1992; Gilliers *et al.*, 2004; De Raedemaeker *et al.*, 2012b). Indeed, the Fulton K provides an integrated assessment of body condition during the last weeks to months before capture

(Caldarone *et al.*, 2012; De Raedemaecker *et al.*, 2012b). Conversely, recent condition indices, such as RNA:DNA (Buckley, 1984; Richard *et al.*, 1991; Ferron and Leggett, 1994) and RG (Maillet, 1989; Molony and Choat, 1990; Peck *et al.*, 2015) are more sensitive to recent environmental changes. In fact, these indices represent the environmental conditions of the sites where fish were captured, as they are less sensitive to the cumulative effect of previous conditions (Caldarone *et al.*, 2012). However, the RNA:DNA was also not correlated to the RG. The RNA:DNA responds within days (Ferron and Leggett, 1994) to changes in feeding conditions and thus faster than RG that can take up to 3 weeks (Molony and Choat, 1990; Selleslagh and Amara, 2013; Peck *et al.*, 2015). Moreover, the recent growth may also integrate past events in fish life, such as growth rates at previous life stages (Chambers *et al.*, 1988; Vigliola and Meekan, 2002; Hurst *et al.*, 2009) and may be affected by nursery habitat quality of the preceding months (Campana and Neilson, 1985; Vinagre *et al.*, 2009a; De Raedemaecker *et al.*, 2011). Indeed, individuals with higher post-settlement growth rates also presented higher recent growth rates (Vigliola *et al.*, 2007). In theory, individuals in better condition can escape predators (Ellis and Gibson, 1995) and capture food with higher efficiency (Sogard, 1997; Wennhage, 2000). In result, they will grow faster and attain size-refuge from predators giving them higher chances of survival (Nash and Geffen, 2012), according to the “bigger-is-better” hypothesis (Houde, 1987; Leggett and Deblois, 1994; Suthers, 1998). Moreover, the stable growth patterns from settlement until capture may reflect the low mobility of the 0-group European flounder (Raffaelli *et al.*, 1990; Le Pape and Cogneux, 2016) and steady habitat conditions. Interindividual variability in growth rates was then driven by small scale variability in habitat quality.

The 0-group European flounder in this study were captured in the late summer when sub-optimal growth has been observed in nurseries for the this species (Jager *et al.*, 1995; Freitas *et al.*, 2012) and the flatfish common sole *Solea solea* (Fonseca *et al.*, 2006; Laffargue *et al.*, 2007; Teal *et al.*, 2008) and plaice *Pleuronectes platessa* (Freitas *et al.*, 2012; Ciotti *et al.*, 2013a). These low growth rates were linked to changes in prey quality and availability (Teal *et al.*, 2008), and competition (Edwards *et al.*, 1970; Laffargue *et al.*, 2007; Ciotti *et al.*, 2013b) that caused food limitation. However, low vacuity index and high abundances of macroinvertebrate prey in the Lima estuary (Sousa *et al.*, 2006b; Mendes *et al.*, 2014) did not support the evidence

of food limitation (Karakiri *et al.*, 1991; Amara *et al.*, 2001; Selleslagh and Amara, 2013). Moreover, the Fulton K of 0-group European flounder was in line with the values reported for the species in other nurseries, such as the Canche (mean = 0.89) and Authie (mean = 1.0) (Amara *et al.*, 2009), Minho, Douro and Mondego (mean = 0.70, Vasconcelos *et al.* 2009). The RG was higher than in the Canche (mean = 5.62), Authie (mean = 5.98) and Seine (mean = 4.40) (Amara *et al.*, 2009). The Lima estuary is located at a lower latitude where higher temperature and longer photoperiod explain these higher growth rates (Henderson and Seaby, 2005; Vinagre *et al.*, 2009a). Conversely, the RNA:DNA was lower than reported for flounder juveniles in the laboratory (O'Neill *et al.*, 2011), and in nurseries located at the same latitude, such as the Minho and Douro estuaries (Vasconcelos *et al.*, 2009). However, methodological differences may limit comparisons of RNA:DNA between studies, even though a standardizing factor (Caldarone *et al.*, 2006) was applied when possible. This study represented the first integrated assessment of 0-group European flounder condition in the Lima nursery combining morphometric and biochemical indices together with otolith growth rates. As the number of the 0-group European flounder sampled was low, future research is needed to support these results. The integration of these indices showed the good individual condition of 0-group European flounder in the Lima nursery in comparison to European flounder juvenile populations throughout the species geographical range (Amara *et al.*, 2009; Vasconcelos *et al.*, 2009). In general, both short and long-term indices supported good habitat quality of the Lima upper estuary to the 0-group European flounder.

5.5. Conclusions

This study showed that: 1) 0-group European flounder was in good condition in the Lima estuary; 2) the condition indices Fulton K and RNA:DNA were not correlated, and these indices were also not correlated to the otolith growth rates PSGR and RG 3) there was a correlation between the otolith based growth rates, namely PSGR and the RG, suggesting that growth patterns were stable throughout the nursery period. Overall, this study reinforced the advantages of integrating condition and growth indices with different temporal responses towards the assessment of nursery habitat quality. The good nursery habitat quality of the Lima estuary was shown by the good condition status and high growth rates of the 0-group European flounder, at the short and long temporal frame.

CHAPTER 6

Final Considerations and Suggestions for Future Research

6.1. Final Considerations

Estuaries support the nursery function for many marine fishes playing a key role in the replenishment of adult populations (Beck *et al.*, 2001). However, estuaries also suffer heavy pressures from anthropogenic activities resulting in habitat loss and modification that may compromise nursery function (Wolanski and Elliott, 2015). Therefore, the identification of nurseries and essential habitats has received much attention in recent years (Beck *et al.*, 2001; Nagelkerken *et al.*, 2015; Sheaves *et al.*, 2015). In this perspective, there is a demand for understanding the complex ecological interactions supporting nursery successful occupation (Sheaves *et al.*, 2015). This includes the integration of habitat units critical for different early life stages, as well as connectivity between these habitat units supported by animal movements (e.g. ontogenetic shifts) across different spatial and temporal scales (Nagelkerken *et al.*, 2015).

The spatial patterns of prey and predator species (Sogard, 1992; Taylor, 2003; Hammerschlag *et al.*, 2010), together with ecosystem hydrodynamics and geomorphology (Baker *et al.*, 2013) are major drivers of habitat use and ontogenetic migrations (Sheaves *et al.*, 2015; Amorim *et al.*, 2018) with effects on growth and recruitment, hence nursery value (Kimirei *et al.*, 2013; Sheaves *et al.*, 2015). This thesis aimed to investigate the role of feeding patterns as a major driver of flounder nursery use, and thus contributing to the understanding of trade-offs underlying nursery value. The Lima estuary was chosen as the study system as it has been identified as important nursery for European flounder (Ramos *et al.*, 2010; Amorim *et al.*, 2018).

Chapter 2 integrated several aspects of flounder early life in the Lima estuarine nursery. The otolith microstructure analysis supported spatial temporal abundance data from this thesis and previous studies in the Lima estuary (Ramos *et al.*, 2010; Amorim *et al.*, 2016). Otolith microstructure showed that 0-group flounder hatched from February to June with peak settlement in May and June. This is in agreement with estuarine colonization in late spring in the Lima estuary (Ramos *et al.*, 2009; Amorim *et al.*, 2016) and other estuaries throughout its geographical range (Summers, 1979; Jager, 1998; Bos, 1999; Martinho *et al.*, 2013). Moreover, 0-group flounder predominantly used the upper estuary where newly-settled were observed mainly during early summer, in agreement with Ramos *et al.* (2010) and Amorim *et al.* (2016),

and as generally observed for the species (van der Veer *et al.*, 1991; Jager *et al.*, 1993; Selleslagh and Amara, 2008; Freitas *et al.*, 2009). The 0-group flounder diet reflected this spatial distribution as the main prey were typical from low salinity Lima upper estuarine zone (Sousa *et al.*, 2006b). A diet shift occurred at 50 mm TL from the insect larvae Chironomidae to the amphipod *Corophium* spp. in agreement with changes in habitat suitability between post-settlement (< 50 mm TL) flounder and older young-of-the-year previously described in the Lima estuary (Amorim *et al.*, 2018). The 0-group flounder presented fast growth rates in the Lima estuary, contradicting the sub-optimal growth hypothesis due to food limitation in late summer observed in Southern European estuaries for flounder and other flatfish, such as *P. platessa* (Amara, 2004; Nash *et al.*, 2007; Freitas *et al.*, 2012) and *S. solea* (Amara, 2004). Moreover, the RG (recent growth rate) was higher in the Lima nursery than in French estuaries where copepods were the main prey of 0-group flounder (Amara *et al.*, 2009). In fact, macrobenthic prey that were the main prey items of the 0-group flounder appear to be more profitable prey leading to higher growth rates than planktonic prey, as copepods. In general, these early life patterns in terms of estuarine colonization, abundance and feeding strategies sustained high otolith post-settlement and recent growth rates, in comparison to other nurseries (Amara *et al.*, 2009) and showed the nursery value of the Lima estuary.

Chapter 3 further explored the flounder feeding patterns at the 0-group and 1-group stages. The diet of 0-group flounder was highly specialized in Chironomidae and *Corophium* which were highly abundant in the upper estuary, corroborating the conclusions from Chapter 2. These macrobenthic prey often integrate the diet of flounder juveniles (Weatherley, 1989; Jager *et al.*, 1993; Nissling *et al.*, 2007; Florin and Lavados, 2010; Selleslagh and Amara, 2015). The 0-group flounder diet in the Lima estuary was consistent at the interannual scale, as data from Chapters 2 and 3 belong to different sampling years (2010 and 2013, respectively). Furthermore, salinity and main prey were associated to 0-group flounder distribution, explaining the concentration of the newly-settled in the Lima upper estuary. As juveniles grew, the diet diversity increased with the incorporation of prey from the lower and middle estuaries. There was an ontogenetic diet shift from 0-group to 1-group flounder which may have been driven by a partial spatial partitioning between these age groups. Diet shifts may minimize niche overlap hence intraspecific competition (Aarnio *et al.*, 1996;

Florin and Lavados, 2010), thereby maintaining individual condition (Andersen *et al.*, 2005b; Mendes *et al.*, 2014). Overall, these diet shifts were present across flounder juveniles stages and may optimize nursery habitat use.

Based on evidence from Chapters 2 and 3, Chapter 4 explored the hypothesis that juvenile abundance patterns were related to feeding strategies, and that spatial partitioning optimized feeding use thereby reducing competition, hence ensuring good condition. The integration of dietary indices and stable isotope analysis showed that upstream areas of the Lima estuary were the main feeding locations of 0-group flounder, and thus explaining the high abundances of these juveniles in this estuarine area. On the other hand, the 0-group flounder in the lower salinity section of the estuary were in a good condition status with higher recent condition associated to feeding in downstream areas. Indeed, low salinities do not appear to have been reported to favour flounder condition and growth (Gutt, 1985; O'Neill *et al.*, 2011). Therefore, there may be a habitat trade-off with high food availability justifying the concentration of 0-group flounder in upstream areas, even though there is a physiological stress associated to low salinity. The 1-group flounder fed both in upstream and downstream areas showing dispersal throughout the estuary. The recent condition of 1-group flounder did not vary between upstream and downstream feeding locations. Overall, feeding locations varied between ontogenetic stages indicating resource partitioning as a strategy to avoid intraspecific competition, and ultimately supporting good individual condition of flounder juveniles.

Condition (Amara *et al.*, 2009; De Raedemaeker *et al.*, 2012b; Parsons *et al.*, 2013) and growth (Selleslagh and Amara, 2012; Cardoso *et al.*, 2016) indices are widely used tools to assess nursery habitat quality. The lag time responses to environmental conditions vary between condition indices (Suthers, 1998). The relationship between early life patterns with an emphasis on feeding use, and condition and growth rates of flounder juveniles in the Lima nursery has been explored throughout this thesis. Chapter 5 aimed to integrate 0-group flounder condition indices and growth rates as a proxy of the Lima nursery quality at short and long temporal scales. High growth rates were maintained throughout the nursery period, as shown by the RG and PSGR. These otolith-based growth rates of 0-group flounder collected in 2014 (PSGR= $14.37 \pm 3.86 \mu\text{m}.\text{day}^{-1}$, RG= $14.524x \pm 4.16 \mu\text{m}.\text{day}^{-1}$, Chapter 5) were higher than the ones

collected in 2013 ($\text{PSGR} = 10.48 \pm 2.00 \mu\text{m}.\text{day}^{-1}$, $\text{RG} = 10.42 \pm 2.75 \mu\text{m}.\text{day}^{-1}$, Chapter 2). However, these results should be compared with caution, since data comprised only two sampling events in August 2014, while 2013 surveys covered a temporal series based on monthly surveys between June and October 2013. On the other hand, morphometric condition assessed by Fulton K was stable throughout the different sampling years, namely 2010 (Chapter 3) and 2014 (Chapters 4 and 5). The Fulton K was within values reported in other flounder nurseries, such as the Canche (mean=0.89) and Authie (mean=1.0) (Amara *et al.*, 2009), Minho, Douro and Mondego (mean=0.70, Vasconcelos *et al.* 2009). The RNA:DNA indicated good nutritional status of flounder juveniles, but was lower than flounder reared in laboratory (O'Neill *et al.*, 2011), and in nurseries located at the same latitude, such as the Minho and Douro estuaries (Vasconcelos *et al.*, 2009). These differences could be the result of methodological differences in the RNA and DNA quantification. Overall, the good nursery habitat quality of the Lima estuary was shown by the good condition status and faster growth rates of the 0-group flounder in the Lima nursery, at the short and long temporal frame.

This thesis shows the differential use of space and food resources by 0-group and 1-group flounder and the need to consider habitat requirements of different life stages for management and conservation purposes. Moreover, it was shown that feeding was a major driver of flounder habitat use, explaining the concentration of 0-group flounder in the upper estuary. Overall, flounder early life patterns that include estuarine colonization, spatial temporal patterns and diet supported good individual condition and high growth rates, showing sustained nursery carrying capacity and habitat quality of the Lima estuary, despite the cumulating habitat loss in the past century (Amorim *et al.*, 2017). This thesis has therefore contributed to further knowledge on flounder lifecycle providing a base tool towards the management of an important nursery habitat for flounder.

6.2. Suggestions for future research

Current approaches in nursery identification and management highlight the dynamic and complex ecological interactions underlying nursery habitat use (Nagelkerken *et al.*, 2015; Potter *et al.*, 2015). However, such approaches are hampered by a lack of basic knowledge on key aspects of species lifecycle and basic processes supporting nursery successful occupation. This thesis has therefore contributed to a more comprehensive understanding of habitat use by the flounder juveniles. However, there are still knowledge gaps on flounder key early life-history strategies.

Few studies have attempted to integrate larval and juvenile phases (Ramos *et al.*, 2010) despite the importance of both phases and the links between them for the complete assessment of nursery function. Hence, the initial aim of this thesis was to integrate spatial-temporal patterns of flounder larvae and juveniles, including abundances, feeding ecology, condition and growth for a complete assessment of habitat use and quality of the Lima nursery. This aim could not be further pursued as only one flounder larvae was captured throughout the fortnightly plankton surveys performed between May and October 2013 among 16 sampling points throughout the Lima estuary. One possible explanation is that estuarine recruitment varied annually. Flounder may deploy variable early life strategies across estuarine nurseries and even within the same nursery (Morais *et al.*, 2011; Daverat *et al.*, 2012). Previous studies have suggested direct settlement of flounder in the Lima (Ramos *et al.*, 2010; Amorim *et al.*, 2018) and other nurseries (Jager, 1998; Bos, 1999), with larvae entering the estuary at late stages and performing settlement inside the estuary. However, other authors have also suggested indirect settlement, i.e. the larvae settle in coastal habitats and then migrate to estuarine nurseries as early juveniles (Primo *et al.*, 2013). Moreover, in the Minho estuary evidence otolith microchemistry studies suggested that flounder completed the lifecycle inside the estuary, including spawning and development of larval and juvenile phases (Morais *et al.*, 2011). Future studies should then include larval surveys to confirm the flounder early life patterns in the Lima estuary initially proposed by Ramos *et al.* (2010).

Settlement habitats are critical bottlenecks of early post-settlement stages (Nagelkerken *et al.*, 2015) as they provide a vital link in the connectivity between planktonic larval to juvenile benthic phase. This thesis and previous studies (e.g.

Ramos *et al.*, 2010; Amorim *et al.*, 2016) have shown occurrence of newly settled in the Lima upper estuary suggesting that this is a potential settlement habitat. However, further studies are required to ascertain the exact location of flounder settlement areas in the Lima estuary through techniques such as otolith microchemistry or dedicated *in-situ* surveys.

The trade-offs driving flounder habitat use and the physiological plasticity of the species allowed the colonization of upstream areas by 0-group flounder. Such evidence could be further explored by integrating the data from the current study on feeding and individual growth rates into dynamic energy budget (DEB) models (Kooijman and Kooijman, 2000; Cardoso *et al.*, 2016). These models compare individual growth rates with predictions of maximum growth i.e. growth at optimal food conditions. Thereby, DEB models could be applied to predict flounder physiological performances at variable environmental conditions (e.g. temperature and prey availability), integrating tolerance limits and growth energetics. The knowledge obtained on the set of abiotic and biotic conditions sustaining higher growth rates would allow the identification of areas providing higher habitat quality.

The major factors driving nursery use may vary across systems and geographical regions (Martinho *et al.*, 2013; Sheaves *et al.*, 2013; Nagelkerken *et al.*, 2015). considering the plasticity of life-cycle strategies (Morais *et al.*, 2011; Daverat *et al.*, 2012) and opportunistic behaviour of the species (Hampel *et al.*, 2005; Martinho *et al.*, 2008). In this light, future research should also integrate habitat use of other nursery systems across the flounder geographical range, shedding insights into the interplay of ecological factors driving nursery use towards effective conservation and management programmes.

References

- Aarnio, K., Bonsdorff, E. & Rosenback, N. (1996). Food and feeding habits of juvenile flounder *Platichthys flesus* (L.), and turbot *Scophthalmus maximus* L. in the Åland archipelago, northern Baltic Sea. *Journal of Sea Research* 36, 311-320. [http://dx.doi.org/10.1016/S1385-1101\(96\)90798-4](http://dx.doi.org/10.1016/S1385-1101(96)90798-4).
- Able, K. W. (1999). Measures of Juvenile Fish Habitat Quality: Examples from a National Estuarine Research Reserve. In *American Fisheries Society Symposium*, pp. 134-147. doi
- Able, K. W. & Fahay, M. P. (1998). *The First Year in the Life of Estuarine Fishes in the Middle Atlantic Bight*: Rutgers University Press.
- Able, K. W. & Fahay, M. P. (2010). *Ecology of Estuarine Fishes: Temperate Waters of the Western North Atlantic*. Baltimore: Johns Hopkins University Press.
- Amara, R. (2004). 0-group flatfish growth conditions on a nursery ground (Bay of Canche, Eastern English Channel). *Hydrobiologia* 518, 23-32. <https://doi.org/10.1023/B:HYDR.0000025053.62966.c>.
- Amara, R., Lagardere, F., Desaunay, Y. & Marchand, J. (2000). Metamorphosis and estuarine colonisation in the common sole, *Solea solea* (L.): implications for recruitment regulation. *Oceanologica Acta* 23, 469-484. [https://doi.org/10.1016/S0399-1784\(00\)00134-1](https://doi.org/10.1016/S0399-1784(00)00134-1).
- Amara, R., Laffargue, P., Dewarumez, J. M., Maryniak, C., Lagardère, F. & Luzac, C. (2001). Feeding ecology and growth of O-group flatfish (sole, dab and plaice) on a nursery ground (Southern Bight of the North Sea). *Journal of Fish Biology* 58, 788-803. 10.1111/j.1095-8649.2001.tb00531.x.
- Amara, R., Selleslagh, J., Billon, G. & Minier, C. (2009). Growth and condition of 0-group European flounder, *Platichthys flesus* as indicator of estuarine habitat quality. *Hydrobiologia* 627, 87. 10.1007/s10750-009-9717-9.
- Amezcu, F. & Nash, R. D. M. (2001). Distribution of the order Pleuronectiformes in relation to the sediment type in the North Irish Sea. *Journal of Sea Research* 45, 293-301. [https://doi.org/10.1016/S1385-1101\(01\)00044-2](https://doi.org/10.1016/S1385-1101(01)00044-2).
- Amorim, E., Ramos, S., Elliott, M. & Bordalo, A. A. (2016). Immigration and early life stages recruitment of the European flounder (*Platichthys flesus*) to an estuarine nursery: The influence of environmental factors. *Journal of Sea Research* 107, 56-66. <https://doi.org/10.1016/j.seares.2015.07.005>.

- Amorim, E., Ramos, S., Elliott, M. & Bordalo, A. A. (2018). Dynamic habitat use of an estuarine nursery seascape: Ontogenetic shifts in habitat suitability of the European flounder (*Platichthys flesus*). *Journal of Experimental Marine Biology and Ecology* 506, 49-60. <https://doi.org/10.1016/j.jembe.2018.05.011>.
- Amorim, E., Ramos, S., Elliott, M., Franco, A. & Bordalo, A. A. (2017). Habitat loss and gain: Influence on habitat attractiveness for estuarine fish communities. *Estuarine, Coastal and Shelf Science* 197, 244-257. <https://doi.org/10.1016/j.ecss.2017.08.043>.
- Andersen, A. K., Schou, J., Sparrevohn, C. R., Nicolajsen, H. & Støttrup, J. G. (2005a). The quality of release habitat for reared juvenile flounder, *Platichthys flesus*, with respect to salinity and depth. *Fisheries Management and Ecology* 12, 211-219. 10.1111/j.1365-2400.2005.00444.x.
- Andersen, B. S., Carl, J. D., Grønkjær, P. & Støttrup, J. G. (2005b). Feeding ecology and growth of age 0 year *Platichthys flesus* (L.) in a vegetated and a bare sand habitat in a nutrient rich fjord. *Journal of Fish Biology* 66, 531-552. doi:10.1111/j.0022-1112.2005.00620.x.
- Anderson, M. J., Gorley, R.N., Clarke, K.R. (2008). PERMANOVA + for PRIMER: Guide to Software and Statistical Methods. Plymouth: Primer-E.
- Ansell, A. D., Comely, C. A. & Robb, L. (1999). Distribution, movements and diet of macrocrustaceans on a Scottish sandy beach with particular reference to predation on juvenile fishes. *Marine Ecology Progress Series* 176, 115-130. 10.3354/meps176115.
- APA (2016). Plano de Gestão da Região Hidrográfica do Minho e Lima (RH1), Plano de Gestão da Região Hidrográfica 2016/2021. Agência Portuguesa do Ambiente.
- APHA (1992). In Standard methods for the examination of water and wastewater. Washington, D.C.: American Public Health Association.
- Armitage, P., Cranston, P. & Pinder, L. (1995). The Chironomidae: the biology and ecology of non-biting midges. London, UK: Chapman and Hall.
- Armitage, P. D., Pinder, L. C. & Cranston, P. (2011). The Chironomidae: Biology and ecology of non-biting midges: Springer Netherlands.
- Arrington, D. A., Winemiller, K. O., Loftus, W. F. & Akin, S. (2002). How often do fishes "run on empty"? *Ecology* 83, 2145-2151. doi:10.1890/0012-9658(2002)083[2145:HODFRO]2.0.CO;2.

- Attrill, M. J. & Power, M. (2004). Partitioning of temperature resources amongst an estuarine fish assemblage. *Estuarine, Coastal and Shelf Science* 61, 725-738. <https://doi.org/10.1016/j.ecss.2004.07.010>.
- Azevedo, I., Ramos, S., Mucha, A. P. & Bordalo, A. A. (2013). Applicability of ecological assessment tools for management decision-making: A case study from the Lima estuary (NW Portugal). *Ocean and Coastal Management* 72, 54-63. 10.1016/j.ocecoaman.2011.08.008.
- Baeta, A., Vieira, L. R., Lírio, A. V., Canhoto, C., Marques, J. C. & Guilhermino, L. (2017). Use of stable isotope ratios of fish larvae as indicators to assess diets and patterns of anthropogenic nitrogen pollution in estuarine ecosystems. *Ecological Indicators* 83, 112-121. 10.1016/j.ecolind.2017.07.062.
- Bailey, K. M., Abookire, A. A. & Duffy-Anderson, J. T. (2008). Ocean transport paths for the early life history stages of offshore-spawning flatfishes: a case study in the Gulf of Alaska. *Fish and Fisheries* 9, 44-66. doi:10.1111/j.1467-2979.2007.00268.x.
- Bailey, K. M., Brown, E. S. & Duffy-Anderson, J. T. (2003). Aspects of distribution, transport and recruitment of Alaska plaice (*Pleuronectes quadrituberculatus*) in the Gulf of Alaska and eastern Bering Sea: comparison of marginal and central populations. *Journal of Sea Research* 50, 87-95. [https://doi.org/10.1016/S1385-1101\(03\)00064-9](https://doi.org/10.1016/S1385-1101(03)00064-9).
- Baker, R., Fry, B., Rozas, L. P. & Minello, T. J. (2013). Hydrodynamic regulation of salt marsh contributions to aquatic food webs. *Marine Ecology Progress Series* 490, 37-52. <https://doi.org/10.3354/meps10442>.
- Barrow, J., Ford, J., Day, R. & Morrongiello, J. (2018). Environmental drivers of growth and predicted effects of climate change on a commercially important fish, *Platycephalus laevigatus*. *Marine Ecology Progress Series* 598, 201-212. doi:10.3354/meps12234.
- Beaumont, W. R. C. & Mann, R. H. K. (1984). The age, growth and diet of a freshwater population of the flounder, *Platichthys flesus* (L.), in southern England. *Journal of Fish Biology* 25, 607-616. 10.1111/j.1095-8649.1984.tb04907.x.
- Beck, M. W., Heck, K. L., Able, K. W., Childers, D. L., Eggleston, D. B., Gillanders, B. M., Halpern, B., Hays, C. G., Hoshino, K., Minello, T. J., Orth, R. J., Sheridan, P. F. & Weinstein, M. P. (2001). The Identification, Conservation, and Management of Estuarine and Marine Nurseries for Fish and Invertebrates. *BioScience* 51, 633-641. 10.1641/0006-3568(2001)051[0633:TICAMO]2.0.CO;2.

- Berghahn, R., Lüdemann, K. & Ruth, M. (1995). Differences in individual growth of newly settled 0-group plaice (*Pleuronectes Platessa* L.) in the intertidal of neighbouring wadden sea areas. *Netherlands Journal of Sea Research* 34, 131-138. [https://doi.org/10.1016/0077-7579\(95\)90021-7](https://doi.org/10.1016/0077-7579(95)90021-7).
- Besyst, B., Cattrijsse, A. & Mees, J. (1999). Feeding ecology of juvenile flatfishes of the surf zone of a sandy beach. *Journal of Fish Biology* 55, 1171-1186. [10.1111/j.1095-8649.1999.tb02068.x](https://doi.org/10.1111/j.1095-8649.1999.tb02068.x).
- Beukers-Stewart, B. D. & Jones, G. P. (2004). The influence of prey abundance on the feeding ecology of two piscivorous species of coral reef fish. *Journal of Experimental Marine Biology and Ecology* 299, 155-184. <https://doi.org/10.1016/j.jembe.2003.08.015>.
- Boesch, D. F. & Turner, R. E. (1984). Dependence of fishery species on salt marshes: The role of food and refuge. *Estuaries* 7, 460-468. [10.2307/1351627](https://doi.org/10.2307/1351627).
- Bœuf, G. & Payan, P. (2001). How should salinity influence fish growth? *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 130, 411-423. [https://doi.org/10.1016/S1532-0456\(01\)00268-X](https://doi.org/10.1016/S1532-0456(01)00268-X).
- Bos, A. R. (1999). Tidal transport of flounder larvae (*Platichthys flesus*) in the Elbe River, Germany. *Arch. Fish. Mar. Res* 47, 47-60.
- Bos, A. R. & Thiel, R. (2006). Influence of salinity on the migration of postlarval and juvenile flounder *Pleuronectes flesus* L. in a gradient experiment. *Journal of Fish Biology* 68, 1411-1420. [doi:10.1111/j.0022-1112.2006.01023.x](https://doi.org/10.1111/j.0022-1112.2006.01023.x).
- Bowen, S. H., Lutz, E. V. & Ahlgren, M. O. (1995). Dietary Protein and Energy as Determinants of Food Quality: Trophic Strategies Compared. *Ecology* 76, 899-907. [doi:10.2307/1939355](https://doi.org/10.2307/1939355).
- Bray, J. R. & Curtis, J. T. (1957). An ordination of the upland forest communities of southern Wisconsin. *Ecology Monographs* 27, 325-349. [doi:10.2307/1942268](https://doi.org/10.2307/1942268).
- Brett, J. R. & Groves, T. D. D. (1979). 6 - Physiological Energetics. In *Fish Physiology* (Hoar, W. S., Randall, D. J. & Brett, J. R., eds.), pp. 279-352: Academic Press.
- Buckley, L., Caldarone, E. & Ong, T.-L. (1999). RNA–DNA ratio and other nucleic acid-based indicators for growth and condition of marine fishes. *Hydrobiologia* 401, 265-277. [10.1023/a:1003798613241](https://doi.org/10.1023/a:1003798613241).

- Buckley, L. J. (1984). RNA-DNA ratio: an index of larval fish growth in the sea. *Marine Biology* 80, 291-298. 10.1007/bf00392824.
- Bulow, F. J. (1970). RNA–DNA Ratios as Indicators of Recent Growth Rates of a Fish. *Journal of the Fisheries Research Board of Canada* 27, 2343-2349. 10.1139/f70-262.
- Burke, J. S. (1995). Role of feeding and prey distribution of summer and southern flounder in selection of estuarine nursery habitats. *Journal of Fish Biology* 47, 355-366. doi:10.1111/j.1095-8649.1995.tb01905.x.
- Burrows, M. T., Gibson, R. N., Robb, L. & Comely, C. A. (1994). Temporal patterns of movement in juvenile flatfishes and their predators: underwater television observations. *Journal of Experimental Marine Biology and Ecology* 177, 251-268. [https://doi.org/10.1016/0022-0981\(94\)90240-2](https://doi.org/10.1016/0022-0981(94)90240-2).
- Burrows, M. T., Gontarek, S. J., Nash, R. D. M. & Gibson, R. N. (2001). Shrimp predation on 0-group plaice: contrasts between field data and predictions of an individual-based model. *Journal of Sea Research* 45, 243-254. [http://dx.doi.org/10.1016/S1385-1101\(01\)00045-4](http://dx.doi.org/10.1016/S1385-1101(01)00045-4).
- Cabral, H. & Costa, M. J. (1999). Differential Use of Nursery Areas Within the Tagus Estuary by Sympatric Soles, *Solea solea* and *Solea senegalensis*. *Environmental Biology of Fishes* 56, 389-397. 10.1023/a:1007571523120.
- Cabral, H. N., Costa, M. J. & Salgado, J. P. (2001). Does the Tagus estuary fish community reflect environmental changes? *Climate Research* 18, 119-126. 10.3354/cr018119.
- Cabral, H. N., Lopes, M. & Loeper, R. (2002). Trophic niche overlap between flatfishes in a nursery area on the Portuguese coast. *Scientia Marina* 66, 293-300. 10.3989/scimar.2002.66n3293.
- Cabral, H. N., Vasconcelos, R., Vinagre, C., França, S., Fonseca, V., Maia, A., Reis-Santos, P., Lopes, M., Ruano, M., Campos, J., Freitas, V., Santos, P. T. & Costa, M. J. (2007). Relative importance of estuarine flatfish nurseries along the Portuguese coast. *Journal of Sea Research* 57, 209-217. <http://dx.doi.org/10.1016/j.seares.2006.08.007>.
- Caldarone, E. M., Clemmesen, C. M., Berdalet, E., Miller, T. J., Folkvord, A., Holt, G. J., Olivar, M. P. & Suthers, I. M. (2006). Intercalibration of four spectrofluorometric protocols for measuring RNA/DNA ratios in larval and juvenile fish. *Limnology and Oceanography: Methods* 4, 153-163. 10.4319/lom.2006.4.153.

- Caldarone, E. M., MacLean, S. A. & Sharack, B. (2012). Evaluation of bioelectrical impedance analysis and Fulton's condition factor as nonlethal techniques for estimating short-term responses in postsmolt Atlantic salmon (*Salmo salar*) to food availability. *Fishery Bulletin* 110.
- Caldarone, E. M., Wagner, M., Onge-Burns, J. S. & Buckley, L. J. (2001). Protocol and Guide for Estimating Nucleic Acids in Larval Fish Using a Fluorescence Microplate Reader. Northeast Fisheries Science Center Reference Document 01-11, 22.
- Camp, A. L., Ryer, C. H., Laurel, B. & Seals, K. (2011). Effect of nursery habitat on density-dependent habitat selection in juvenile flatfish. *Journal of Experimental Marine Biology and Ecology* 404, 15-20. <https://doi.org/10.1016/j.jembe.2011.05.001>.
- Campana, S. E. & Neilson, J. D. (1985). Microstructure of Fish Otoliths. *Canadian Journal of Fisheries and Aquatic Sciences* 42, 1014-1032. 10.1139/f85-127.
- Cardoso, J. F. M. F., Freitas, V., de Paoli, H., Witte, J. I. J. & van der Veer, H. W. (2016). Growth conditions of 0-group plaice *Pleuronectes platessa* in the western Wadden Sea as revealed by otolith microstructure analysis. *Journal of Sea Research* 111, 88-96. <https://doi.org/10.1016/j.seares.2016.01.010>.
- Cardoso, R., Araújo, M. d. F., Freitas, M. d. C. & Fatela, F. (2008). Geochemical characterisation of sediments from marginal environments of Lima Estuary (NW Portugal). *e-Terra* 5, 1-11.
- Castillo-Rivera, B. M., Kobelkowsky, A. & Chávez, A. M. (2000). Feeding biology of the flatfish *Citharichthys spilopterus* (Bothidae) in a tropical estuary of Mexico. *Journal of Applied Ichthyology* 16, 73-78. doi:10.1046/j.1439-0426.2000.00151.x.
- Chambers, R. C., Leggett, W. C. & Brown, J. A. (1988). Variation in and among early life history traits of laboratory-reared winter flounder *Pseudopleuronectes americanus*. *Marine Ecology Progress Series* 47, 1-15. 10.3354/meps047001.
- Chícharo, M. & Chícharo, L. (2008). RNA:DNA Ratio and Other Nucleic Acid Derived Indices in Marine Ecology. *International Journal of Molecular Sciences* 9, 1453-1471. 10.3390/ijms9081453.
- Ciotti, B. J., Targett, T. E. & Burrows, M. T. (2013a). Decline in growth rate of juvenile European plaice (*Pleuronectes platessa*) during summer at nursery beaches along the west coast of Scotland. *Canadian Journal of Fisheries and Aquatic Sciences* 70, 720-734. 10.1139/cjfas-2012-0331.

- Ciotti, B. J., Targett, T. E. & Burrows, M. T. (2013b). Spatial variation in growth rate of early juvenile European plaice *Pleuronectes platessa*. Marine Ecology Progress Series 475, 213-232. 10.3354/meps10087.
- Ciotti, B. J., Targett, T. E., Nash, R. D. M., Batty, R. S., Burrows, M. T. & Geffen, A. J. (2010). Development, validation and field application of an RNA-based growth index in juvenile plaice *Pleuronectes platessa*. Journal of Fish Biology 77, 2181-2209. 10.1111/j.1095-8649.2010.02786.x.
- Ciotti, B. J., Targett, T. E., Nash, R. D. M. & Burrows, M. T. (2013b). Small-scale spatial and temporal heterogeneity in growth and condition of juvenile fish on sandy beaches. Journal of Experimental Marine Biology and Ecology 448, 346-359. <http://dx.doi.org/10.1016/j.jembe.2013.08.003>.
- Claridge, P. N., Potter, I. C. & Hardisty, M. W. (1986). Seasonal Changes in Movements, Abundance, Size Composition and Diversity of the Fish Fauna of the Severn Estuary. Journal of the Marine Biological Association of the United Kingdom 66, 229-258. 10.1017/S002531540003976X.
- Clarke, K. R. & Gorley, R. N. (2006). Primer V6: User Manual - Tutorial: Plymouth Marine Laboratory.
- Clarke, K. R. & Warwick, R. (2001). Change in marine communities. An approach to statistical analysis and interpretation.
- Clemmesen, C. (1988). A RNA and DNA fluorescence technique to evaluate the nutritional condition of marine fish larvae. Meeresforschung-Reports on Marine Research 32, 134-143.
- Clemmesen, C. (1994). The effect of food availability, age or size on the RNA/DNA ratio of individually measured herring larvae: laboratory calibration. Marine Biology 118, 377-382. 10.1007/bf00350294.
- Communities, E. (2000). Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy. p. 75: Official Journal of the European Communities.
- Costa-Dias, S., Sousa, R. & Antunes, C. (2010). Ecological quality assessment of the lower Lima Estuary. Marine Pollution Bulletin 61, 234-239. 10.1016/j.marpolbul.2010.02.019.

- Costa, M. J. & Bruxelas, A. (1989). The structure of fish communities in the Tagus Estuary, Portugal, and its role as a nursery for commercial fish species. *Scientia Marina* 53, 561-566.
- Courrat, A., Lobry, J., Nicolas, D., Laffargue, P., Amara, R., Lepage, M., Girardin, M. & Le Pape, O. (2009). Anthropogenic disturbance on nursery function of estuarine areas for marine species. *Estuarine, Coastal and Shelf Science* 81, 179-190. <https://doi.org/10.1016/j.ecss.2008.10.017>.
- Dahlgren, C. P., Kellison, G. T., Adams, A. J., Gillanders, B. M., Kendall, M. S., Layman, C. A., Ley, J. A., Nagelkerken, I. & Serafy, J. E. (2006). Marine nurseries and effective juvenile habitats: concepts and applications. *Marine Ecology Progress Series* 312, 291-295. 10.3354/meps312291.
- Dando, P. R. (2011). Site fidelity, homing and spawning migrations of flounder *Platichthys flesus* in the Tamar estuary, South West England. *Marine Ecology Progress Series* 430, 183-196. <https://doi.org/10.3354/meps09116>.
- Darnaude, A. M., Harmelin-Vivien, M. L. & Salen-Picard, C. (2001). Food partitioning among flatfish (Pisces: Pleuronectiforms) juveniles in a Mediterranean coastal shallow sandy area. *Journal of the Marine Biological Association of the United Kingdom* 81, 119-127. 10.1017/S0025315401003460.
- Darnaude, A. M., Salen-Picard, C. & Harmelin-Vivien, M. L. (2004). Depth variation in terrestrial particulate organic matter exploitation by marine coastal benthic communities off the Rhone River delta (NW Mediterranean). *Marine Ecology Progress Series* 275, 47-57. 10.3354/meps275047.
- Daverat, F., Morais, P., Dias, E., Babaluk, J., Martin, J., Eon, M., Fablet, R., Pécheyran, C. & Antunes, C. (2012). Plasticity of European flounder life history patterns discloses alternatives to catadromy. *Marine Ecology Progress Series* 465, 267-280. 10.3354/meps09910.
- Day, J. W., Kemp, W. M., Yanez-Arancibia, A. & Crump, B. C. (2012). *Estuarine Ecology*. New Jersey: John Wiley & Sons, Inc.
- De Groot, S. J. (1971). On the interrelationships between morphology of the alimentary tract, food and feeding behaviour in flatfishes (pisces: pleuronectiformes). *Netherlands Journal of Sea Research* 5, 121-196. [http://dx.doi.org/10.1016/0077-7579\(71\)90008-1](http://dx.doi.org/10.1016/0077-7579(71)90008-1).
- De Raedemaeker, F., Brophy, D., O'Connor, I. & Comerford, S. (2012a). Habitat characteristics promoting high density and condition of juvenile flatfish at nursery grounds on

the west coast of Ireland. *Journal of Sea Research* 73, 7-17.
<https://doi.org/10.1016/j.seares.2012.04.013>.

De Raedemaeker, F., Brophy, D., O'Connor, I. & O'Neill, B. (2012b). Dependence of RNA:DNA ratios and Fulton's K condition indices on environmental characteristics of plaice and dab nursery grounds. *Estuarine, Coastal and Shelf Science* 98, 60-70.
<https://doi.org/10.1016/j.ecss.2011.11.033>.

De Raedemaeker, F., Keating, J., Brophy, D., O'Connor, I. & Mc Grath, D. (2011). Spatial variability in diet, condition and growth of juvenile plaice (*Pleuronectes platessa*) at sandy beach nursery grounds on the south-west coast of Ireland. *Journal of the Marine Biological Association of the United Kingdom* 91, 1215-1223. 10.1017/S0025315410001505.

DeNiro, M. J. & Epstein, S. (1978). Influence of diet on the distribution of carbon isotopes in animals. *Geochimica et Cosmochimica Acta* 42, 495-506. [http://dx.doi.org/10.1016/0016-7037\(78\)90199-0](http://dx.doi.org/10.1016/0016-7037(78)90199-0).

Deniro, M. J. & Epstein, S. (1981). Influence of diet on the distribution of nitrogen isotopes in animals. *Geochimica et Cosmochimica Acta* 45, 341-351. [https://doi.org/10.1016/0016-7037\(81\)90244-1](https://doi.org/10.1016/0016-7037(81)90244-1).

Dias, E., Morais, P., Leopold, M., Campos, J. & Antunes, C. (2012). Natural born indicators: Great cormorant *Phalacrocorax carbo* (Aves: Phalacrocoracidae) as monitors of river discharge influence on estuarine ichthyofauna. *Journal of Sea Research* 73, 101-108.
<https://doi.org/10.1016/j.seares.2012.06.012>.

Dinis, M. T. (1986). Quatre Soleidae de l'estuaire du Tage. Reproduction et croissance. Essai d'élevage de *Solea senegalensis* Kaup. Brest, France: Université de Bretagne Occidentale.

Dörner, H. & Wagner, A. (2003). Size-dependent predator–prey relationships between perch and their fish prey. *Journal of Fish Biology* 62, 1021-1032. doi:10.1046/j.1095-8649.2003.00092.x.

Edgar, G. J. & Shaw, C. (1995). The production and trophic ecology of shallow-water fish assemblages in southern Australia III. General relationships between sediments, seagrasses, invertebrates and fishes. *Journal of Experimental Marine Biology and Ecology* 194, 107-131.
[https://doi.org/10.1016/0022-0981\(95\)00085-2](https://doi.org/10.1016/0022-0981(95)00085-2).

- Edwards, R. & Steele, J. H. (1968). The ecology of 0-group plaice and common dabs at Loch Ewe I. Population and food. *Journal of Experimental Marine Biology and Ecology* 2, 215-238. [http://dx.doi.org/10.1016/0022-0981\(68\)90017-8](http://dx.doi.org/10.1016/0022-0981(68)90017-8).
- Edwards, R. R. C., Steele, J. H. & Trevallion, A. (1970). The ecology of 0-group plaice and common dabs in loch ewe. III. Prey-predator experiments with plaice. *Journal of Experimental Marine Biology and Ecology* 4, 156-173. [http://dx.doi.org/10.1016/0022-0981\(70\)90022-5](http://dx.doi.org/10.1016/0022-0981(70)90022-5).
- Elliott, M., Burdon, D., Hemingway, K. L. & Apitz, S. E. (2007a). Estuarine, coastal and marine ecosystem restoration: Confusing management and science – A revision of concepts. *Estuarine, Coastal and Shelf Science* 74, 349-366. <http://dx.doi.org/10.1016/j.ecss.2007.05.034>.
- Elliott, M. & Dewailly, F. (1995). The structure and components of European estuarine fish assemblages. *Netherland Journal of Aquatic Ecology* 29, 397-417. 10.1007/BF02084239.
- Elliott, M. & Hemingway, K. L. (2002). *Fishes in estuaries*: John Wiley & Sons.
- Elliott, M., Mander, L., Mazik, K., Simenstad, C., Valesini, F., Whitfield, A. & Wolanski, E. (2016). Ecoengineering with Ecohydrology: Successes and failures in estuarine restoration. *Estuarine, Coastal and Shelf Science* 176, 12-35. <http://dx.doi.org/10.1016/j.ecss.2016.04.003>.
- Elliott, M. & Quintino, V. (2007). The Estuarine Quality Paradox, Environmental Homeostasis and the difficulty of detecting anthropogenic stress in naturally stressed areas. *Marine Pollution Bulletin* 54, 640-645. <https://doi.org/10.1016/j.marpolbul.2007.02.003>.
- Elliott, M. & Whitfield, A. K. (2011). Challenging paradigms in estuarine ecology and management. *Estuarine, Coastal and Shelf Science* 94, 306-314. <https://doi.org/10.1016/j.ecss.2011.06.016>.
- Elliott, M., Whitfield, A. K., Potter, I. C., Blaber, S. J. M., Cyrus, D. P., Nordlie, F. G. & Harrison, T. D. (2007b). The guild approach to categorizing estuarine fish assemblages: a global review. *Fish and Fisheries* 8, 241-268. doi:10.1111/j.1467-2679.2007.00253.x.
- Ellis, T. & Gibson, R. N. (1995). Size-selective predation of 0-group flatfishes on a Scottish coastal nursery ground. *Marine Ecology Progress Series* 127, 27-37. doi: 10.3354/meps127027.

- Evans, S. (1983). Production, Predation and Food Niche Segregation in a Marine Shallow Soft-Bottom Community. *Marine Ecology Progress Series* 10, 147-157. [10.3354/meps010147](https://doi.org/10.3354/meps010147).
- Fairbridge, R. W. (1980). The estuary: Its definition and geodynamic cycle. In *Chemistry and biogeochemistry of estuaries* (Olausson, E. & Cato, I., eds.), pp. 1-35. Chichester: Wiley.
- Falcão, A. P., Mazzolari, A., Gonçalves, A. B., Araújo, M. A. V. C. & Trigo-Teixeira, A. (2013). Influence of elevation modelling on hydrodynamic simulations of a tidally-dominated estuary. *Journal of Hydrology* 497, 152-164. <https://doi.org/10.1016/j.jhydrol.2013.05.045>.
- FAO (2018). <http://www.fao.org/fishery/species/2550/en>.
- Fauchald, K. & Jumars, P. A. (1979). The diet of worms: a study of polychaete feeding guilds. *Oceanography and Marine Biology, An Annual Review* 17, 193–284.
- Ferron, A. & Leggett, W. C. (1994). An Appraisal of Condition Measures for Marine Fish Larvae. *Advances in Marine Biology* 30, 217-303. [http://dx.doi.org/10.1016/S0065-2881\(08\)60064-4](http://dx.doi.org/10.1016/S0065-2881(08)60064-4).
- Florin, A.-B. & Lavados, G. (2010). Feeding habits of juvenile flatfish in relation to habitat characteristics in the Baltic Sea. *Estuarine, Coastal and Shelf Science* 86, 607-612. <https://doi.org/10.1016/j.ecss.2009.11.028>.
- Fonds, M., Cronie, R., Vethaak, A. D. & Van Der Puyl, P. (1992). Metabolism, food consumption and growth of plaice (*Pleuronectes platessa*) and flounder (*Platichthys flesus*) in relation to fish size and temperature. *Netherlands Journal of Sea Research* 29, 127-143. [https://doi.org/10.1016/0077-7579\(92\)90014-6](https://doi.org/10.1016/0077-7579(92)90014-6).
- Fonseca, V. F., Vinagre, C. & Cabral, H. N. (2006). Growth variability of juvenile soles *Solea solea* and *Solea senegalensis*, and comparison with RNA : DNA ratios in the Tagus estuary, Portugal. *Journal of Fish Biology* 68, 1551-1562. [10.1111/j.0022-1112.2006.001042.x](https://doi.org/10.1111/j.0022-1112.2006.001042.x).
- França, S., Vasconcelos, R. P., Tanner, S., Máguas, C., Costa, M. J. & Cabral, H. N. (2011). Assessing food web dynamics and relative importance of organic matter sources for fish species in two Portuguese estuaries: A stable isotope approach. *Marine Environmental Research* 72, 204-215. <http://dx.doi.org/10.1016/j.marenvres.2011.09.001>.
- Franco, A., Elliott, M., Franzoi, P. & Torricelli, P. (2008). Life strategies of fishes in European estuaries: the functional guild approach. *Marine Ecology Progress Series* 354, 219-228. <https://doi.org/10.3354/meps07203>.

- Freitas, V., Costa-Dias, S., Campos, J., Bio, A., Santos, P. & Antunes, C. (2009). Patterns in abundance and distribution of juvenile flounder, *Platichthys flesus*, in Minho estuary (NW Iberian Peninsula). *Aquatic Ecology* 43, 1143. 10.1007/s10452-009-9237-8.
- Freitas, V., Kooijman, S. & van der Veer, H. W. (2012). Latitudinal trends in habitat quality of shallow-water flatfish nurseries. *Marine Ecology Progress Series* 471, 203-214. <https://doi.org/10.3354/meps10025>.
- Fry, B. (2002). Conservative mixing of stable isotopes across estuarine salinity gradients: A conceptual framework for monitoring watershed influences on downstream fisheries production. *Estuaries* 25, 264-271. 10.1007/bf02691313.
- Fry, B. & Sherr, E. B. (1989). $\delta^{13}\text{C}$ Measurements as Indicators of Carbon Flow in Marine and Freshwater Ecosystems. In *Stable Isotopes in Ecological Research* (Rundel, P. W., Ehleringer, J. R. & Nagy, K. A., eds.), pp. 196-229. New York, NY: Springer New York.
- Geffen, A. J., van der Veer, H. W. & Nash, R. D. M. (2007). The cost of metamorphosis in flatfishes. *Journal of Sea Research* 58, 35-45. <https://doi.org/10.1016/j.seares.2007.02.004>.
- Gerdol, V. & Hughes, R. (1994). Feeding behaviour and diet of *Corophium volutator* in an estuary in southeastern England. *Marine Ecology-progress Series* 114, 103-103.
- Gibson, R. N. (1973). Tidal and circadian activity rhythms in juvenile plaice, *Pleuronectes platessa*. *Marine Biology* 22, 379-386. 10.1007/BF00391398.
- Gibson, R. N. (1994). Impact of habitat quality and quantity on the recruitment of juvenile flatfishes. *Netherlands Journal of Sea Research* 32, 191-206. [http://dx.doi.org/10.1016/0077-7579\(94\)90040-X](http://dx.doi.org/10.1016/0077-7579(94)90040-X).
- Gibson, R. N., Nash, R. D., Geffen, A. J. & Van der Veer, H. W. (2005). *Flatfishes: biology and exploitation*: John Wiley & Sons.
- Gibson, R. N., Pihl, L., Burrows, M. T., Modin, J., Wennhage, H. & Nickell, L. A. (1998). Diel movements of juvenile plaice *Pleuronectes platessa* in relation to predators, competitors, food availability and abiotic factors on a microtidal nursery ground. *Marine Ecology Progress Series* 165, 145-159. 10.3354/meps165145.
- Gibson, R. N., Robb, L., Burrows, M. T. & Ansell, A. D. (1996). Tidal, diel and longer term changes in the distribution of fishes on a Scottish sandy beach. *Marine Ecology Progress Series* 130, 1-17. :10.3354/meps130001.

- Gibson, R. N., Robb, L., Wennhage, H. & Burrows, M. T. (2002). Ontogenetic changes in depth distribution of juvenile flatfishes in relation to predation risk and temperature on a shallow-water nursery ground. *Marine Ecology Progress Series* 229, 233-244. 10.3354/meps229233.
- Gilliers, C., Amara, R. & Bergeron, J.-P. (2004). Comparison of growth and condition indices of juvenile flatfish in different coastal nursery grounds. *Environmental Biology of Fishes* 71, 189-198. 10.1007/s10641-004-0090-2.
- Glass, L. A., Rooker, J. R., Kraus, R. T. & Holt, G. J. (2008). Distribution, condition, and growth of newly settled southern flounder (*Paralichthys lethostigma*) in the Galveston Bay Estuary, TX. *Journal of Sea Research* 59, 259-268. <https://doi.org/10.1016/j.seares.2008.02.006>.
- Gning, N., Vidy, G. & Thiaw, O. T. (2008). Feeding ecology and ontogenic diet shifts of juvenile fish species in an inverse estuary: The Sine-Saloum, Senegal. *Estuarine, Coastal and Shelf Science* 76, 395-403. <https://doi.org/10.1016/j.ecss.2007.07.018>.
- Google Earth V 7.3.2.5491. (October 10, 2018). The Lima River estuary, Portugal. 41°40'49.71"N, 8°50'30.53"W. <http://www.earth.google.com> [October 10, 2018].
- Graeb, B. D. S., Mangan, M. T., Jolley, J. C., Wahl, D. H. & Dettmers, J. M. (2006). Ontogenetic Changes in Prey Preference and Foraging Ability of Yellow Perch: Insights Based on Relative Energetic Return of Prey. *Transactions of the American Fisheries Society* 135, 1493-1498. 10.1577/T05-063.1.
- Gravato, C., Guimarães, L., Santos, J., Faria, M., Alves, A. & Guilhermino, L. (2010). Comparative study about the effects of pollution on glass and yellow eels (*Anguilla anguilla*) from the estuaries of Minho, Lima and Douro Rivers (NW Portugal). *Ecotoxicology and Environmental Safety* 73, 524-533. <https://doi.org/10.1016/j.ecoenv.2009.11.009>.
- Green, B. C., Smith, D. J., Grey, J. & Underwood, G. J. C. (2012). High site fidelity and low site connectivity in temperate salt marsh fish populations: a stable isotope approach. *Oecologia* 168, 245-255. 10.1007/s00442-011-2077-y.
- Greenwood, M. F. D. & Hill, A. S. (2003). Temporal, spatial and tidal influences on benthic and demersal fish abundance in the Forth estuary. *Estuarine, Coastal and Shelf Science* 58, 211-225. [https://doi.org/10.1016/S0272-7714\(03\)00071-4](https://doi.org/10.1016/S0272-7714(03)00071-4).
- Grioche, A., Harlay, X., Koubbi, P. & Lago, L. F. (2000). Vertical migrations of fish larvae: Eulerian and Lagrangian observations in the Eastern English Channel. *Journal of Plankton Research* 22, 1813-1828. 10.1093/plankt/22.10.1813.

- Grioche, A., Koubbi, P. & Sautour, B. (1997). Ontogenic migration of *Pleuronectes flesus* larvae in the eastern English Channel. *Journal of Fish Biology* 51, 385-396. doi:10.1111/j.1095-8649.1997.tb06110.x.
- Grol, M. G. G., Rypel, A. L. & Nagelkerken, I. (2014). Growth potential and predation risk drive ontogenetic shifts among nursery habitats in a coral reef fish. *Marine Ecology Progress Series* 502, 229-244. <https://doi.org/10.3354/meps10682>.
- Grønkjær, P., Carl, J. D., Rasmussen, T. H. & Hansen, K. W. (2007). Effect of habitat shifts on feeding behaviour and growth of 0 year-group flounder *Platichthys flesus* (L.) transferred between macroalgae and bare sand habitats. *Journal of Fish Biology* 70, 1587-1605. doi:10.1111/j.1095-8649.2007.01447.x.
- Gutt, J. (1985). The growth of juvenile flounders (*Platichthys flesus* L.) at salinities of 0, 5, 15 and 35‰. *Journal of Applied Ichthyology* 1, 17-26. 10.1111/j.1439-0426.1985.tb00406.x.
- Gwak, W. S. & Tanaka, M. (2001). Developmental change in RNA: DNA ratios of fed and starved laboratory-reared Japanese flounder larvae and juveniles, and its application to assessment of nutritional condition for wild fish. *Journal of Fish Biology* 59, 902-915. 10.1111/j.1095-8649.2001.tb00160.x.
- Haedrich, R. L. (1983). Estuarine fishes. In *Estuaries and enclosed seas* (Ketchum, B. H., ed.), pp. 183-207. Amsterdam: Elsevier.
- Hammerschlag, N., Heithaus, M. R. & Serafy, J. E. (2010). Influence of predation risk and food supply on nocturnal fish foraging distributions along a mangrove–seagrass ecotone. *Marine Ecology Progress Series* 414, 223-235. <https://doi.org/10.3354/meps08731>.
- Hampel, H., Cattrijsse, A. & Elliott, M. (2005). Feeding habits of young predatory fishes in marsh creeks situated along the salinity gradient of the Schelde estuary, Belgium and The Netherlands. *Helgoland Marine Research* 59, 151-162. 10.1007/s10152-004-0214-2.
- Harmelin-Vivien, M., Loizeau, V., Mellon, C., Beker, B., Arlhac, D., Bodiguel, X., Ferraton, F., Hermand, R., Philippon, X. & Salen-Picard, C. (2008). Comparison of C and N stable isotope ratios between surface particulate organic matter and microphytoplankton in the Gulf of Lions (NW Mediterranean). *Continental Shelf Research* 28, 1911-1919. <http://dx.doi.org/10.1016/j.csr.2008.03.002>.
- Harter, S. L. & Heck, K. L. (2006). Growth rates of juvenine pinfish (*Lagodon rhomboides*): Effects of habitat and predation risk. *Estuaries and Coasts* 29, 318-327. 10.1007/bf02782000.

- Haynes, P. S., Brophy, D., De Raedemaeker, F. & McGrath, D. (2011). The feeding ecology of 0 year-group turbot *Scophthalmus maximus* and brill *Scophthalmus rhombus* on Irish west coast nursery grounds. *Journal of Fish Biology* 79, 1866-1882. 10.1111/j.1095-8649.2011.03128.x.
- Hemmer-Hansen, J., Nielsen, E. E., Grønkvær, P. & Loeschcke, V. (2007). Evolutionary mechanisms shaping the genetic population structure of marine fishes; lessons from the European flounder (*Platichthys flesus* L.). *Molecular Ecology* 16, 3104-3118. doi:10.1111/j.1365-294X.2007.03367.x.
- Henderson, P. A. & Holmes, R. H. A. (1991). On the population dynamics of dab, sole and flounder within Bridgwater bay in the lower Severn estuary, England. *Netherlands Journal of Sea Research* 27, 337-344. [http://dx.doi.org/10.1016/0077-7579\(91\)90036-Z](http://dx.doi.org/10.1016/0077-7579(91)90036-Z).
- Henderson, P. A. & Seaby, R. M. (2005). The role of climate in determining the temporal variation in abundance, recruitment and growth of sole *Solea solea* in the Bristol Channel. *Journal of the Marine Biological Association of the United Kingdom* 85, 197-204. 10.1017/S0025315405011069h.
- Herzka, S. Z. (2005). Assessing connectivity of estuarine fishes based on stable isotope ratio analysis. *Estuarine, Coastal and Shelf Science* 64, 58-69. <https://doi.org/10.1016/j.ecss.2005.02.006>.
- Hoffman, J. C., Kelly, J. R., Peterson, G. S. & Cotter, A. M. (2015). Landscape-Scale Food Webs of Fish Nursery Habitat Along a River-Coast Mixing Zone. *Estuaries and Coasts* 38, 1335-1349. 10.1007/s12237-014-9880-8.
- Houde, E. (1987). Fish early life dynamics and recruitment variability. *American Fisheries Society Symposium* 2, 17-29.
- Hovenkamp, F. (1991). Immigration of larval plaice (*Pleuronectes platessa* L.) into the western wadden sea: A question of timing. *Netherlands Journal of Sea Research* 27, 287-296. [https://doi.org/10.1016/0077-7579\(91\)90031-U](https://doi.org/10.1016/0077-7579(91)90031-U).
- Hovenkamp, F. & Witte, J. I. (1991). Growth, otolith growth and RNA/DNA ratios of larval plaice *Pleuronectes platessa* in the North Sea 1987 to 1989. *Marine Ecology Progress Series* 70, 105-116. 10.3354/meps070105.

- Hughes, R. G. (1988). Dispersal by Benthic Invertebrates: The in Situ Swimming Behaviour of the Amphipod *Corophium Volutator*. *Journal of the Marine Biological Association of the United Kingdom* 68, 565-579. 10.1017/S002531540002871X.
- Hugie, D. M. & Dill, L. M. (1994). Fish and game: a game theoretic approach to habitat selection by predators and prey. *Journal of Fish Biology* 45, 151-169. doi:10.1111/j.1095-8649.1994.tb01090.x.
- Hurst, T. P., Abookire, A. A. & Knoth, B. (2009). Quantifying thermal effects on contemporary growth variability to predict responses to climate change in northern rock sole (*Lepidopsetta polyxystra*). *Canadian Journal of Fisheries and Aquatic Sciences* 67, 97-107. 10.1139/F09-171.
- Hutchinson, S. & Hawkins, L. E. (2004). The relationship between temperature and the size and age of larvae and peri-metamorphic stages of *Pleuronectes flesus*. *Journal of Fish Biology* 65, 448-459. doi:10.1111/j.0022-1112.2004.00462.x.
- Hyslop, E. J. (1980). Stomach contents analysis—a review of methods and their application. *Journal of Fish Biology* 17, 411-429. 10.1111/j.1095-8649.1980.tb02775.x.
- ICES (2008). Report of the 2nd Workshop on Age Reading of Flounder (WKARFLO), 26-29 May 2008. Rostock, Germany: ICES.
- Jager, Z. (1998). Accumulation of flounder larvae (*Platichthys flesus* L.) in the Dollard (Ems estuary, Wadden Sea). *Journal of Sea Research* 40, 43-57. [https://doi.org/10.1016/S1385-1101\(98\)00010-0](https://doi.org/10.1016/S1385-1101(98)00010-0).
- Jager, Z. (1999). Selective Tidal Stream Transport of Flounder Larvae (*Platichthys flesus* L.) in the Dollard (Ems Estuary). *Estuarine, Coastal and Shelf Science* 49, 347-362. <https://doi.org/10.1006/ecss.1999.0504>.
- Jager, Z., Kleef, H. L. & Tydeman, P. (1993). The distribution of 0-group flatfish in relation to abiotic factors on the tidal flats in the brackish Dollard (Ems Estuary, Wadden Sea). *Journal of Fish Biology* 43, 31-43. doi:10.1111/j.1095-8649.1993.tb01177.x.
- Jager, Z., Kleef, H. L. & Tydeman, P. (1995). Mortality and growth of 0-group flatfish in the brackish dollard (Ems Estuary, Wadden Sea). *Netherlands Journal of Sea Research* 34, 119-129. [http://dx.doi.org/10.1016/0077-7579\(95\)90020-9](http://dx.doi.org/10.1016/0077-7579(95)90020-9).
- Jobling, M. (1994). *Fish Bioenergetics*. London: Chapman & Hall.

- Juanes, F., Buckel, J. A. & Scharf, F. S. (2001). Predatory behaviour and selectivity of a primary piscivore: comparison of fish and non-fish prey. *Marine Ecology Progress Series* 217, 157-165. [10.3354/meps217157](https://doi.org/10.3354/meps217157).
- Juanes, F., Jeffrey, A. B. & Scharf, F. S. (2008). Feeding Ecology of Piscivorous Fishes. In *Handbook of Fish Biology and Fisheries* (Hart, P. J. & Reynolds, J. D., eds.).
- Karakiri, M., Berghahn, R. & van der Veer, H. W. (1991). Variations in settlement and growth of 0-group plaice (*Pleuronectes platessa* L.) in the dutch wadden sea as determined by otolith microstructure analysis. *Netherlands Journal of Sea Research* 27, 345-351. [https://doi.org/10.1016/0077-7579\(91\)90037-2](https://doi.org/10.1016/0077-7579(91)90037-2).
- Karakiri, M., Berghahn, R. & von Westernhagen, H. (1989). Growth differences in 0-group plaice *Pleuronectes platessa* as revealed by otolith microstructure analysis. *Marine Ecology Progress Series* 55, 15-22. [10.3354/meps055015](https://doi.org/10.3354/meps055015).
- Keast, A. & Webb, D. (1966). Mouth and Body Form Relative to Feeding Ecology in the Fish Fauna of a Small Lake, Lake Opinicon, Ontario. *Journal of the Fisheries Research Board of Canada* 23, 1845-1874. [10.1139/f66-175](https://doi.org/10.1139/f66-175).
- Kennedy, P., Kennedy, H. & Papadimitriou, S. (2005). The effect of acidification on the determination of organic carbon, total nitrogen and their stable isotopic composition in algae and marine sediment. *Rapid Communications in Mass Spectrometry* 19, 1063-1068. [10.1002/rcm.1889](https://doi.org/10.1002/rcm.1889).
- Kerstan, M. (1991). The importance of rivers as nursery grounds for 0- and 1-group flounder (*Platichthys flesus* L.) in comparison to the Wadden sea. *Netherlands Journal of Sea Research* 27, 353-366. [http://dx.doi.org/10.1016/0077-7579\(91\)90038-3](http://dx.doi.org/10.1016/0077-7579(91)90038-3).
- Kimirei, I. A., Nagelkerken, I., Trommelen, M., Blankers, P., van Hoytema, N., Hoeijmakers, D., Huijbers, C. M., Mgaya, Y. D. & Rypel, A. L. (2013). What Drives Ontogenetic Niche Shifts of Fishes in Coral Reef Ecosystems? *Ecosystems* 16, 783-796. <https://doi.org/10.1007/s10021-013-9645-4>.
- Kooijman, S. A. L. M. & Kooijman, S. A. L. M. (2000). *Dynamic energy and mass budgets in biological systems*: Cambridge university press.
- Kopp, D., Le Bris, H., Grimaud, L., Nérot, C. & Brind'Amour, A. (2013). Spatial analysis of the trophic interactions between two juvenile fish species and their preys along a coastal–estuarine gradient. *Journal of Sea Research* 81, 40-48. <https://doi.org/10.1016/j.seares.2013.03.013>.

- Kostecki, C., Roussel, J. M., Desroy, N., Roussel, G., Lanshere, J., Le Bris, H. & Le Pape, O. (2012). Trophic ecology of juvenile flatfish in a coastal nursery ground: contributions of intertidal primary production and freshwater particulate organic matter. *Marine Ecology Progress Series* 449, 221-232. <https://doi.org/10.3354/meps09563>.
- Koubbi, P., Loots, C., Cotonnec, G., Harlay, X., Grioche, A., Vaz, S., Martin, C., Walkey, M. & Carpentier, A. (2006). Spatial patterns and GIS habitat modelling of *Solea solea*, *Pleuronectes flesus* and *Limanda limanda* fish larvae in the eastern English Channel during the spring. *Scientia Marina* 70S2, 147-157.
- Laffargue, P., Lagardère, F., Rijnsdorp, A. D., Fillon, A. & Amara, R. (2007). Growth performances of juvenile sole *Solea solea* under environmental constraints of embayed nursery areas. *Aquatic Living Resources* 20, 213-221. 10.1051/alr:2007034.
- Laurel, B. J., Danley, C. & Haines, S. (2014). The effects of temperature on growth, development and settlement of northern rock sole larvae (*Lepidopsetta polyxystra*). *Fisheries Oceanography* 23, 495-505. doi:10.1111/fog.12084.
- Le Pape, O., Baulier, L., Cloarec, A., Martin, J., Le Loc'h, F. & Désaunay, Y. (2007). Habitat suitability for juvenile common sole (*Solea solea*, L.) in the Bay of Biscay (France): A quantitative description using indicators based on epibenthic fauna. *Journal of Sea Research* 57, 126-136. <https://doi.org/10.1016/j.seares.2006.08.011>.
- Le Pape, O. & Bonhommeau, S. (2015). The food limitation hypothesis for juvenile marine fish. *Fish and Fisheries* 16, 373-398. 10.1111/faf.12063.
- Le Pape, O. & Cognez, N. (2016). The range of juvenile movements of estuarine and coastal nursery dependent flatfishes: estimation from a meta-analytical approach. *Journal of Sea Research* 107, 43-55. <https://doi.org/10.1016/j.seares.2015.06.001>.
- Le Pape, O., Modéran, J., Beaunée, G., Riera, P., Nicolas, D., Savoye, N., Harmelin-Vivien, M., Darnaude, A. M., Brind'Amour, A., Le Bris, H., Cabral, H., Vinagre, C., Pasquaud, S., França, S. & Kostecki, C. (2013). Sources of organic matter for flatfish juveniles in coastal and estuarine nursery grounds: A meta-analysis for the common sole (*Solea solea*) in contrasted systems of Western Europe. *Journal of Sea Research* 75, 85-95. <https://doi.org/10.1016/j.seares.2012.05.003>.
- Le Pichon, C., Trancart, T., Lambert, P., Daverat, F. & Rochard, E. (2014). Summer habitat use and movements of late juvenile European flounder (*Platichthys flesus*) in tidal freshwaters:

- Results from an acoustic telemetry study. *Journal of Experimental Marine Biology and Ecology* 461, 441-448. <https://doi.org/10.1016/j.jembe.2014.09.015>.
- Leggett, W. C. & DeBlois, E. (1994). Recruitment in marine fishes: Is it regulated by starvation and predation in the egg and larval stages? *Netherlands Journal of Sea Research* 32, 119-134. [https://doi.org/10.1016/0077-7579\(94\)90036-1](https://doi.org/10.1016/0077-7579(94)90036-1).
- Leopold, M. F., van Damme, C. J. G. & van der Veer, H. W. (1998). Diet of cormorants and the impact of cormorant predation on juvenile flatfish in the Dutch Wadden Sea. *Journal of Sea Research* 40, 93-107. [https://doi.org/10.1016/S1385-1101\(98\)00028-8](https://doi.org/10.1016/S1385-1101(98)00028-8).
- Link, J. S., Bolles, K. & Milliken, C. G. (2002). The feeding ecology of flatfish in the Northwest Atlantic. *Journal of Northwest Atlantic Fishery Science* 30, 1-17.
- Lonsdale, J.-A., Weston, K., Barnard, S., Boyes, S. J. & Elliott, M. (2015). Integrating management tools and concepts to develop an estuarine planning support system: A case study of the Humber Estuary, Eastern England. *Marine Pollution Bulletin* 100, 393-405. <http://dx.doi.org/10.1016/j.marpolbul.2015.08.017>.
- Maes, J., Taillieu, A., Van Damme, P. A., Cottenie, K. & Ollevier, F. (1998). Seasonal Patterns in the Fish and Crustacean Community of a Turbid Temperate Estuary (Zeeschelde Estuary, Belgium). *Estuarine, Coastal and Shelf Science* 47, 143-151. <https://doi.org/10.1006/ecss.1998.0350>.
- Magnhagen, C. & Wiederholm, A.-M. (1982). Food selectivity versus prey availability: a study using the marine fish *Pomatoschistus microps*. *Oecologia* 55, 311-315. [10.1007/bf00376917](https://doi.org/10.1007/bf00376917).
- Maillet, G. (1989). Effects of starvation on the frequency of formation and width of growth increments in sagittae of laboratory-reared Atlantic menhaden *Brevoortia tyrannus* larvae. *Fishery Bulletin* 88, 155-165.
- Malloy, K. D. & Targett, T. E. (1994). The use of RNA:DNA ratios to predict growth limitation of juvenile summer flounder (*Paralichthys dentatus*) from Delaware and North Carolina estuaries. *Marine Biology* 118, 367-375. [10.1007/BF00350293](https://doi.org/10.1007/BF00350293).
- Marchand, J. (1991). The influence of environmental conditions on settlement, distribution and growth of 0-group sole (*Solea solea* (L.)) in a macrotidal estuary (Vilaine, France). *Netherlands Journal of Sea Research* 27, 307-316. [https://doi.org/10.1016/0077-7579\(91\)90033-W](https://doi.org/10.1016/0077-7579(91)90033-W).

- Marshall, S. & Elliott, M. (1997). A comparison of univariate and multivariate numerical and graphical techniques for determining inter- and intraspecific feeding relationships in estuarine fish. *Journal of Fish Biology* 51, 526-545. 10.1111/j.1095-8649.1997.tb01510.x.
- Marshall, S. & Elliott, M. (1998). Environmental Influences on the Fish Assemblage of the Humber Estuary, U.K. *Estuarine, Coastal and Shelf Science* 46, 175-184. <http://dx.doi.org/10.1006/ecss.1997.0268>.
- Martinho, F., Leitão, R., Neto, J. M., Cabral, H., Lagardère, F. & Pardal, M. A. (2008). Estuarine colonization, population structure and nursery functioning for 0-group sea bass (*Dicentrarchus labrax*), flounder (*Platichthys flesus*) and sole (*Solea solea*) in a mesotidal temperate estuary. *Journal of Applied Ichthyology* 24, 229-237. 10.1111/j.1439-0426.2007.01049.x.
- Martinho, F., Leitão, R., Neto, J. M., Cabral, H. N., Marques, J. C. & Pardal, M. A. (2007). The use of nursery areas by juvenile fish in a temperate estuary, Portugal. *Hydrobiologia* 587, 281-290. 10.1007/s10750-007-0689-3.
- Martinho, F., van der Veer, H. W., Cabral, H. N. & Pardal, M. A. (2013). Juvenile nursery colonization patterns for the European flounder (*Platichthys flesus*): A latitudinal approach. *Journal of Sea Research* 84, 61-69. <https://doi.org/10.1016/j.seares.2013.07.014>.
- Martinsson, J. & Nissling, A. (2011). Nursery area utilization by turbot (*Psetta maxima*) and flounder (*Platichthys flesus*) at Gotland, central Baltic Sea. *Boreal Environment Research* 16, 60-70.
- Mattila, J. & Bonsdorff, E. (1998). Predation by juvenile flounder (*Platichthys flesus* L.): a test of prey vulnerability, predator preference, switching behaviour and functional response. *Journal of Experimental Marine Biology and Ecology* 227, 221-236. [https://doi.org/10.1016/S0022-0981\(97\)00272-4](https://doi.org/10.1016/S0022-0981(97)00272-4).
- McBride, R. S., MacDonald, T. C., Richard Jr, E., Rydene, D. A. & Hood, P. B. (2001). Nursery habitats for ladyfish, *Elops saurus*, along salinity gradients in two Florida estuaries. *Fishery Bulletin* 99, 443-443.
- McConnaughey, R. A. & Smith, K. R. (2000). Associations between flatfish abundance and surficial sediments in the eastern Bering Sea. *Canadian Journal of Fisheries and Aquatic Sciences* 57, 2410-2419. 10.1139/f00-219.
- McCullagh, P. & Nelder, J. A. (1989). *Generalized Linear Models*, Second Edition: Taylor & Francis.

- McDowall, R. M. (1988). Diadromy in fishes: migrations between freshwater and marine environments: Croom Helm.
- McLusky, D. S. (1967). Some Effects of Salinity on the Survival, Moulting, and Growth of *Corophium Volutator* [Amphipoda]. *Journal of the Marine Biological Association of the United Kingdom* 47, 607-617. 10.1017/S0025315400035220.
- McLusky, D. S. & Elliott, M. (2004). The estuarine ecosystem: ecology, threats and management: Oxford University Press on Demand.
- McLusky, D. S. & Elliott, M. (2007). Transitional waters: A new approach, semantics or just muddying the waters? *Estuarine, Coastal and Shelf Science* 71, 359-363. <https://doi.org/10.1016/j.ecss.2006.08.025>.
- Mendes, C., Ramos, S. & Bordalo, A. A. (2014). Feeding ecology of juvenile flounder *Platichthys flesus* in an estuarine nursery habitat: Influence of prey–predator interactions. *Journal of Experimental Marine Biology and Ecology* 461, 458-468. <http://dx.doi.org/10.1016/j.jembe.2014.09.016>.
- Meyer, S., Caldarone, E. M., Chícharo, M. A., Clemmesen, C., Faria, A. M., Faulk, C., Folkvord, A., Holt, G. J., Høie, H., Kanstinger, P., Malzahn, A., Moran, D., Petereit, C., Støttrup, J. G. & Peck, M. A. (2012). On the edge of death: Rates of decline and lower thresholds of biochemical condition in food-deprived fish larvae and juveniles. *Journal of Marine Systems* 93, 11-24. <https://doi.org/10.1016/j.jmarsys.2011.09.010>.
- Mikaela, A. J. B., Mark, G. M., Robertson, D. R. & McCormick, M. I. (2002). Larval Growth Predicts the Recruitment Success of a Coral Reef Fish. *Oecologia* 131, 521-525. <https://doi.org/10.1007/s00442-002-0918-4>.
- Minagawa, M. & Wada, E. (1984). Stepwise enrichment of ^{15}N along food chains: Further evidence and the relation between $\delta^{15}\text{N}$ and animal age. *Geochimica et Cosmochimica Acta* 48, 1135-1140. [http://dx.doi.org/10.1016/0016-7037\(84\)90204-7](http://dx.doi.org/10.1016/0016-7037(84)90204-7).
- Modin, J. & Pihl, L. (1996). Small-scale distribution of juvenile plaice and flounder in relation to predatory shrimp in a shallow Swedish bay. *Journal of Fish Biology* 49, 1070-1085. 10.1111/j.1095-8649.1996.tb01779.x.
- Molinero, A. & Flos, R. (1992). Influence of season on the feeding habits of the common sole *Solea solea*. *Marine Biology* 113, 499-507. 10.1007/BF00349177.

- Molony, B. W. & Choat, J. H. (1990). Otolith increment widths and somatic growth rate: the presence of a time-lag. *Journal of Fish Biology* 37, 541-551. 10.1111/j.1095-8649.1990.tb05887.x.
- Morais, P., Dias, E., Babaluk, J. & Antunes, C. (2011). The migration patterns of the European flounder *Platichthys flesus* (Linnaeus, 1758) (Pleuronectidae, Pisces) at the southern limit of its distribution range: Ecological implications and fishery management. *Journal of Sea Research* 65, 235-246. <https://doi.org/10.1016/j.seares.2010.11.001>.
- Mucha, A. P., Vasconcelos, M. T. S. D. & Bordalo, A. A. (2005). Spatial and seasonal variations of the macrobenthic community and metal contamination in the Douro estuary (Portugal). *Marine Environmental Research* 60, 531-550. <https://doi.org/10.1016/j.marenvres.2004.12.004>.
- Munk, P. & Nielsen, J. G. (2005). Eggs and Larvae of North Sea Fishes: Biofolia.
- Muus, B. J. (1967). The Fauna of Danish Estuaries and Lagoons: Distribution and Ecology of Dominating Species in the Shallow Reaches of the Mesohaline Zone: A. F. Høst.
- Muus, B. J. & Nielsen, J. G. (1999). Sea fish. Scandinavian Fishing Year Book. Hedehusene, Denmark.
- Nagelkerken, I., Sheaves, M., Baker, R. & Connolly, R. M. (2015). The seascape nursery: a novel spatial approach to identify and manage nurseries for coastal marine fauna. *Fish and Fisheries* 16, 362-371. doi:10.1111/faf.12057.
- Nash, R. D. M. & Geffen, A. J. (2012). Mortality through the early life-history of fish: What can we learn from European plaice (*Pleuronectes platessa* L.)? *Journal of Marine Systems* 93, 58-68. <https://doi.org/10.1016/j.jmarsys.2011.09.009>.
- Nash, R. D. M., Geffen, A. J., Burrows, M. T. & Gibson, R. N. (2007). Dynamics of shallow-water juvenile flatfish nursery grounds: application of the self-thinning rule. *Marine Ecology Progress Series* 344, 231-244. <https://doi.org/10.3354/meps06933>.
- Ng, J. S. S., Wai, T.-C. & Williams, G. A. (2007). The effects of acidification on the stable isotope signatures of marine algae and molluscs. *Marine Chemistry* 103, 97-102. <http://dx.doi.org/10.1016/j.marchem.2006.09.001>.
- Nicolas, D., Le Loc'h, F., Désaunay, Y., Hamon, D., Blanchet, A. & Le Pape, O. (2007). Relationships between benthic macrofauna and habitat suitability for juvenile common sole

- (*Solea solea*, L.) in the Vilaine estuary (Bay of Biscay, France) nursery ground. *Estuarine, Coastal and Shelf Science* 73, 639-650. <https://doi.org/10.1016/j.ecss.2007.03.006>.
- Nielsen, J. G. (1986). Pleuronectidae. In *Fishes of the North-Eastern Atlantic and Mediterranean* (Whitehead, P. J. P., Bauchot, M. L., Hureau, J. C., Nielsen, J. & Tortonese, E., eds.), pp. 1299-1307. Paris: UNESCO.
- Nissling, A. & Dahlman, G. (2010). Fecundity of flounder, *Pleuronectes flesus*, in the Baltic Sea — Reproductive strategies in two sympatric populations. *Journal of Sea Research* 64, 190-198. <https://doi.org/10.1016/j.seares.2010.02.001>.
- Nissling, A., Jacobsson, M. & Hallberg, N. (2007). Feeding ecology of juvenile turbot *Scophthalmus maximus* and flounder *Pleuronectes flesus* at Gotland, Central Baltic Sea. *Journal of Fish Biology* 70, 1877-1897. doi:10.1111/j.1095-8649.2007.01463.x.
- Nissling, A., Westin, L. & Hjerne, O. (2002). Reproductive success in relation to salinity for three flatfish species, dab (*Limanda limanda*), plaice (*Pleuronectes platessa*), and flounder (*Pleuronectes flesus*), in the brackish water Baltic Sea. *ICES Journal of Marine Science* 59, 93-108. 10.1006/jmsc.2001.1134.
- O'Neill, B., De Raedemaeker, F., McGrath, D. & Brophy, D. (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. <http://dx.doi.org/10.1016/j.jembe.2011.10.007>.
- Odum, E. P., Odum, H. T. & Andrews, J. (1971). *Fundamentals of ecology*: Saunders Philadelphia.
- Palmer, S. C. F., Coulon, A. & Travis, J. M. J. (2014). Inter-individual variability in dispersal behaviours impacts connectivity estimates. *Oikos* 123, 923-932. 10.1111/oik.01248.
- Pannella, G. (1971). Fish Otoliths: Daily Growth Layers and Periodical Patterns. *Science* 173, 1124-1127. 10.1126/science.173.4002.1124.
- Paperno, R., Targett, T. E. & Greco, P. A. (2000). Spatial and temporal variation in recent growth, overall growth, and mortality of juvenile weakfish (*Cynoscion regalis*) in Delaware Bay. *Estuaries* 23, 10-20. 10.2307/1353221.

- Parnell, A. C., Inger, R., Bearhop, S. & Jackson, A. L. (2010). Source Partitioning Using Stable Isotopes: Coping with Too Much Variation. *PLoS ONE* 5, e9672. <https://doi.org/10.1371/journal.pone.0009672>.
- Parnell, A. C., Phillips, D. L., Bearhop, S., Semmens, B. X., Ward, E. J., Moore, J. W., Jackson, A. L., Grey, J., Kelly, D. J. & Inger, R. (2013). Bayesian stable isotope mixing models. *Environmetrics* 24, 387-399. 10.1002/env.2221.
- Parsons, D. M., Morrison, M. A., Thrush, S. F., Middleton, C., Smith, M., Spong, K. T. & Buckthought, D. (2013). The influence of habitat structure on juvenile fish in a New Zealand estuary. *Marine Ecology* 34, 492-500. 10.1111/maec.12050.
- Pasquaud, S., Elie, P., Jeantet, C., Billy, I., Martinez, P. & Girardin, M. (2008). A preliminary investigation of the fish food web in the Gironde estuary, France, using dietary and stable isotope analyses. *Estuarine, Coastal and Shelf Science* 78, 267-279. <https://doi.org/10.1016/j.ecss.2007.12.014>.
- Peck, M. A., Baumann, H., Clemmesen, C., Herrmann, J.-P., Moyano, M. & Temming, A. (2015). Calibrating and comparing somatic-, nucleic acid-, and otolith-based indicators of growth and condition in young juvenile European sprat (*Sprattus sprattus*). *Journal of Experimental Marine Biology and Ecology* 471, 217-225. <https://doi.org/10.1016/j.jembe.2015.06.011>.
- Pepin, P. (1991). Effect of Temperature and Size on Development, Mortality, and Survival Rates of the Pelagic Early Life History Stages of Marine Fish. *Canadian Journal of Fisheries and Aquatic Sciences* 48, 503-518. 10.1139/f91-065.
- Peterson, B. J. & Fry, B. (1987). Stable Isotopes in Ecosystem Studies. *Annual Review of Ecology and Systematics* 18, 293-320. 10.1146/annurev.es.18.110187.001453.
- Phelan, B. A., Manderson, J. P., Stoner, A. W. & Bejda, A. J. (2001). Size-related shifts in the habitat associations of young-of-the-year winter flounder (*Pseudopleuronectes americanus*): field observations and laboratory experiments with sediments and prey. *Journal of Experimental Marine Biology and Ecology* 257, 297-315. [https://doi.org/10.1016/S0022-0981\(00\)00340-3](https://doi.org/10.1016/S0022-0981(00)00340-3).
- Phillips, D. L., Inger, R., Bearhop, S., Jackson, A. L., Moore, J. W., Parnell, A. C., Semmens, B. X. & Ward, E. J. (2014). Best practices for use of stable isotope mixing models in food-web studies. *Canadian Journal of Zoology* 92, 823-835. 10.1139/cjz-2014-0127.

- Piet, G. J., Pfisterer, A. B. & Rijnsdorp, A. D. (1998). On factors structuring the flatfish assemblage in the southern North Sea. *Journal of Sea Research* 40, 143-152. [https://doi.org/10.1016/S1385-1101\(98\)00008-2](https://doi.org/10.1016/S1385-1101(98)00008-2).
- Pihl, L., Cattrijsse, A., Codling, I., Mathieson, S., McLusky, D. S. & Roberts, C. (2007). Habitat use by Fishes in Estuaries and Other Brackish Areas. In *Fishes in Estuaries*, pp. 10-53: Blackwell Science Ltd.
- Pinkas, L. (1971). Food habits of albacore, bluefin tuna and bonito in California waters. *California Fish and Game* 152, 1-105.
- Post, D. M. (2002). Using stable isotopes to estimate trophic position: models, methods, and assumptions. *Ecology* 83, 703-718. 10.1890/0012-9658(2002)083[0703:USITET]2.0.CO;2.
- Post, M. H. M., Blom, E., Chen, C., Bolle, L. J. & Baptist, M. J. (2017). Habitat selection of juvenile sole (*Solea solea* L.): Consequences for shoreface nourishment. *Journal of Sea Research* 122, 19-24. <https://doi.org/10.1016/j.seares.2017.02.011>.
- Potter, I. C., Beckley, L. E., Whitfield, A. K. & Lenanton, R. C. J. (1990). Comparisons between the roles played by estuaries in the life cycles of fishes in temperate Western Australia and Southern Africa. *Environmental Biology of Fishes* 28, 143-178. 10.1007/BF00751033.
- Potter, I. C., Tweedley, J. R., Elliott, M. & Whitfield, A. K. (2015). The ways in which fish use estuaries: a refinement and expansion of the guild approach. *Fish and Fisheries* 16, 230-239. 10.1111/faf.12050.
- Power, M., Attrill, M. J. & Thomas, R. M. (2000). Environmental factors and interactions affecting the temporal abundance of juvenile flatfish in the Thames Estuary. *Journal of Sea Research* 43, 135-149. [https://doi.org/10.1016/S1385-1101\(00\)00010-1](https://doi.org/10.1016/S1385-1101(00)00010-1).
- Preston, T. & Owens, N. J. P. (1983). Interfacing an automatic elemental analyser with an isotope ratio mass spectrometer: the potential for fully automated total nitrogen and nitrogen-15 analysis. *Analyst* 108, 971-977. 10.1039/AN9830800971.
- Primo, A. L., Azeiteiro, U. M., Marques, S. C., Martinho, F., Baptista, J. & Pardal, M. A. (2013). Colonization and nursery habitat use patterns of larval and juvenile flatfish species in a small temperate estuary. *Journal of Sea Research* 76, 126-134. <https://doi.org/10.1016/j.seares.2012.08.002>.

- Pritchard, D. W. (1967). Estuaries. In What is an estuary: a physical viewpoint. (Lauff, G. H., ed.), pp. 3-5. Washington, D.C: American Association for the Advancement of Science.
- Pyke, G. H. (1984). Optimal Foraging Theory: A Critical Review. *Annual Review of Ecology and Systematics* 15, 523-575. 10.1146/annurev.es.15.110184.002515.
- R Development Core Team (2007). R: A language and environment for statistical computing. R foundation for Statistical Computing. Vienna, Austria.
- Raffaelli, D., Conacher, A., McLachlan, H. & Emes, C. (1989). The role of epibenthic crustacean predators in an estuarine food web. *Estuarine, Coastal and Shelf Science* 28, 149-160. [https://doi.org/10.1016/0272-7714\(89\)90062-0](https://doi.org/10.1016/0272-7714(89)90062-0).
- Raffaelli, D., Richner, H., Summers, R. & Northcott, S. (1990). Tidal migrations in the flounder (*Platichthys flesus*). *Marine Behaviour and Physiology* 16, 249-260. 10.1080/10236249009378753.
- Ramos, S. (2007). Ichthyoplankton of the Lima estuary (NW Portugal): ecology of the early life stages of Pleuronectiformes. p. 228. Porto: University of Porto.
- Ramos, S., Cowen, R. K., Paris, C., Ré, P. & Bordalo, A. A. (2006a). Environmental forcing and larval fish assemblage dynamics in the Lima River estuary (northwest Portugal). *Journal of Plankton Research* 28, 275-286. 10.1093/plankt/fbi104.
- Ramos, S., Cowen, R. K., Ré, P. & Bordalo, A. A. (2006b). Temporal and spatial distributions of larval fish assemblages in the Lima estuary (Portugal). *Estuarine, Coastal and Shelf Science* 66, 303-314. <https://doi.org/10.1016/j.ecss.2005.09.012>.
- Ramos, S., Paris, C. B. & Angélico, M. M. (2017). Larval fish dispersal along an estuarine–ocean gradient. *Canadian Journal of Fisheries and Aquatic Sciences* 74, 1462-1473. 10.1139/cjfas-2016-0325.
- Ramos, S., Ré, P. & Bordalo, A. A. (2009). Environmental control on early life stages of flatfishes in the Lima Estuary (NW Portugal). *Estuarine, Coastal and Shelf Science* 83, 252-264. <https://doi.org/10.1016/j.ecss.2009.03.031>.
- Ramos, S., Ré, P. & Bordalo, A. A. (2010). Recruitment of flatfish species to an estuarine nursery habitat (Lima estuary, NW Iberian Peninsula). *Journal of Sea Research* 64, 473-486. <https://doi.org/10.1016/j.seares.2010.01.010>.

- Raventos, N. & Macpherson, E. (2005). Effect of pelagic larval growth and size-at-hatching on post-settlement survivorship in two temperate labrid fish of the genus *Symphodus*. *Marine Ecology Progress Series* 285, 205-211. 10.3354/meps285205.
- Reichert, M. J. M. (2003). Diet, consumption, and growth of juvenile fringed flounder (*Etropus crossotus*); a test of the 'maximum growth/optimum food hypothesis' in a subtropical nursery area. *Journal of Sea Research* 50, 97-116. [https://doi.org/10.1016/S1385-1101\(03\)00081-9](https://doi.org/10.1016/S1385-1101(03)00081-9).
- Ribeiro, H., de Sousa, T., Santos, J. P., Sousa, A. G. G., Teixeira, C., Monteiro, M. R., Salgado, P., Mucha, A. P., Almeida, C. M. R., Torgo, L. & Magalhães, C. (2018). Potential of dissimilatory nitrate reduction pathways in polycyclic aromatic hydrocarbon degradation. *Chemosphere* 199, 54-67. <https://doi.org/10.1016/j.chemosphere.2018.01.171>.
- Richard, P., Bergeron, J.-P., Boulhic, M., Galois, R. & Person-Le Ruyet, J. (1991). Effect of starvation on RNA, DNA and protein content of laboratory-reared larvae and juveniles of *Solea solea*. *Marine Ecology Progress Series* 72, 69-77. 10.3354/meps072069.
- Ricker, W. E. (1975). *Computation and Interpretation of Biological Statistics of Fish Populations*. Ottawa: Bulletin of the Fisheries Research Board of Canada, Bulletin 191.
- Rijnsdorp, A. D., Berghahn, R., Miller, J. M. & Van Der Veer, H. W. (1995). Recruitment mechanisms in flatfish: What did we learn and where do we go? *Netherlands Journal of Sea Research* 34, 237-242. [https://doi.org/10.1016/0077-7579\(95\)90031-4](https://doi.org/10.1016/0077-7579(95)90031-4).
- Riley, J. D., Symonds, D. J. & Woolner, L. (1981). On the factors influencing the distribution of 0-group demersal fish in coastal waters. In *Rapports et Proces-verbaux des Réunions*, pp. 223-228.
- Rogers, S. I. (1992). Environmental factors affecting the distribution of sole (*Solea solea* (L.)) within a nursery area. *Netherlands Journal of Sea Research* 29, 153-161. [https://doi.org/10.1016/0077-7579\(92\)90016-8](https://doi.org/10.1016/0077-7579(92)90016-8).
- Russo, T., Pulcini, D., O'Leary, Á., Cataudella, S. & Mariani, S. (2008). Relationship between body shape and trophic niche segregation in two closely related sympatric fishes. *Journal of Fish Biology* 73, 809-828. 10.1111/j.1095-8649.2008.01964.x.
- Ryer, C. H., Laurel, B. J. & Stoner, A. W. (2010). Testing the shallow water refuge hypothesis in flatfish nurseries. *Marine Ecology Progress Series* 415, 275-282. <https://doi.org/10.3354/meps08732>.

- Ryer, C. H., Stoner, A. W., Spencer, M. L. & Abookire, A. A. (2007). Presence of larger flatfish modifies habitat preference by Age-0 northern rock sole *Lepidopsetta polyxystra*. Marine Ecology Progress Series 342, 227-238. 10.3354/meps342227.
- Scaps, P. (2002). A review of the biology, ecology and potential use of the common ragworm *Hediste diversicolor* (O.F. Müller) (Annelida: Polychaeta). Hydrobiologia 470, 203-218. 10.1023/a:1015681605656.
- Scharf, F. S., Juanes, F. & Rodney, A. R. (2000). Predator size-prey size relationships of marine fish predators: interspecific variation and effects of ontogeny and body size on trophic-niche breadth. Marine Ecology Progress Series 208, 229-248. 10.3354/meps208229.
- Schoener, T. W. (1970). Nonsynchronous Spatial Overlap of Lizards in Patchy Habitats. Ecology 51, 408-418. 10.2307/1935376.
- Searcy, S. P. & Sponaugle, S. (2001). Selective mortality during the larval-juvenile transition in two coral reef fishes. Ecology 82, 2452-2470. doi:10.1890/0012-9658(2001)082[2452:SMDTLJ]2.0.CO;2.
- Selleslagh, J. & Amara, R. (2008). Inter-season and interannual variations in fish and macrocrustacean community structure on a eastern English Channel sandy beach: Influence of environmental factors. Estuarine, Coastal and Shelf Science 77, 721-730. <https://doi.org/10.1016/j.ecss.2007.11.004>.
- Selleslagh, J. & Amara, R. (2013). Effect of starvation on condition and growth of juvenile plaice *Pleuronectes platessa*: nursery habitat quality assessment during the settlement period. Journal of the Marine Biological Association of the United Kingdom 93, 479-488. 10.1017/S0025315412000483.
- Selleslagh, J. & Amara, R. (2015). Are Estuarine Fish Opportunistic Feeders? The Case of a Low Anthropized Nursery Ground (the Canche Estuary, France). Estuaries and Coasts 38, 252-267. 10.1007/s12237-014-9787-4.
- Selleslagh, J., Blanchet, H., Bachelet, G. & Lobry, J. (2015). Feeding Habitats, Connectivity and Origin of Organic Matter Supporting Fish Populations in an Estuary with a Reduced Intertidal Area Assessed by Stable Isotope Analysis. Estuaries and Coasts 38, 1431-1447. 10.1007/s12237-014-9911-5.
- Shannon, C. E. & Weaver, W. (1949). The Mathematical Theory of Communication. Illinois, Urbana, IL: University of Illinois Press.

- Sheaves, M. (2009). Consequences of ecological connectivity: the coastal ecosystem mosaic. *Marine Ecology Progress Series* 391, 107-115. <https://doi.org/10.3354/meps08121>.
- Sheaves, M., Baker, R., Nagelkerken, I. & Connolly, R. M. (2015). True Value of Estuarine and Coastal Nurseries for Fish: Incorporating Complexity and Dynamics. *Estuaries and Coasts* 38, 401-414. 10.1007/s12237-014-9846-x.
- Sheaves, M., Johnston, R., Johnson, A., Baker, R. & Connolly, R. M. (2013). Nursery Function Drives Temporal Patterns in Fish Assemblage Structure in Four Tropical Estuaries. *Estuaries and Coasts* 36, 893-905. 10.1007/s12237-013-9610-7.
- Simpson, S., Batley, G., Scientific, C. & Organization, I. R. (2016). *Sediment Quality Assessment: A Practical Guide*: CSIRO Publishing.
- Sogard, S. M. (1992). Variability in growth rates of juvenile fishes in different estuarine habitats. *Marine Ecology Progress Series* 85, 35-53. 10.3354/meps085035.
- Sogard, S. M. (1997). Size-Selective Mortality in the Juvenile Stage of Teleost Fishes: A Review. *Bulletin of Marine Science* 60, 1129-1157.
- Sogard, S. M. (2011). Interannual Variability in Growth Rates of Early Juvenile Sablefish and the Role of Environmental Factors. *Bulletin of Marine Science* 87, 857-872. 10.5343/bms.2010.1045.
- Sousa, R., Antunes, C. & Guilhermino, L. (2006a). Factors influencing the occurrence and distribution of *Corbicula fluminea* (Müller, 1774) in the River Lima estuary. *Annales de Limnologie - International Journal of Limnology* 42, 165-171. 10.1051/limn/2006017.
- Sousa, R., Dias, S. & Antunes, J. C. (2006b). Spatial Subtidal Macrobenthic Distribution in Relation to Abiotic Conditions in the Lima Estuary, NW of Portugal. *Hydrobiologia* 559, 135-148. 10.1007/s10750-005-1371-2.
- Sousa, R., Dias, S. & Antunes, C. (2007). Subtidal macrobenthic structure in the lower Lima estuary, NW of Iberian Peninsula. *Annales Zoologici Fennici* 44, 303-313.
- Souza, A. T., Dias, E., Nogueira, A., Campos, J., Marques, J. C. & Martins, I. (2013). Population ecology and habitat preferences of juvenile flounder *Platichthys flesus* (Actinopterygii: Pleuronectidae) in a temperate estuary. *Journal of Sea Research* 79, 60-69. <https://doi.org/10.1016/j.seares.2013.01.005>.

- Stierhoff, K. L., Targett, T. E. & Power, J. H. (2009). Hypoxia-induced growth limitation of juvenile fishes in an estuarine nursery: assessment of small-scale temporal dynamics using RNA:DNA. *Canadian Journal of Fisheries and Aquatic Sciences* 66, 1033-1047. 10.1139/F09-066.
- Strauss, R. E. (1979). Reliability Estimates for Ivlev's Electivity Index, the Forage Ratio, and a Proposed Linear Index of Food Selection. *Transactions of the American Fisheries Society* 108, 344-352. doi:10.1577/1548-8659(1979)108<344:REFIEI>2.0.CO;2.
- Summers, R. W. (1979). Life cycle and population ecology of the flounder *Platichthys flesus* (L.) in the Ythan estuary, Scotland. *Journal of Natural History* 13, 703-723. 10.1080/00222937900770531.
- Summers, R. W. (1980). The diet and feeding behaviour of the flounder *Platichthys flesus* (L.) in the Ythan estuary, Aberdeenshire, Scotland. *Estuarine and Coastal Marine Science* 11, 217-232. [https://doi.org/10.1016/S0302-3524\(80\)80042-9](https://doi.org/10.1016/S0302-3524(80)80042-9).
- Suthers, I. (1992). The use of condition indices in larval fish. In *Larval biology*. Australian Society for Fish Biology workshop, Hobart. Australian Government Publishing Service, Canberra, p. 49.
- Suthers, I. M. (1998). Bigger? Fatter? Or is faster growth better? Considerations on condition in larval and juvenile coral-reef fish. *Australian Journal of Ecology* 23, 265-273. 10.1111/j.1442-9993.1998.tb00730.x.
- Suthers, I. M., Frank, K. T. & Campana, S. E. (1989). Spatial Comparison of Recent Growth in Postlarval Atlantic Cod (*Gadus morhua*) off Southwestern Nova Scotia: Inferior Growth in a Presumed Nursery Area. *Canadian Journal of Fisheries and Aquatic Sciences* 46, s113-s124. 10.1139/f89-283.
- Suthers, I. M., Fraser, A. & Frank, K. T. (1992). Comparison of lipid, otolith and morphometric condition indices of pelagic juvenile cod *Gadus morhua* from the Canadian Atlantic. *Marine Ecology Progress Series* 84, 31-40. 10.3354/meps084031.
- Suzuki, K. W., Kasai, A., Isoda, T., Nakayama, K. & Tanaka, M. (2008). Distinctive stable isotope ratios in important zooplankton species in relation to estuarine salinity gradients: Potential tracer of fish migration. *Estuarine, Coastal and Shelf Science* 78, 541-550. <https://doi.org/10.1016/j.ecss.2008.01.014>.

- Tableau, A., Brind'Amour, A., Woillez, M. & Le Bris, H. (2016a). Influence of food availability on the spatial distribution of juvenile fish within soft sediment nursery habitats. *Journal of Sea Research* 111, 76-87. <https://doi.org/10.1016/j.seares.2015.12.004>.
- Tanaka, M., Goto, T., Tomiyama, M. & Sudo, H. (1989). Immigration, settlement and mortality of flounder (*Paralichthys olivaceus*) larvae and juveniles in a nursery ground, Shijiki bay, Japan. *Netherlands Journal of Sea Research* 24, 57-67. [https://doi.org/10.1016/0077-7579\(89\)90170-1](https://doi.org/10.1016/0077-7579(89)90170-1).
- Taylor, D. L. (2003). Size-dependent predation on post-settlement winter flounder *Pseudopleuronectes americanus* by sand shrimp *Crangon septemspinosa*. *Marine Ecology Progress Series* 263, 197-215. 10.3354/meps263197.
- Teal, L. R., de Leeuw, J. J., van der Veer, H. W. & Rijnsdorp, A. D. (2008). Effects of climate change on growth of 0-group sole and plaice. *Marine Ecology Progress Series* 358, 219-230. <https://doi.org/10.3354/meps07367>
- Teixeira, C. M., Batista, M. I. & Cabral, H. N. (2010). Diet, growth and reproduction of four flatfishes on the Portuguese coast. 2010 74, 11. 10.3989/scimar.2010.74n2223.
- Thiel, R. & Potter, I. C. (2001). The ichthyofaunal composition of the Elbe Estuary: an analysis in space and time. *Marine Biology* 138, 603-616. 10.1007/s002270000491.
- Tomiyama, T., Uehara, S. & Kurita, Y. (2013). Feeding relationships among fishes in shallow sandy areas in relation to stocking of Japanese flounder. *Marine Ecology Progress Series* 479, 163-175. <https://doi.org/10.3354/meps10191>.
- Toole, C. L. (1980). Intertidal recruitment and feeding in relation to optimal utilization of nursery areas by juvenile English sole (*Parophrys vetulus*: Pleuronectidae). *Environmental Biology of Fishes* 5, 383-390. 10.1007/bf00005192.
- Treml, E. A., Roberts, J. J., Chao, Y., Halpin, P. N., Possingham, H. P. & Riginos, C. (2012). Reproductive Output and Duration of the Pelagic Larval Stage Determine Seascape-Wide Connectivity of Marine Populations. *Integrative and Comparative Biology* 52, 525-537. 10.1093/icb/ics101.
- Uzars, D., Ustups, D., Müller-Karulis, B. & Urtans, E. (2003). Size structure and feeding ecology of fish communities in the surf zone of the Eastern Baltic. In ICES Annual Science Conference. Tallin, Estonia.

- van der Veer, H. W., Berghahn, R., Miller, J. M. & Rijnsdorp, A. D. (2000). Recruitment in flatfish, with special emphasis on North Atlantic species: Progress made by the Flatfish Symposia. *ICES Journal of Marine Science* 57, 202-215. 10.1006/jmsc.1999.0523.
- van der Veer, H. W. & Bergman, M. J. N. (1987). Predation by crustaceans on a newly settled 0-group plaice *Pleuronectes platessa* population in the western Wadden Sea. *Marine Ecology Progress Series* 35, 203-215. 10.3354/meps035203.
- van der Veer, H. W., Bergman, M. J. N., Dapper, R. & Witte, J. I. J. (1991). Population dynamics of an intertidal 0-group flounder *Platichthys flesus* population in the western Dutch Wadden Sea. *Marine Ecology Progress Series* 73, 141-148. 10.3354/meps073141.
- van der Veer, H. W., Freitas, V., Koot, J., Witte, J. I. J. & Zuur, A. F. (2010). Food limitation in epibenthic species in temperate intertidal systems in summer: analysis of 0-group plaice *Pleuronectes platessa*. *Marine Ecology Progress Series* 416, 215-227. 10.3354/meps08786
- van der Veer, H. W., Kooijman, S. A. L. M. & van der Meer, J. (2001). Intra- and interspecies comparison of energy flow in North Atlantic flatfish species by means of dynamic energy budgets. *Journal of Sea Research* 45, 303-320. [https://doi.org/10.1016/S1385-1101\(01\)00061-2](https://doi.org/10.1016/S1385-1101(01)00061-2).
- van der Veer, H. W. & Witte, J. I. J. (1993). The 'maximum growth/optimal food condition' hypothesis: a test for 0-group plaice *Pleuronectes platessa* in the Dutch Wadden Sea. *Marine Ecology Progress Series* 101, 81-90. 10.3354/meps101081.
- Vander Zanden, M. J., Cabana, G. & Rasmussen, J. B. (1997). Comparing trophic position of freshwater fish calculated using stable nitrogen isotope ratios ($\delta^{15}\text{N}$) and literature dietary data. *Canadian Journal of Fisheries and Aquatic Sciences* 54, 1142-1158. 10.1139/f97-016.
- Vasconcelos, R. P., Reis-Santos, P., Fonseca, V., Ruano, M., Tanner, S., Costa, M. J. & Cabral, H. N. (2009). Juvenile fish condition in estuarine nurseries along the Portuguese coast. *Estuarine, Coastal and Shelf Science* 82, 128-138. <https://doi.org/10.1016/j.ecss.2009.01.002>.
- Vasconcelos, R. P., Reis-Santos, P., Maia, A., Fonseca, V., França, S., Wouters, N., Costa, M. J. & Cabral, H. N. (2010). Nursery use patterns of commercially important marine fish species in estuarine systems along the Portuguese coast. *Estuarine, Coastal and Shelf Science* 86, 613-624. <https://doi.org/10.1016/j.ecss.2009.11.029>.

- Vaslet, A., Phillips, D.L., France, C.A.M., Feller, I.C. & Baldwin, C.C. (2015). Trophic behaviour of juvenile reef fishes inhabiting interlinked mangrove-seagrass habitats in offshore-mangrove islets. *Journal of Fish Biology* 87:256-273. <https://doi.org/10.1111/jfb.12715>.
- Vigliola, L., Doherty, P. J., Meekan, M. G., Drown, D. M., Jones, M. E. & Barber, P. H. (2007). Genetic identity determines risk of post-settlement mortality of a marine fish. *Ecology* 88, 1263-1277. 10.1890/06-0066.
- Vigliola, L. & Meekan, M. G. (2002). Size at hatching and planktonic growth determine post-settlement survivorship of a coral reef fish. *Oecologia* 131, 89-93. 10.1007/s00442-001-0866-4.
- Vinagre, C., Cabral, H. & Costa, M. J. (2008). Prey selection by flounder, *Platichthys flesus*, in the Douro estuary, Portugal. *Journal of Applied Ichthyology* 24, 238-243. doi:10.1111/j.1439-0426.2008.01055.x.
- Vinagre, C., Ferreira, T., Matos, L., Costa, M. J. & Cabral, H. N. (2009a). Latitudinal gradients in growth and spawning of sea bass, *Dicentrarchus labrax*, and their relationship with temperature and photoperiod. *Estuarine, Coastal and Shelf Science* 81, 375-380. <https://doi.org/10.1016/j.ecss.2008.11.015>.
- Vinagre, C., França, S., Costa, M. J. & Cabral, H. N. (2005). Niche overlap between juvenile flatfishes, *Platichthys flesus* and *Solea solea*, in a southern European estuary and adjacent coastal waters. *Journal of Applied Ichthyology* 21, 114-120. doi:10.1111/j.1439-0426.2004.00639.x.
- Vinagre, C., Maia, A., Reis-Santos, P., Costa, M. J. & Cabral, H. N. (2009b). Small-scale distribution of *Solea solea* and *Solea senegalensis* juveniles in the Tagus estuary (Portugal). *Estuarine, Coastal and Shelf Science* 81, 296-300. <https://doi.org/10.1016/j.ecss.2008.11.008>.
- Vinagre, C., Salgado, J., Cabral, H. N. & Costa, M. J. (2011). Food Web Structure and Habitat Connectivity in Fish Estuarine Nurseries—Impact of River Flow. *Estuaries and Coasts* 34, 663-674. 10.1007/s12237-010-9315-0.
- Vizzini, S., Sarà, G., Michener, R. H. & Mazzola, A. (2002). The role and contribution of the seagrass *Posidonia oceanica* (L.) Delile organic matter for secondary consumers as revealed by carbon and nitrogen stable isotope analysis. *Acta Oecologica* 23, 277-285. [https://doi.org/10.1016/S1146-609X\(02\)01156-6](https://doi.org/10.1016/S1146-609X(02)01156-6).

- Wallace, R. K. & Ramsey, J. S. (1983). Reliability in Measuring Diet Overlap. *Canadian Journal of Fisheries and Aquatic Sciences* 40, 347-351. 10.1139/f83-050.
- Watai, M., Ishihara, T., Abe, O., Ohshimo, S. & Strussmann, C. A. (2017). Evaluation of growth-dependent survival during early stages of Pacific bluefin tuna using otolith microstructure analysis. *Marine and Freshwater Research* 68, 2008-2017. <https://doi.org/10.1071/MF16337>.
- Weatherley, N. S. (1989). The diet and growth of O-group flounder, *Platichthys flesus* (L.), in the River Dee, North Wales. *Hydrobiologia* 178, 193-198. 10.1007/bf00006026.
- Wennhage, H. (2000). Vulnerability of settling plaice *Pleuronectes platessa* to predation: effects of developmental stage and alternative prey. *Marine Ecology Progress Series* 203, 289-299. 10.3354/meps203289.
- Wennhage, H. & Gibson, R. N. (1998). Influence of food supply and a potential predator (*Crangon crangon*) on settling behaviour of plaice (*Pleuronectes platessa*). *Journal of Sea Research* 39, 103-112. [https://doi.org/10.1016/S1385-1101\(97\)00011-7](https://doi.org/10.1016/S1385-1101(97)00011-7).
- Whitfield, A. K. (1994). Fish species diversity in southern African estuarine systems: an evolutionary perspective. *Environmental Biology of Fishes* 40, 37-48. 10.1007/BF00002181.
- Whitfield, A. K. (1999). Ichthyofaunal assemblages in estuaries: A South African case study. *Reviews in Fish Biology and Fisheries* 9, 151-186. 10.1023/a:1008994405375.
- Wirjoatmodjo, S. & Pitcher, T. J. (1984). Flounders follow the tides to feed: Evidence from ultrasonic tracking in an estuary. *Estuarine, Coastal and Shelf Science* 19, 231-241. [https://doi.org/10.1016/0272-7714\(84\)90067-2](https://doi.org/10.1016/0272-7714(84)90067-2).
- Witting, D. A. & Able, K. W. (1993). Effects of body size on probability of predation for juvenile summer and winter flounder based on laboratory experiments. *Fishery Bulletin* 91, 3.
- Wolanski, E. & Elliott, M. (2015). *Estuarine Ecohydrology: an introduction*. Amsterdam: Elsevier.
- Woodland, R. J., Secor, D. H., Fabrizio, M. C. & Wilberg, M. J. (2012). Comparing the nursery role of inner continental shelf and estuarine habitats for temperate marine fishes. *Estuarine, Coastal and Shelf Science* 99, 61-73. <https://doi.org/10.1016/j.ecss.2011.12.019>.
- Zar, J. H. (1996). *Biostatistical Analysis*. New Jersey: Prentice Hall International Editions.

- Złoch, I. & Sapota, M. (2010). Trophic interactions between preadult and adult *Pomatoschistus minutus* and *Pomatoschistus microps* and young *Platichthys flesus* occurring in inshore waters of the Gulf of Gdańsk (Southern Baltic). *Oceanological & Hydrobiological Studies* 39, 37-53. 10.2478/v10009-010-0018-4.
- Zucchetta, M., Franco, A., Torricelli, P. & Franzoi, P. (2010). Habitat distribution model for European flounder juveniles in the Venice lagoon. *Journal of Sea Research* 64, 133-144. <https://doi.org/10.1016/j.seares.2009.12.003>.

APPENDICES

Published papers



Feeding ecology of juvenile flounder *Platichthys flesus* in an estuarine nursery habitat: Influence of prey–predator interactions

C. Mendes^{a,b,*}, S. Ramos^b, A.A. Bordalo^{a,b}

^a Laboratory of Hydrobiology and Ecology, Institute of Biomedical Sciences, University of Porto (ICBAS-UP), Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

^b Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), Rua dos Bragas, 289, 4050-123 Porto, Portugal



ARTICLE INFO

Article history:

Received 6 December 2013

Received in revised form 24 September 2014

Accepted 25 September 2014

Available online xxxx

Keywords:

Feeding

Habitat selection

Nursery grounds

Platichthys flesus

Predator–prey interactions

ABSTRACT

The present study aimed to investigate the feeding ecology and influence of prey–predator interactions on juvenile flounder *Platichthys flesus* in an Atlantic estuarine nursery area (Lima estuary, NW Portugal), focusing on prey selection and ontogenetic shifts in the diet. The relationship between prey availability and flounder distribution was also investigated. Juvenile flounder diet included 21 taxa of macroinvertebrates and fishes, sand and plant debris. According to numerical, occurrence, and gravimetric dietary indices, macroinvertebrates, namely Chironomidae and *Corophium* spp. were the main prey items. The diet diversity tended to increase as juveniles grew, although some dietary overlap occurred between the early juveniles (50–149 mm total length (TL)). In fact, the diet diversity of the newly settled juveniles (<50 mm TL) was particularly low, evidencing the importance of Chironomidae. Moreover, an ontogenetic shift was evident, since older juveniles (1⁺) presented a distinct diet, including new items absent from the diet of the 0⁺ juveniles, namely Teleostei, *Carcinus maenas*, and Nemertea. The juvenile flounder presented an overall generalist behavior, feeding on the most abundant macroinvertebrates namely Chironomidae and *Corophium* spp., as evidenced by the Strauss linear index. The spatial distribution of the 0⁺ flounder in the Lima estuary was associated with salinity and prey (Chironomidae and *Corophium* spp.). These preys were characteristic of the upper estuary where most of the juveniles, especially the newly settled, were found. Hence, this study reinforces the importance of both abiotic and biotic factors as environmental driven controls of habitat use during the early phases of the demersal life of European flounder.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

One of the most important roles that estuaries provide to fishes is the nursery function (Kerstan, 1991; McLusky and Elliott, 2004; Woodland et al., 2012). A nursery habitat may be described as a restricted area where early development stages of a species spend a limited period of their life cycle, during which they are spatially and temporally separated from the adults (Beck et al., 2001), even when spatial overlap occurs. In these areas, the survival of early development stages is enhanced through superior conditions for feeding, growth, and/or predation refuge (Beck et al., 2001; Pihl et al., 2002).

Habitat selection in the nursery areas results from a compromise between different environmental factors, both biotic and abiotic (Burrows, 1994; Hugie and Dill, 1994). The influence of each factor varies throughout the ontogenetic development (Phelan et al., 2001), and also at a variety of temporal and spatial scales (Gibson et al., 1996). For example, as diet and main predators change throughout ontogeny, juveniles may reorganize their distribution around the nursery habitat (Burke, 1995; Castillo-Rivera et al., 2000; Modin and Pihl,

1996). Furthermore, both differences in ontogenetic state and seasonal fluctuations in the abiotic and biotic factors act together to produce characteristic distribution patterns and differential habitat use at different spatial and temporal scales (Gibson et al., 2002).

Flatfish, including the European flounder *Platichthys flesus*, are among the species that use estuaries as nursery areas. The distribution of *P. flesus* is commonly described as ranging from the coasts of northern Europe to the Mediterranean, constituting an important component of demersal fish assemblages economically exploited (Maes et al. 1998; Thiel and Potter, 2001). However, it has been recently suggested that the present southern distribution limit of this species is at the coast of Portugal (Cabral et al., 2001). This euryhaline species tolerate salinities from 0 to 35, although the juveniles preferentially use low salinity areas as nursery grounds (Hemmer-Hanson et al., 2007; Zuccheta et al., 2010). This broad distribution and tolerance to a wide range of environments, makes *P. flesus* particularly interesting to study the mechanisms underlying habitat use. Abiotic factors affecting *P. flesus* distribution include salinity (Ramos et al., 2009), temperature (Power et al., 2000), depth (Cabral et al., 2007; Vasconcelos et al., 2010), dissolved oxygen (Maes et al., 2007; Power et al., 2000), turbidity, and sediment composition (Kerstan, 1991; Zuccheta et al., 2010). The correlation between abiotic factors and the abundance of juveniles does not

* Corresponding author. Tel.: +351 22 042 819 5.

E-mail address: claudiavrmendes@gmail.com (C. Mendes).

always imply that the former has a direct effect on the distribution patterns of the latter. Instead, abiotic factors may be used by fishes to locate habitats with favorable biotic conditions, such as reduced risk of predation or high food availability (Gibson, 2005).

Prey availability has been shown to affect the distribution of juvenile flatfish in nursery habitats (Le Pape et al., 2007; Nicolas et al., 2007; Vinagre et al., 2006). In fact, as the diet changes throughout the ontogenetic development (Aarnio et al., 1996; Andersen et al., 2005; Florin and Lavados, 2010), prey distribution may be responsible for differential ontogenetic distribution of the juveniles (Burke, 1995). The ontogenetic shifts may also contribute to reduce the trophic overlap between the different development stages (Aarnio et al., 1996). Thus, this resource partitioning strategy, either through spatial segregation or variations in the type of prey consumed may be important to minimize niche overlap and hence competition for food in nurseries (Cabral et al., 2002), where high densities of juveniles are typically observed. Consequently, survival may be enhanced, possibly increasing recruitment to adult populations (Russo et al., 2008). However, few studies have investigated prey–predator interactions influence on flatfish (Burke, 1995; Le Pape et al., 2007), and in particular flounder (Vinagre et al., 2008) nursery habitats, comparatively to the vast number of studies regarding abiotic factors (e.g. Cabral et al., 2007; Power et al., 2000; Zucchetto et al., 2010).

The Lima estuary (NW Portugal) has been previously identified as an important nursery for the flounder *P. flesus* (Ramos et al., 2010), and juvenile flounder spatial distribution has been related with abiotic features, namely the salinity regime and sediment type (Ramos et al., 2009). Thus, the present study aimed to investigate the feeding ecology and influence of prey–predator interactions on the juvenile flounder in this nursery habitat, answering the following questions: (1) Do juveniles exhibit prey selectivity?; (2) Are there ontogenetic shifts in the diet?; (3) Is there a relationship between prey availability and juvenile distribution? The study represents a step forward towards the understanding of the early life of the European flounder at its southern distribution limit. This information will help the detection of eventual changes in the habitat use of the species in this area, and consequently meet the management challenges regarding their conservation.

2. Material and methods

2.1. Study area

The Lima estuary, located in the NW Atlantic coast of Portugal, is a small open estuary with a semidiurnal and mesotidal regime (3.7 m). Salt intrusion can extend up to 20 km upstream, with an average flushing rate of 0.4 m s^{-1} , and a residence time of 9 days (Ramos et al., 2006). The river mouth is partially obstructed by a 2 km long jetty, causing a deflection of the river flow to the south, against the Coriolis effect. For this study, nine sampling stations covering the lower, middle and upper estuary were established (Fig. 1A). The lower estuary (stations L1–L3, average depth = 3.4 m), located in the initial 2.5 km, is a narrow, 9-m deep navigational channel, industrialized, with walled banks. It includes a shipyard, a commercial seaport, and a fishing harbor. The middle estuary (stations L4–L6, average depth = 4.2 m) comprises a broad shallow intertidal saltmarsh zone, mainly colonized by the common rush (*Juncus* spp.). The upper estuary (stations L7–L9) is a narrow shallow channel (<3 m), less disturbed, with natural banks and small sand islands. The average depth of the sampling sites in this estuarine section was 2.4 m.

2.2. Data collection

2.2.1. Macroinvertebrates

Macroinvertebrates are one of the main prey items of flounder juveniles as evidenced by diet studies (e.g. Link et al., 2002; Martinho et al., 2008). Thus, sediment grain characterization and macroinvertebrate community were surveyed seasonally, in February, April, July and October of 2010, representing winter, spring, summer and autumn, respectively. For each survey, triplicate sediment samples were retrieved at each sampling site by means of a Petit Ponar grab with an area of 0.023 m^2 both for sediment and macroinvertebrate community characterization. Sediment samples for macroinvertebrate analysis were immediately fixed in 5% buffered formalin stained with Rose Bengal, and stored for further laboratory analysis (Mucha et al., 2005).

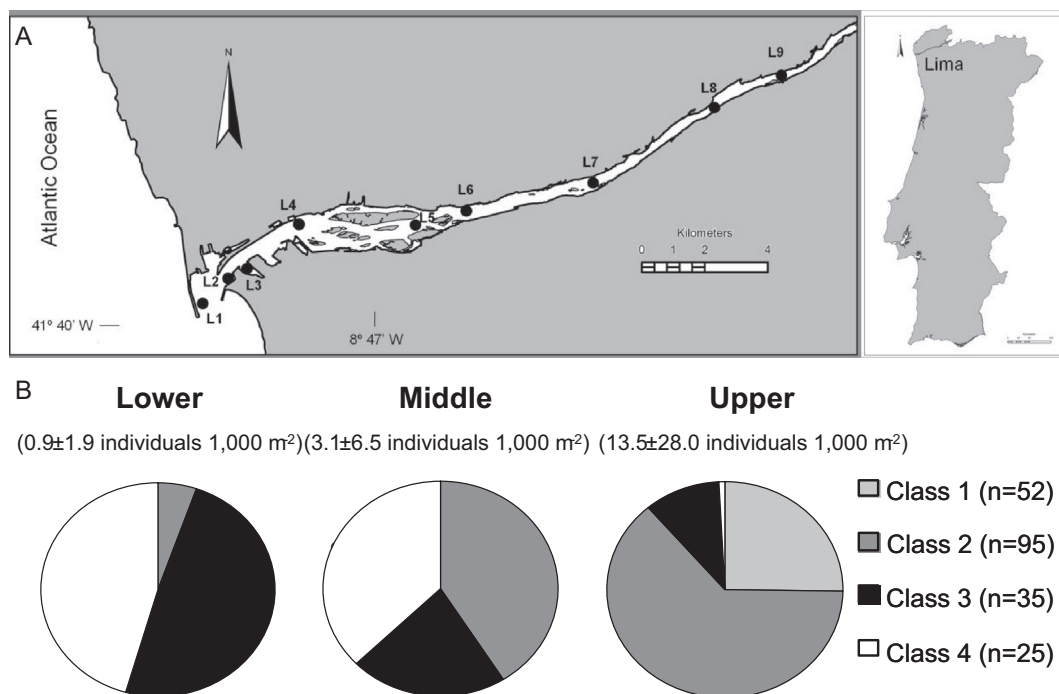


Fig. 1. Lima estuary with the location of sampling stations (A) (L1, L2, L3 – lower estuary; L4, L5, L6 – middle estuary; L7, L8, L9 – upper estuary); and average (individuals m^{-2}) and relative abundances (%) of *P. flesus* juveniles of the lower, middle and upper sections of the Lima estuary (B) (in brackets: total number of fishes sampled per size class).

2.2.2. Fishes

Flounder juveniles were collected monthly during nightly ebb tides, between September 2009 and October 2010, with a 2 m beam trawl, with a mesh size of 5 mm in the cod end and a tickler chain. Trawls were made at a constant speed (3 m s^{-2}) and lasted 10 min. The samples were refrigerated in boxes with ice and transported to the laboratory where they were frozen until sorting. Geographic location of the sampling stations and the distance traveled during each tow was measured with a Magellan 315 GPS. At each site, vertical profiles of temperature and salinity were obtained by means of a multi-parameter water quality probe YSI 6820.

2.3. Laboratory procedures

2.3.1. Sediment characterization

Unfixed sediments were treated in order to determine the percentage of organic matter, by drying the samples at 105°C (24 h), and then by loss on ignition at 500°C (4 h; APHA, 1992). Sediments were dried at 100°C , and grain size analysis was performed by wet (fraction $< 0.063 \text{ mm}$), and dry (other fractions) sieving (CISA Sieve Shaker Mod. RP.08). The sediments were divided into four fractions: silt and clay ($< 0.063 \text{ mm}$), fine sand ($0.063\text{--}0.250 \text{ mm}$), sand ($0.250\text{--}1.000 \text{ mm}$), and gravel ($> 1.000 \text{ mm}$). Each fraction was weighed and expressed as a percentage of the total weight.

2.3.2. Macroinvertebrates

Sediment samples were sieved on a 0.5 mm mesh size, and the macroinvertebrates were kept in 70% alcohol until sorting (Mucha et al., 2005). Macroinvertebrates were then counted and identified to the species level whenever possible, using a binocular microscope (Leica MZ12-5). Whenever individuals were fragmented, only the heads were considered for counting purposes.

2.3.3. Fish

Fishes were measured for total length (TL, 1 mm precision) and weight (WW, wet weight, 0.01 g precision). Considering that the flounder length at first sexual maturity is 200 mm TL (Dinis, 1986), fishes measuring less than 200 mm TL were considered juveniles. Maximum mouth gape width was determined by carefully opening the mouth of the juveniles with the aid of fine-tipped forceps and then measuring across the distance of the gape, using an ictiometer. Stomachs were excised, contents removed and preserved in alcohol 70%, for further prey identification. Each prey item was identified to the lowest taxonomic level possible, using a binocular microscope (Leica MZ12-5), counted, allowed to drip on a tissue paper and weighed (wet weight to 0.001 g). As mentioned above, whenever individuals were fragmented, only the heads were considered for counting purposes. Additionally, the length (mm) of each prey item was also determined whenever possible taking in consideration the preservation state of the prey.

2.4. Data analysis

2.4.1. Macroinvertebrate community

Macroinvertebrate abundance data was standardized as the number of individuals per m^2 of sediment. The most abundant taxa were determined by the average of each taxon abundance per estuarine section. Diversity of macrobenthos was expressed by the Shannon–Winner index (H') (Shannon and Weaver, 1949). Two-way ANOVA was performed to assess spatial and temporal differences on the macrofauna abundance and diversity (H'), with estuarine sections and seasons as fixed factors. Abundance data was log transformed ($\log(x + 1)$). Furthermore, in the event of significance, a posteriori Fisher test was used to determine which means were significantly different at a 0.05 level of probability (Zar, 1996). These analyses were performed with Statistica software (version 10.0, Statsoft Inc., Tulsa, OK, USA). Two-

way crossed analysis of similarity (ANOSIM) was used to investigate seasonal and spatial variations in the macrofauna structure. Similarity percentage analysis (SIMPER) was used to assess which species contributed more to the dissimilarities observed. Tests were based on a Bray–Curtis similarity matrix calculated based on $\log(x + 1)$ transformed abundance data. These analyses were performed with the software package PRIMER v6 (Plymouth Routines Multivariate Ecological Research) (Clarke and Warwick, 2001).

2.4.2. Flounder diet

Trawl opening (2 m) and distance traveled (determined by GPS) were used to estimate the sampled area. Therefore, densities were standardized as the number of individuals per 1000 m^2 swept area. Fishes were divided into four size classes according to their total length: class 1 ($0\text{--}49 \text{ mm}$), class 2 ($50\text{--}99 \text{ mm}$), class 3 ($100\text{--}149 \text{ mm}$), and class 4 ($150\text{--}199 \text{ mm}$). Classes 1, 2 and 3 correspond to 0^+ juveniles and class 4 to 1^+ juveniles, according to Teixeira et al. (2010). Fish condition was assessed by the Fulton's condition factor, K (Ricker, 1975). Feeding activity was evaluated by the vacuity index (I_v), defined as the percent of empty stomachs (Hyslop, 1980). The relative contribution of the different prey taxa was assessed by the percent of numerical abundance (NI), occurrence in the stomachs (OI) and weight (GI) (Hyslop, 1980). These dietary indices were also calculated for each size class. Hierarchical agglomerative clustering with group average was used to investigate dietary variations throughout the flounder juvenile development. SIMPROF test was applied to assess the significance of the clusters produced (Clarke and Warwick, 2001). Analyses were based on Bray–Curtis similarity (Bray and Curtis, 1957) matrix calculated using $\log(x + 1)$ transformed NI, OI and GI data. Multivariate analyses were performed with the software package PRIMER v6 (Plymouth Routines Multivariate Ecological Research) (Clarke and Warwick, 2001).

2.4.3. Prey–predator interactions

For prey selection analysis, only macroinvertebrates were considered since these were the main prey items of flounder juveniles. The samples were divided according to the estuarine section: lower, middle and upper, and season: autumn (September–November), winter (December–February), spring (March–May), and summer (June–August). Prey selection by flounder juveniles was quantified for each size class by the Strauss linear index L (Strauss, 1979):

$$L = r_i - p_i$$

where r_i is the relative frequency of the item i in the diet, and p_i is the relative frequency of the item i in the environment (Lima macroinvertebrate community). This index compares the proportion of a prey item in the diet with its proportion in the environment. It ranges from -1 to $+1$, with negative values indicating avoidance or inaccessibility and positive values indicating preference. Zero indicates random feeding. Extreme values occur when a prey is rare but consumed almost exclusively or is highly abundant but rarely consumed. In order to avoid false results, for example when a given prey type and fish size class does not co-occur in the estuary, prey selection was only analyzed in the most abundant section of the estuary as the following: i) for 0^+ flounder (classes 1, 2 and 3) that concentrated in the upper section, only data from the upper Lima was considered for the Strauss index; ii) for 1^+ juveniles, only the lower estuary was considered, where most of the stomach contents with macroinvertebrate prey were found. The Shannon–Wiener diversity index H' (Shannon and Weaver, 1949) was used to evaluate the diversity of each size class diet. Additionally, the potential diet overlap between the four size classes was measured by the Schoener index (SI) (Schoener, 1970):

$$SI = 1 - 0.5 \left(\sum_{i=1}^n |P_{iA} - P_{iB}| \right)$$

where P_{iA} and P_{iB} are the numerical or gravimetric proportions of the item i in the size class A and B, respectively. Values of the diet overlap vary between 0, when no food was shared and 1, when there was the same proportional use of all food resources. Wallace and Ramsey (1983) suggested that values higher than 0.6 indicate biologically significant overlap.

The influence of prey size on the flounder diet was investigated through the relationship between the prey length and fish length. Data and residuals were plotted and inspected for trends in order to assess if the underlying assumptions of independence, linearity, homoscedasticity, and normality were met. The relationship between fish total length and minimum, maximum and mean prey length for the overall individuals, and for each size class were determined through linear regressions, using R software (R Development Core Team, 2007). Differences in prey size between different juvenile flounder size classes were assessed by non-parametric test Kruskal–Wallis and multiple comparison procedures. These analyses were performed with Statistica software (version 10.0, Statsoft Inc., Tulsa, OK, USA).

The influence of environmental variables in the juvenile flounder distribution in the Lima estuary was investigated. Biotic factors like prey availability tend to act on a finer scale (Le Pape et al., 2007), where abiotic factors like salinity are favorable to fish occurrence. Thus, the environmental variables investigated comprised both water parameters and prey availability. Generalized linear models (GLM) were applied to analyze juvenile flounder distribution patterns, using a Gamma regression with a log link (McCullagh and Nelder, 1989) of the positive abundance values, therefore excluding null values. Since the flounder juveniles in the Lima estuary were mostly 0⁺, only those individuals were used to the model. Main prey *Corophium* spp. and Chironomidae, as well as the physical–chemical variables temperature, salinity and sediment composition were considered as explanatory variables. In order to evaluate potential variables, a stepwise procedure was applied where significance of predictor variables was tested and first order interactions were included. The significance of the difference in the deviance between two models resulting from the addition of a new variable was assessed by a chi-square test. The goodness of fit was evaluated by the percentage of total deviance explained and relative contribution of each variable. Statistical analyses were performed using R software (R Development Core Team, 2007). A significance level of 0.05 was considered for all the statistical procedures.

3. Results

3.1. Environmental parameters

During the study period, the water column temperature ranged between 9.1 °C and 24.9 °C, with a mean of 14.9 ± 2.4 °C. It followed the usual seasonal pattern with lower values during winter. The typical estuarine horizontal salinity gradient was always present, with salinity decreasing upstream. On average, the lower estuarine zone was in the euryhaline range (32.3 ± 5.4), while the middle estuary was in the polyhaline (29.3 ± 9.1), and the upper section was in the mesohaline range (10.3 ± 10.9).

Sediments composition varied across the estuary. The lower estuary was mainly composed by sand (47.1%) and fine sand (32%), while in the upper estuarine section gravel (65.6%) was the dominant fraction, as expected. The middle estuary presented the most equitative distribution of different types of sediment, with a prevalence of gravel (38.2%) and sand (32.7%). There was a trend for an upstream increase of the gravel and a decrease of the silt and clay fractions (lower estuary: 14.8%; upper estuary: 0.2%). The organic matter content showed throughout the year a general trend to decrease from the lower (36.9 mg g^{-1}), to the middle (27.6 mg g^{-1}), and upper (7.8 mg g^{-1}) estuarine sections following the increase in grain size.

3.2. Macroinvertebrate community

A total of 3601 individuals were identified in the macroinvertebrate community, belonging to 63 taxa. Oligochaeta, *Corophium* spp., and *Hediste diversicolor* were the most abundant taxa (Fig. 2), corresponding to 29.6%, 21.3% and 10.3% of the total macrofauna, respectively. The abundance of macrofauna averaged 1788 ± 2597 individuals m^{-2} . No significant seasonal ($F = 2.8$, $p = 0.06$), or spatial ($F = 2.1$, $p = 0.15$) variations of abundance were observed. In general, the lower estuary tended to comprise more species, with an average of 17 species, followed by the middle (average of 13), and upper estuarine sections (average of 8). Diversity (H') presented a significant spatial variation ($F = 3.9$, $p < 0.05$), but did not vary seasonally ($F = 2.5$, $p = 0.08$). Similarly to the number of species, diversity was generally higher in the lower ($H' = 1.9$) and middle estuarine sections ($H' = 1.5$), comparatively to the upper estuary ($H' = 1.3$). According to ANOSIM results, the structure of the macroinvertebrate community varied significantly ($R = 0.6$, $p < 0.05$) between the estuarine sections, but no significant ($R = 0.1$, $p = 0.24$) seasonal variation was observed. In fact, the macroinvertebrate community found in the upper estuary was significantly different from the one observed in the lower ($R = 0.7$, $p < 0.05$) or middle ($R = 0.7$, $p < 0.05$) sections (Table 1). SIMPER results identified Oligochaeta, particularly abundant in the lower estuary (Fig. 2), and *Corophium* spp. and Chironomidae, more abundant in the upper estuary (Fig. 2), being responsible for 43% of the average dissimilarity observed between the macrobenthic community of the lower and the upper sections of the estuary (Table 1). Regarding the differences between the middle and upper estuary, *H. diversicolor*, which was more abundant in the upper estuary (Fig. 2), and *Capitella* spp. and Oligochaeta, which were more abundant in the middle estuary (Fig. 2), contributed together to 43% of the total dissimilarity (Table 1).

3.3. *P. flesus* juvenile distribution

A total of 207 flounder juveniles were collected (Table 2), with the total length ranging from 19 to 197 mm, and total weight varying between 0.1 and 84.6 g. The average density of flounder was 6.0 ± 17.4 individuals 1000 m^{-2} . Class 1 juveniles, with an overall abundance of 1.7 ± 8.6 individuals 1000 m^{-2} were restricted to the upper estuary (Fig. 1B) and were only present in the Spring (4.53 ± 8.00 individuals 1000 m^{-2}), Summer (0.55 ± 1.38 individuals 1000 m^{-2}), and Autumn 2 (0.22 ± 0.67 individuals 1000 m^{-2}). Class 2 was the most abundant (3.0 ± 14.1 individuals 1000 m^{-2}), occurring throughout the year, with densities ranging from 0.48 ± 0.99 in the Summer and 10.45 ± 29.60 individuals 1000 m^{-2} in the Autumn 1. This class tended to occur mostly in the upper estuary although their presence was also noticed in other estuarine sections (Fig. 1B). A similar spatial pattern was observed for class 3 flounders, whose densities peaked in the Autumn 1 (1.53 ± 3.69 individuals 1000 m^{-2}), while the lowest densities were observed in the Spring (0.50 ± 0.79 individuals 1000 m^{-2}). The 1⁺ juveniles (class 4) with an overall abundance 0.5 ± 1.5 individuals 1000 m^{-2} were more frequent in the middle section of the Lima estuary (Fig. 1B) and were present in all seasons except Spring. Abundances of this class ranged from 0.56 ± 0.88 to 0.87 ± 1.20 individuals 1000 m^{-2} in the Winter and Autumn, respectively.

3.4. Diet of *P. flesus* juveniles

The overall percentage of empty stomachs reached 33.3%, and tended to increase with fish size, with class 1 presenting the lowest value (11.5%), followed by classes 2 (30.5%), 3 (51.4%), and 4 (64.0%). The condition of the flounder juveniles, expressed by Fulton's k factor varied between 0.3 (class 2) and 1.6 (class 2), and presented an average value of 1.0, not varying between size classes.

The diet composition of the overall *P. flesus* juveniles analyzed included 21 different taxa, containing macroinvertebrates, fishes, plant

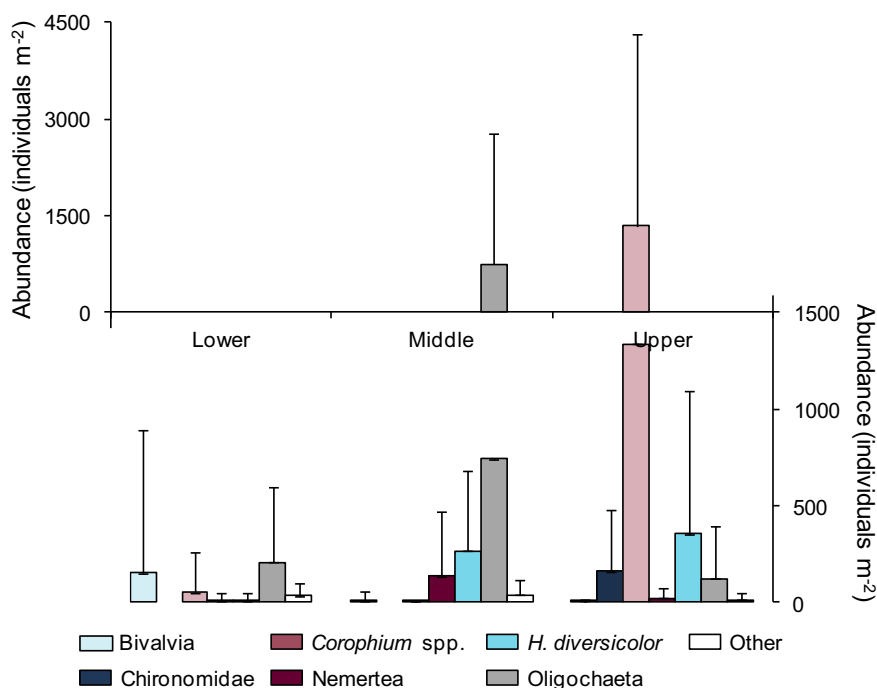


Fig. 2. Average abundance (individuals m^{-2}) per estuarine section (lower, middle and upper) of the main taxa of the Lima estuary macroinvertebrate community including the main prey of juvenile flounder.

debris and sand. In terms of number and occurrence, the diet of flounder juveniles was mainly composed by macroinvertebrate preys, namely Chironomidae, *Corophium* spp., and Elmidae. Gravimetrically, *Corophium* spp., and the brown shrimp *Crangon crangon* were the most important items.

The diet of the newly settled flounders (class 1) included 1453 items. Chironomidae were the dominant item, both in terms of number (NI = 92.4%), occurrence (OI = 68.2%), and weight (GI = 64.0%) (Fig. 3). *Corophium* spp. was important in terms of occurrence (OI = 12.7%), and weight (GI = 21.8%). Elmidae appeared as a minor item (NI = 5.3%, OI = 3.2%, GI = 9.7%), as well as Bivalvia, Ephemeroptera, and Caenidae. The diet of class 2 juveniles was more diversified, including Amphipoda, Polychaeta, Bivalvia, Mysidacea, and Insecta larvae, with a total of 550 items. *Corophium* spp. was the main prey item in any of the three applied dietary indices (NI = 55.5%, OI = 47.2%, GI = 75.1%) (Fig. 3). The diet also comprised Chironomidae and Elmidae, which were important in terms of number (NI = 22.5%; 16.0%), and occurrence (OI = 10.1%; 22.5%). Moreover, *C. crangon* was gravimetrically important (GI = 8.5%). The diet of class 3 comprised

63 items. Similarly to class 2, *Corophium* spp. was the main item of class 3, according to all dietary indices (NI = 50.8%; OI = 31.3%; GI = 63.1%) (Fig. 3). Chironomidae and the gastropod *Ecrobia truncata* were numerically important (NI = 19.0%; 17.5%), as well as in terms of occurrence (OI = 12.5%; 12.5%), while *C. crangon* assumed a great importance in terms of weight (GI = 34.3%), and occurrence (OI = 18.8%). Other items were also present in the diet of this size class, namely Bivalvia, Polychaeta, the gastropod *Potamopyrgus jenkinsi*, and Simuliidae, but with low values. The diet of older juveniles (class 4) consisted of only 5 taxa, and a total of 20 items. Nemertea (NI = 40.0) and *Corophium* spp. (NI = 30.0%) were the main items according to the numerical index, while Teleostea (GI = 5.6%) and Crustacea (GI = 68.4%), including *Carcinus maenas* (GI = 29.5%) and *Corophium* spp. (GI = 9.25%), were the most important items in terms of weight (Fig. 3). All items presented similar values of occurrence (OI = 14.3%).

Macroinvertebrates were the only prey items preserved enough to allow the measurement of the respective total length. Thus, macroinvertebrate prey total length varied significantly ($H = 29.42$, $p < 0.05$) between different juvenile flounder size classes. Class 1 prey size (mean = 2.23 ± 0.87 mm) was significantly lower than prey size of classes 2 (mean = 5.31 ± 1.77 mm) and 3 (mean = 7.92 ± 3.88 mm), but not from class 4 (mean = 4.71 ± 3.12 , $p = 0.31$). The minimum prey size observed for classes 1 and 2 was 1 mm, while the maximum prey length was 7 mm for class 1, and 13 mm for class 2. The prey length of class 3 ranged between 3 and 28 mm, while for class 4 values ranged between 2 and 9 mm. The minimum ($R^2 = 0.9$), mean ($R^2 = 0.9$), and maximum ($R^2 = 0.5$) prey length (Table 3)

Table 1

Results of ANOSIM (R values and significance levels) and SIMPER analyses on abundance of macroinvertebrate taxa (SIMPER results for the three most important taxa contributing to dissimilarities are shown).

Groups	ANOSIM		Average dissimilarity (%)	SIMPER discriminating taxa	Cumulative contribution (%)
	R	P			
Lower vs. middle	0.5	0.06	69.8	<i>Hediste diversicolor</i>	15.3
				Oligochaeta ni	30.4
				Nemertea ni	30.9
				Oligochaeta ni	15.1
Lower vs. upper	0.7	0.03 ^a	83.4	<i>Corophium</i> spp.	30.1
				Chironomidae ni	43.3
				<i>H. diversicolor</i>	14.9
				<i>Capitella</i> spp.	29.3
Middle vs. upper	0.7	0.03 ^a	78.4	Oligochaeta ni	43.4

^a Significant value.

Table 2

Number of *P. flesus* juveniles sampled per size class, mean total length (mm) and mean total weight (g).

Class	Class 1	Class 2	Class 3	Class 4
Number	52	95	35	25
Mean total length (mm)	29.1 ± 8.1	71.9 ± 12.6	121.3 ± 16.1	172.8 ± 13.3
Mean total weight (g)	0.3 ± 0.3	4.3 ± 2.1	19.7 ± 8.9	56.7 ± 14.6

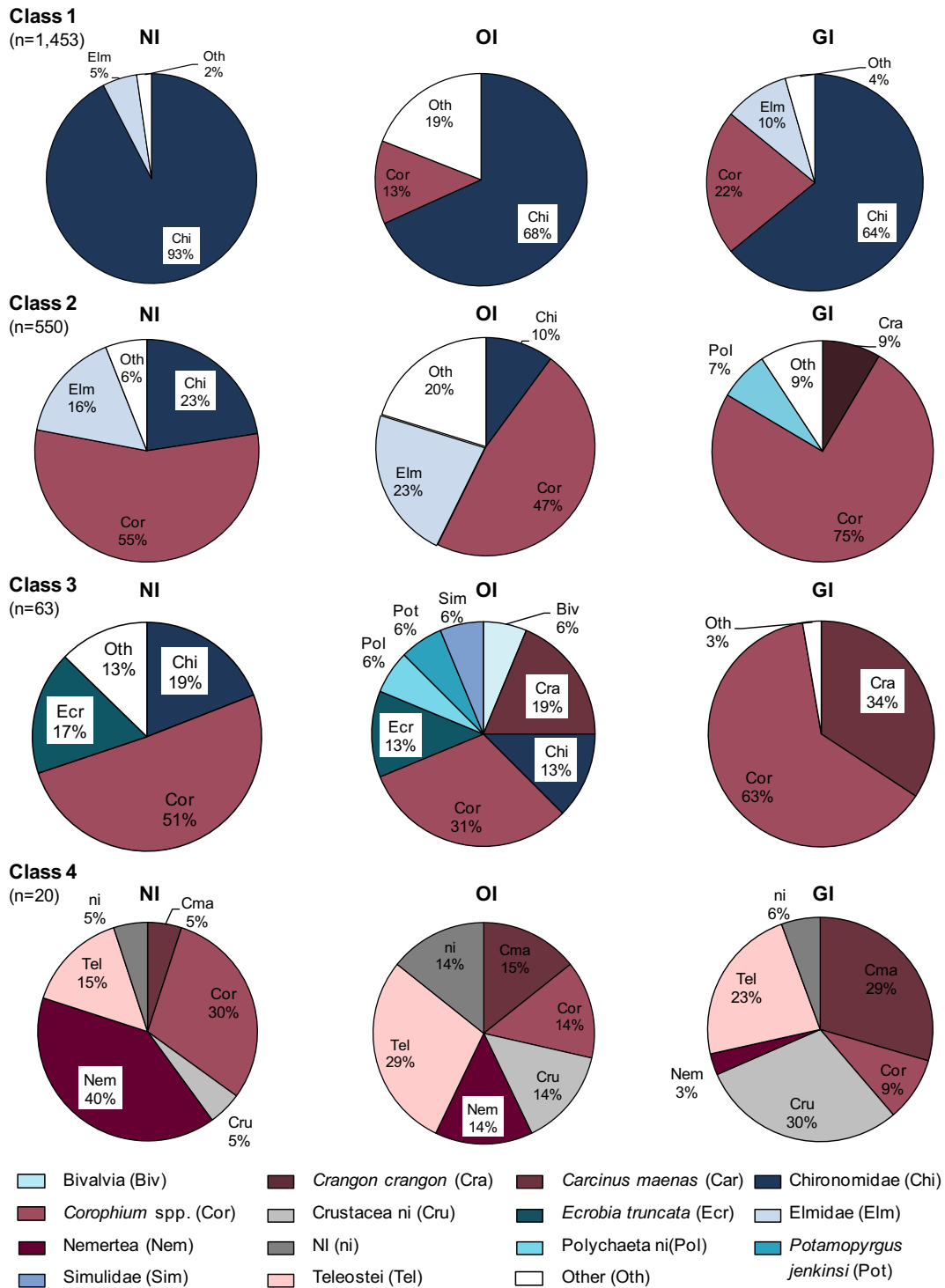


Fig. 3. Numerical (NI), occurrence (OI) and gravimetric (GI) indices for stomach contents of *P. flesus* juveniles for each size class (in brackets: number of prey items per size class).

significantly ($p < 0.05$) increased with total length of class 3 individuals. However, such trends were not always observed when considering the remaining classes (Table 3). When considering NI and GI, there was a diet overlap between the 0^+ juveniles of classes 2 and 3 ($SI = 0.7$). In fact, cluster analysis showed that the diet of classes 2 and 3 clustered at a level between 50% and 60% of similarity, based on NI, OI and GI (Fig. 4). In contrast, the diet of the 1^+ juvenile flounder (class 4) differed significantly from the diet of the 0^+ juveniles (classes 1–3), as shown by the SIMPROF analysis ($p < 0.05$).

3.5. Prey selection

The diet diversity increased along the fish size classes, according to the Shannon–Wiener index, from $H' = 0.3$ for class 1, $H' = 1.3$ for class 2, $H' = 1.4$ for class 3 and $H' = 1.5$ for class 4. Diet diversity index revealed a non-specialized character of the diet, with an overall value of $H' = 0.97$.

According to the Strauss linear index (L), each size class exhibited a different prey selection pattern (Fig. 5). For example, class 1 individuals

Table 3

Statistics for linear regression analysis (R^2 and p value) on minimum, mean and maximum prey length and juvenile flounder total length, according to fish size class.

Size class	Prey length					
	Minimum		Mean		Maximum	
	R^2	p	R^2	p	R^2	p
All fishes	0.26	$p < 0.05$	0.43	$p < 0.05$	0.17	$p < 0.05$
Class 1	0.12	$p < 0.05$	0.04	$p = 0.07$	0.03	$p = 0.27$
Class 2	0.02	$p = 0.46$	0.20	$p < 0.05$	0.06	$p = 0.13$
Class 3	0.94	$p < 0.05$	0.87	$p < 0.05$	0.51	$p < 0.05$
Class 4	0.42	$p = 0.40$	0.85	$p = 0.36$	0.26	$p = 0.49$

presented high L values for Chironomidae during spring and summer (Fig. 5B), and negative values during autumn that coincided with the absence of this taxon among the macroinvertebrate community of the upper estuary (Fig. 5A). However, near zero and negative values were observed for *Corophium* spp. during summer and autumn (Fig. 5B) respectively, although its abundance in the upper estuary was high during these periods (Fig. 5A). L values for Bivalvia and Isopoda were near zero, indicating random feeding on these items (Fig. 5B). Class 2 L values were high for Chironomidae during winter and spring (Fig. 5C). A negative L value occurred in summer, concomitant with a decrease in the respective abundance (Fig. 5A), and an increase in the proportion of *Corophium* spp. in the diet. Concerning class 3, positive L values for

Chironomidae were only observed during winter, while for *Corophium* spp., positive values occurred in spring and autumn (Fig. 5D), the later corresponding to its peak abundance in the macroinvertebrate community (Fig. 5A). Class 4 individuals consumed *Corophium* spp. and Nemertea during winter, presenting positive L values for these items (Fig. 5E). The juveniles from this class caught during other seasons presented always empty stomachs. Polychaetes, although dominant in the macrobenthic community, were nearly absent from the diet of all the fish size classes. Thus, L values for polychaetes were always negative. Some important prey items, namely Elmidae and *E. truncata* were not considered due to their absence in the macrobenthos samples.

Generalized linear models showed that salinity ($p < 0.05$, Table 4) together with the interaction between Chironomidae and *Corophium* spp. ($p < 0.05$, Table 4) were the main factors associated with the 0^+ flounder abundance in the Lima estuary. Accordingly, higher densities of 0^+ flounder were observed in the upper estuary where both Chironomidae and *Corophium* spp. were more abundant than in the other areas where only *Corophium* spp. or none of these main preys were present. Chironomidae were also significantly ($p < 0.05$) associated with these flounders, although with a minor contribution to percentage of deviance explained by the model (Table 4). The model presented an explanatory value of 62% of variability (Table 4).

4. Discussion

The main prey items of flounder juveniles in the Lima estuary included the macroinvertebrates Chironomidae and *Corophium* spp. Chironomidae commonly occur in the flounder diet (Nissling et al., 2007), particularly of smaller juveniles (Florin and Lavados, 2010; Weatherley, 1989). Indeed, Chironomidae dominated the diet of the newly settled juveniles (class 1) in the Lima estuary and was also a major item of classes 2 and 3. *Corophium* spp. was an important item across all size classes of juveniles, similarly to several studies (Hampel et al., 2005; Summers, 1979), including in Portuguese estuaries (Costa and Bruxelas, 1989; Vinagre et al., 2005). Moreover, the inclusion of Elmidae in the diet of classes 1 and 2 emerged as a characteristic behavior of juvenile flounder of the Lima estuary, since no reports of Elmidae integrating the diet of flounder were found in other studies. Paradoxically, this taxon was absent from the macroinvertebrate community. Since these organisms are typical of freshwater environments, juveniles could have been feeding further upstream of the sampling area.

Although polychaetes dominated the macroinvertebrate community in the Lima estuary, they were only present as minor prey items of all flounder size classes. The large size and burrowing ability of polychaetes represents a high handling time and energy cost that may limit their capture by the small flounder (Vinagre et al., 2008). However, polychaetes were not an important item of older 0^+ juveniles (class 3) as reported elsewhere (Selleslagh and Amara, 2014; Vinagre et al., 2008). Specifically, the polychaete *H. diversicolor* and oligochaetes were totally absent in the diet of flounder juveniles, despite being dominant taxa in the macroinvertebrate community and common preys of juvenile flounder (Costa and Bruxelas, 1989; Hampel et al., 2005; Weatherley, 1989). While the burrowing behavior of oligochaetes may prevent their capture by the flounder juveniles (Andersen et al., 2005), *H. diversicolor* is active on the sediment surface (Fauchald and Jumars, 1979; Muus, 1967; Scaps, 2002). Moreover, *H. diversicolor* individuals found in the Lima macroinvertebrate community were within the range of the prey consumed by all the juvenile classes, including the newly settled. Thus, the present results seem to indicate that prey size was not the reason for its absence in the flounder diet. Gastropods *E. truncata* and *P. jenkinsi*, although common items in the diet, were not observed in the benthic samples during this study. However, in the context of other studies, these species have been frequently observed in the Lima estuary (data not shown), explaining their occurrence in the flounder diet.

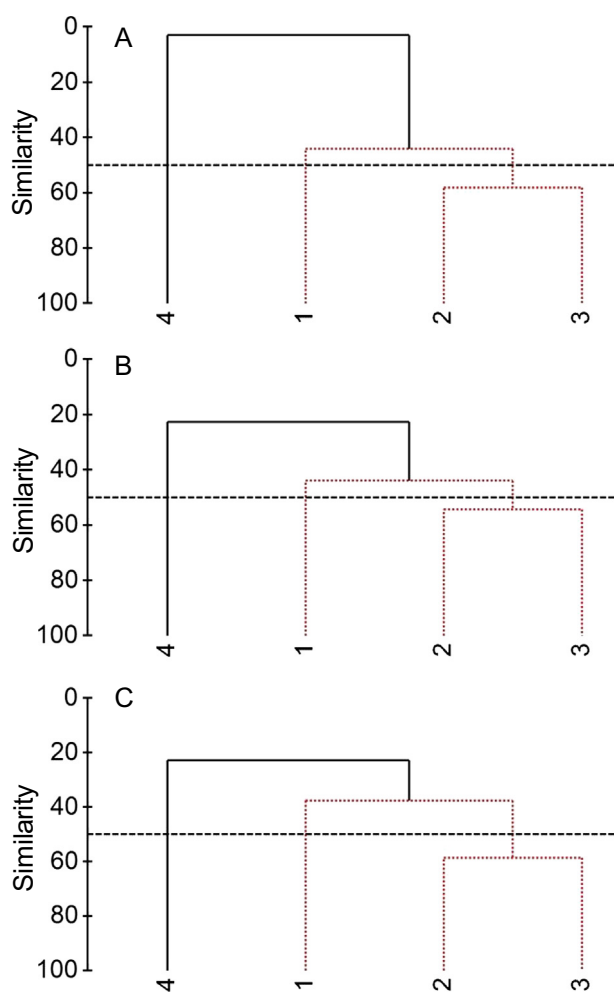


Fig. 4. Cluster analysis of the four *P. flesus* size classes, based on numerical index (NI) (A), occurrence index (OI) (B) and gravimetric index (GI) (C). Significant clusters according to SIMPROF are shown in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

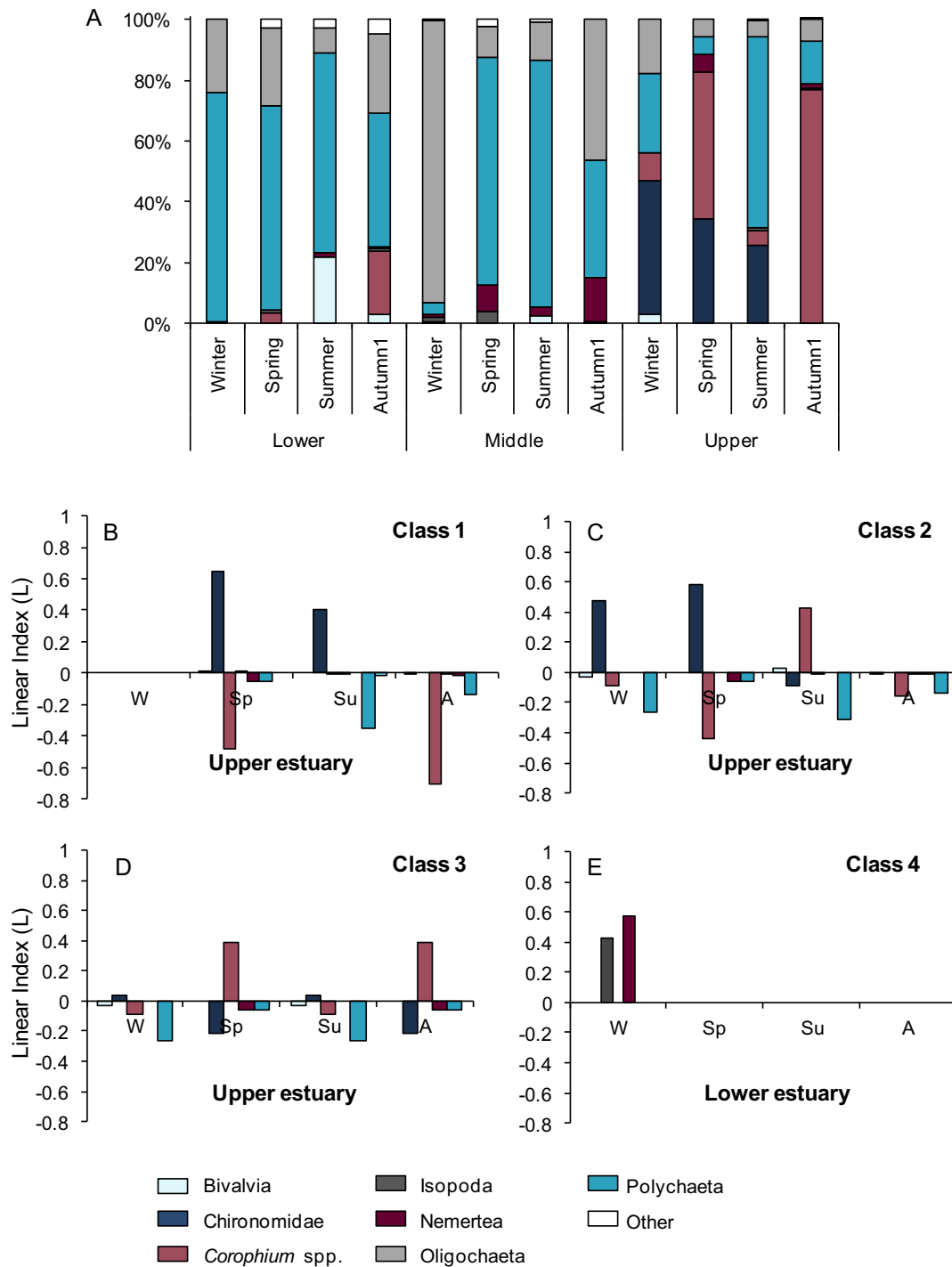


Fig. 5. a) Seasonal abundance of macrobenthos prey in the Lima estuary (A); Strauss linear index values for the main prey items of *P. flesus* size classes: 1 (B), 2 (C), 3 (D) and 4 (E) (W – winter; Sp – spring; Su – summer; A – autumn).

A general increase of prey length was observed along flounder juvenile development, a pattern commonly associated with the increase of the mouth gape width (Dorner and Wagner, 2003; Juanes, 1994; Keast and Webb, 1966). Noteworthy, the preys consumed by 1⁺ juveniles did not present a significantly ($p > 0.05$) higher total length in comparison to the prey consumed by the newly settled flounders. This feature might have been a consequence of the low number of prey analyzed for this class and their advanced digestion state that prevented the measurement of larger prey like Teleostei and unidentified crustaceans that were also fragmented. Moreover, despite the ability to ingest larger prey, larger flounder diet continued to include small food items

(Vinagre et al., 2008) such as Nemertea. The increase in vacuity also observed along the size classes suggests a higher feeding activity of the smaller flounders, as they tend to present higher consumption rates when compared to the older ones (Fonds et al., 1992). Fish consumption rates depend on the energy content, particle size and conversion efficiency of their prey (Arrington et al., 2002; Bowen et al., 1995; Brett and Groves, 1979). Accordingly, the consumption of larger and energetically more profitable prey by the older juveniles may have enabled an increase of the feeding intervals. Furthermore, the ingestion of larger preys may also explain why older juveniles (class 4) presented such reduced number of prey items and similar condition factor of the younger

Table 4

Statistics for the Gamma regression models fitted to 0^+ *P. flesus* densities in the Lima estuary (residual deviance, deviance, percentage of the total deviance explained by each factor and p value).

	Residual deviance	Deviance	% explained	p value
Null	26.31			$p < 0.05$
Salinity	15.99	10.32	39.23	$p < 0.05$
<i>Corophium</i> spp.	15.97	0.02	0.06	$p = 0.84$
Chironomidae	15.85	0.12	0.46	$p < 0.05$
<i>Corophium</i> spp.: Chironomidae	9.95	5.90	22.42	$p < 0.05$
Total			62.17	

flounder. These results suggest that fishes maintained the same nutritional state throughout their juvenile phase. Moreover, the flounder condition in the Lima estuary ($k = 1.0$) was within the range of the results obtained for other European estuaries ($k_{\min} = 0.73$, $k_{\max} = 1.20$, Amara et al., 2009), but slightly higher than for other Iberian estuaries, namely Minho, Douro and Mondego ($k = 0.73$) (Vasconcelos et al., 2009).

Flounders are capable of an adaptive feeding behavior, optimizing the use of the food resources (Andersen et al., 2005). In fact, flounder feeding becomes more specialized when preys are highly available, as in sandy habitats, in contrast to vegetated habitats (Andersen et al., 2005). The upper section of the Lima estuary, where most of juveniles concentrated, was mainly composed of gravel, being the vegetation scarce. Thus, the low diversity of the flounder juvenile diet may have resulted from the presence of *Corophium* spp., an active and abundant prey that has been indicated as main item in these types of habitats (Grønkvær et al., 2007). Nevertheless, diet diversity remained within the range of values reported for flounder juveniles (Aarnio et al., 1996; Andersen et al., 2005; Hampel et al., 2005). The diet diversity was particularly low for the newly settled juveniles (class 1), since their diet was mostly based on Chironomidae (NI = 92.4%), and increased along the size classes, in agreement with other studies (Aarnio et al., 1996). This feature might reflect the ability of larger fish to ingest larger and consequently, a wider range of prey. However, the high diversity of the class 4 diet needs to be interpreted with caution, since the stomach contents analyzed comprised only 20 prey items belonging to 5 taxa. Thus, the high diet diversity of class 4 resulted from an equilibrated contribution of the prey items in the diet and should not be interpreted as more diverse in terms of including more prey items.

The diet of 0^+ juveniles of classes 2 and 3 denoted a relevant diet overlap. Indeed, *Corophium* spp. and Chironomidae in terms of NI, and *C. crangon* in terms of GI comprised the main prey items of these classes. The diet overlap may be the result of a common distribution pattern along the middle and upper estuaries where the same kind of prey items was available. The diet of 1^+ juveniles (class 4) differed from the young flounder diet, indicating an ontogenetic shift in the diet. In the nursery grounds, where high densities of flatfish juveniles of different species can occur, both inter- and intraspecific competition may arise (Martinsson and Nissling, 2011). Ontogenetic shifts in the diet have been reported (Florin and Lavados, 2010), enabling resource partitioning between different life stages, and minimizing niche overlap, hence intraspecific competition. The diet shift observed for 1^+ juveniles resulted from the incorporation of new items such as *C. maenas*, Nemertea and Teleostea. Moreover, this feature may be related to the ability of older juveniles to consume larger prey, as the diet shift was coincident with an important increase of the prey size of the larger 0^+ (class 3). Uzars et al. (2003) reported a similar diet shift to large amphipods, decapods, and fishes, but at a lower fish size (90–150 mm TL). Noteworthy, Nemertea which is a small food item was not observed in the diet of smaller classes. This item was included in the diet of flounder juveniles caught in the lower estuary, while the smaller juveniles were concentrated in the upper estuary where other small prey, namely

Chironomidae and *Corophium* spp. were more abundant. Thus, the ontogenetic shift in the diet may also have been driven by a partial spatial partitioning of 0^+ and 1^+ flounder in the Lima estuary.

A relationship between flounder juvenile distribution and salinity, sediment characteristics and prey availability was observed in several Portuguese estuaries, using GLM (Vasconcelos et al., 2010). In the Lima estuary, the spatial distribution of flounder juveniles was also negatively correlated with salinity (Table 4). Indeed, younger juveniles (class 1 and most of class 2) were restricted to the upper estuary in agreement with Ramos et al. (2010), suggesting a preference for low salinity waters (Bos and Thiel, 2006). Furthermore, older juveniles (classes 3 and 4) assumed a broader distribution throughout the estuary as they developed as regularly observed in other estuarine habitats (Kerstan, 1991). However, salinity tolerance may not be enough to explain these distribution patterns, as older 0^+ and 1^+ flounders have also been commonly found in estuarine low salinity areas (Freitas et al., 2009; Martinho et al., 2007). Moreover, laboratory experiments showed that growth (Gutt, 1985) and condition (Gutt, 1985; O'Neill et al., 2011) of newly settled were not enhanced in low salinity conditions, suggesting that other factors such as high food supply, low predation, and competition (Beaumont and Mann, 1984; Bos, 1999), may also be related to the newly settled preference for upstream areas. Accordingly, a relationship between prey abundance and the distribution of flatfish juveniles has been described (Le Pape et al., 2007; Vinagre et al., 2009), including for flounder (Vasconcelos et al., 2010). Furthermore, environmental variables such as sediment composition and salinity may only act indirectly (Gibson, 2005), by influencing the distribution of the macroinvertebrate prey (Amezcuca and Nash, 2001; Gibson, 1994; McConnaughey and Smith, 2000). In fact, Ramos et al. (2010) showed that the spatial distribution of *P. flesus* juveniles was related to the sediment composition in the Lima estuary possibly through its effect on prey abundance. Indeed, the 0^+ flounder distribution in the Lima estuary was also related to the abundance of the main preys Chironomidae and *Corophium* spp. (Table 4), highly abundant and characteristic of the upper estuary. Chironomidae are typical of freshwater, often being the dominant group of insects in these environments (Armitage et al., 1995). On the other hand, *Corophium* spp. is highly tolerant to a wide range of salinities, from 2 to 50‰ (McLusky, 1967), and in the Lima estuary, *Corophium multisetosum*, one of the recorded species, showed a preference for low salinities (Sousa et al., 2007). Thus, although highly available preys such *Corophium* spp. were present throughout the estuary, the 0^+ flounder concentrated in the upper estuary where abundances of this prey were higher and Chironomidae were also available. In conclusion, salinity was the main environmental drive of the juvenile flounder spatial distribution, which jointly with high food availability of the upper section of the Lima estuary, explained the concentration of the newly settled flounders in that section of the estuary. Our results seem to support that both abiotic and biotic factors affect the suitability of fish habitats. However, the contribution of these preys to the 0^+ flounder distribution model may be related to their co-occurrence in the upper estuary, especially when considering the feeding opportunist behavior reported for flounder (De Groot, 1971; Martinho et al., 2008), and the high densities of Chironomidae and *Corophium* spp. in the upper estuary. Further studies are required to test these hypotheses, by comparing areas with similar salinity and distinct *Corophium* spp. and Chironomidae abundances, what was not possible to find in our dataset of the Lima estuary.

Variations of the proportions of the main prey items in the diet of flounder juveniles, namely Chironomidae and *Corophium* spp. tended to reflect fluctuations in the Lima estuarine macroinvertebrate community. The diet of class 1 presented a high proportion of Chironomidae during Spring and Summer when this item was highly available in the macroinvertebrate community. Nevertheless, Chironomidae abundances may be higher than those reported, as these organisms are also present in the bottom water column (Walton, 1979) where newly settled may feed. As a result, an overestimation of Chironomidae selection

may have occurred. On the other hand, the decrease of *Corophium* spp. in the diet of class 2 coincided with its peak abundance in the macroinvertebrate community during autumn. At this time of year, Chironomidae were not observed in the macroinvertebrate community, whereas Elmidae emerged in the diet as a major item. As stated above, Elmidae was not observed in the macroinvertebrate community, and therefore prey selection for this item was not evaluated. Hence, seasonal variations of the main prey items revealed that juvenile flounder adapted their diet according to the availability of prey (Aarnio et al., 1996), feeding on abundant items.

5. Conclusions

The present study showed that:

- (1) Juvenile flounder in the Lima estuary fed mainly on macroinvertebrates, and the diet became more diverse as the juveniles grew, including other groups and larger size preys.
- (2) 0⁺ flounder fed on highly available items (Chironomidae and *Corophium* spp.), exhibiting a highly specialized diet.
- (3) The newly settled flounders were restricted to the Lima upper estuary, and fed almost exclusively on Chironomidae and *Corophium* spp., typical species of this estuarine section.
- (4) An ontogenetic shift in the diet occurred from 0⁺ to 1⁺ juveniles.
- (5) Salinity and preys Chironomidae and *Corophium* spp. were the main factors associated with 0⁺ flounder distribution in the Lima estuary.

Acknowledgments

This study was partially supported by the Portuguese Science and Technology Foundation (FCT) through a PhD fellowship to Cláudia Mendes (SFRH/BD/86325/2012) and a post-doc fellowship (SFRH/BPD/26782/2006) to Sandra Ramos, and by the European Regional Development Fund (ERDF) through the COMPETE – Operational Competitiveness Programme and national funds through FCT – Foundation for Science and Technology, under the project PEst-C/MAR/LA0015/2011. The authors would like to thank all the people that helped in the field surveys and sorting. [RH]

References

- Aarnio, K., Bonsdorff, E., Rosenback, N., 1996. Food and feeding habits of juvenile flounder *Platichthys flesus* (L.), and turbot *Scophthalmus maximus* L. in the Åland archipelago, northern Baltic Sea. *J. Sea Res.* 36 (3–4), 311–320.
- Amara, R., Selleslagh, J., Billon, G., Minier, C., 2009. Growth and condition of 0-group European flounder, *Platichthys flesus* as an indicator of estuarine habitat quality. *Hydrobiologia* 627, 87–98.
- Amezcu, F., Nash, R.D.M., 2001. Distribution of the order Pleuronectiformes in relation to sediment type in the North Irish Sea. *J. Sea Res.* 45, 293–301.
- Andersen, B.S., Carl, J.D., Grønkjær, P., Støttrup, J.C., 2005. Feeding ecology and growth of age 0 year *Platichthys flesus* (L.) in a vegetated and a bare sand habitat in a nutrient rich fjord. *J. Fish Biol.* 66, 531–552.
- APHA, 1992. Standard methods for the examination of water and wastewater, 18th ed. American Public Health Association, Washington, D.C.
- Armitage, P., Cranston, P.S., Pinder, C.V., 1995. The Chironomidae: The Biology and Ecology of Non-biting Midges. Chapman & Hall, London (572 pp.).
- Arrington, D.A., Winemiller, K.O., Loftus, W.F., Akin, S., 2002. How often do fishes “run on empty”? *Ecology* 83 (8), 2145–2151.
- Beaumont, W.R.C., Mann, R.H.K., 1984. The age, growth and diet of a freshwater population of the flounder, *Platichthys flesus* (L.), in southern England. *J. Fish Biol.* 25, 607–616.
- Beck, M.W., Heck, K.L.J., Able, K.W., Childers, D.L., Eggleston, D.B., Gillanders, B.M., Halpern, B., Hays, C.G., Hoshino, K., Minello, T.J., Orth, R.J., Sheridan, P.F., Weinstein, M.P., 2001. The identification, conservation, and management of estuarine and marine nurseries for fish and invertebrates. *Bioscience* 51 (8), 633–641.
- Bos, A., 1999. Tidal transport of flounder larvae (*Pleuronectes flesus*) in the Elbe River, Germany. *Arch. Fish. Mar. Res.* 47, 47–60.
- Bos, A.R., Thiel, R., 2006. Influence of salinity on the migration of postlarval and juvenile flounder *Pleuronectes flesus* L. in a gradient experiment. *J. Fish Biol.* 68, 1411–1420.
- Bowen, S.H., Lutz, E.V., Ahlgren, M.O., 1995. Dietary protein and energy as determinants of food quality: trophic strategies compared. *Ecology* 76, 899–907.
- Bray, J.R., Curtis, J.T., 1957. An ordination of the upland forest communities of southern Wisconsin. *Ecol. Monogr.* 27, 325–349.
- Brett, J.R., Groves, T.D.D., 1979. Physiological energetics. In: Hoar, W.S., Randall, D.J., Brett, J.R. (Eds.), *Fish Physiology Bioenergetics and Growth* vol. 8. Academic Press, New York, pp. 279–352.
- Burke, J.S., 1995. Role of feeding and prey distribution of summer and southern flounder in selection of estuarine nursery habitats. *J. Fish Biol.* 47, 355–366.
- Burrows, M.T., 1994. An optimal foraging and migration model for juvenile plaice. *Evol. Ecol.* 8 (2), 125–149.
- Cabral, H.N., Costa, M.J., Salgado, J.P., 2001. Does the Tagus estuary fish community reflect environmental changes? *Clim. Res.* 18, 119–126.
- Cabral, H.N., Lopes, M., Loeper, R., 2002. Trophic niche overlap between flatfishes in a nursery area on the Portuguese coast. *Sci. Mar.* 66 (3), 293–300.
- Cabral, H.N., Vasconcelos, R., Vinagre, C., França, S., Fonseca, V., Maia, A., Reis-Santos, P., Lopes, M., Ruano, M., Campos, V., Santos, P.T., Costa, M.J., 2007. Relative importance of estuarine flatfish nurseries along the Portuguese coast. *J. Sea Res.* 57, 209–217.
- Castillo-Rivera, B.M., Kobelkowsky, A., Chávez, A.M., 2000. Feeding biology of the flatfish *Citharichthys spilopterus* (Bothidae) in a tropical estuary of Mexico. *J. Appl. Ichthyol.* 16 (2), 73–78.
- Clarke, K.R., Warwick, R.M., 2001. Change in Marine Communities: An Approach to Statistical Analysis and Interpretation, 2nd edition. PRIMER-E, Plymouth.
- Costa, M.J., Bruxelas, A., 1989. The structure of fish communities in the Tagus estuary, Portugal, and its role as a nursery for commercial fish species. *Sci. Mar.* 53, 561–566.
- De Groot, S.J., 1971. On the interrelationships between morphology of the alimentary tract, food and feeding behaviour in flatfishes (Pisces: Pleuronectiformes). *Neth. J. Sea Res.* 5, 121–196.
- Development Core Team, 2007. R: A language and environment for statistical computing. R foundation for statistical computing, Vienna, Austria.
- Dinis M.T., 1986. Quatre soleidae de l'estuaire du Tage. Reproduction et croissance. Essai de l'évage de *Solea senegalensis* Kaup. Université de Bretagne Occidentale: Brest, France (PhD Thesis).
- Dorner, H., Wagner, A., 2003. Size-dependent predator–prey relationships between perch and their fish prey. *J. Fish Biol.* 62, 1021–1032.
- Fauchald, K., Jumars, P.A., 1979. The diet of worms: a study of polychaete feeding guilds. *Oceanogr. Mar. Biol. Annu. Rev.* 17 (193–284), 1979.
- Florin, A.B., Lavados, G., 2010. Feeding habits of juvenile flatfish in relation to habitat characteristics in the Baltic Sea. *Estuar. Coast. Shelf Sci.* 86 (4), 607–612.
- Fonds, M., Cronie, R., Vethaak, A.D., Van Der Puyl, P., 1992. Metabolism, food consumption and growth of plaice (*Pleuronectes platessa*) and flounder (*Platichthys flesus*) in relation to fish size and temperature. *Neth. J. Sea Res.* 29 (1–3), 127–143.
- Freitas, V., Costa-Dias, S., Campos, J., Bio, A., Santos, P., Antunes, C., 2009. Patterns in abundance and distribution of juvenile flounder *Platichthys flesus*, in Minho estuary (NW Iberian Peninsula). *Aquat. Ecol.* 43 (4), 1143–1153.
- Gibson, R.N., 1994. Impact of habitat quality and quantity on the recruitment of juvenile flatfishes. *Neth. J. Sea Res.* 32 (2), 191–206.
- Gibson, R.N., 2005. Flatfishes: Biology and Exploitation. Blackwell Publishing, Oxford (391 pp.).
- Gibson, R.N., Robb, L., Burrows, M.T., Ansell, A.D., 1996. Tidal, diel and longer term changes in the distribution of fishes on a Scottish sandy beach. *Mar. Ecol. Prog. Ser.* 130, 1–17.
- Gibson, R.N., Rob, L., Wennhage, H., Burrows, M.T., 2002. Ontogenetic changes in depth distribution of juvenile flatfishes in relation to predation risk and temperature on a shallow-water nursery ground. *Mar. Ecol. Prog. Ser.* 229, 233–244.
- Grønkjær, P., Carl, J.D., Rasmussen, T.H., Hansen, K.W., 2007. Effect of habitat shifts on feeding behaviour and growth of 0 year-group flounder *Platichthys flesus* (L.) transferred between macroalgae and bare sand habitats. *J. Fish Biol.* 70 (5), 1587–1605.
- Gutt, J., 1985. The growth of juvenile flounders (*Platichthys flesus* L.) at salinities of 0, 5, 15 and 35‰. *J. Appl. Ichthyol.* 1, 17–26.
- Hampel, H., Cattrijsse, A., Elliot, M., 2005. Feeding habits of young predatory fishes in marsh creeks situated along the salinity gradient of the Schelde estuary, Belgium and The Netherlands. *Helgol. Mar. Res.* 59 (2), 151–162.
- Hemmer-Hanson, J., Nielsen, E.E.G., Grønkjær, P., Loeschcke, V., 2007. Evolutionary mechanisms shaping the genetic population structure of marine fishes; lessons from the European flounder (*Platichthys flesus* L.). *Mol. Ecol.* 16 (15), 3104–3118.
- Hugie, D.M., Dill, L.M., 1994. Fish and game: a game theoretic approach to habitat selection by predators and prey. *J. Fish Biol.* 45, 151–169.
- Hyslop, E.J., 1980. Stomach content analysis – a review of methods and their application. *J. Fish Biol.* 17, 411–429.
- Juanes, F., 1994. What determines prey size selectivity in piscivorous fishes? In: Stouder, D.J., Fresh, K.L., Feller, R.J. (Eds.), *Theory and Application in Fish Feeding Ecology*. University of South Carolina Press, Columbia, S.C., pp. 79–100.
- Keast, A., Webb, D., 1966. Mouth and body form relative to feeding ecology in fish fauna of a small lake, Lake Opinicon, Ontario. *J. Fish. Res. Board Can.* 23, 1845–1874.
- Kerstan, M., 1991. The importance of rivers as nursery grounds for 0- and 1-group flounder (*Platichthys flesus* L.) in comparison to the Wadden Sea. *Neth. J. Sea Res.* 27, 353–366.
- Le Pape, O., Baulier, L., Cloarec, A., Martin, J., Le Loc'h, F., Désaunay, Y., 2007. Habitat suitability for juvenile common sole (*Solea solea*, L.) in the Bay of Biscay (France): a quantitative description using indicators based on epibenthic fauna. *J. Sea Res.* 57 (2–3), 126–136.
- Link, J.S., Bolles, K., Milliken, C.G., 2002. The feeding ecology of flatfish in the Northwest Atlantic. *J. Northwest Atl. Fish. Sci.* 30, 1–17.
- Maes, J., Taillieu, A., Van Damme, P.A., Cottenie, K., Ollevier, F., 1998. Seasonal Patterns in the Fish and Crustacean community of a turbid temperate estuary. *Est. Coast. Shelf Sci.* 47, 143–151.
- Martinho, F., Leitão, R., Neto, J.M., Cabral, H.N., Marques, J.C., Pardal, M.A., 2007. The use of nursery areas by juvenile fish in a temperate estuary, Portugal. *Hydrobiologia* 587, 281–290.

- Martinho, F., Leitão, R., Neto, J.M., Cabral, H., Lagardère, F., Pardal, M.A., 2008. Estuarine colonization, population structure and nursery functioning for 0-group sea bass (*Dicentrarchus labrax*), flounder (*Platichthys flesus*) and sole (*Solea solea*) in a mesotidal temperate estuary. *J. Appl. Ichthyol.* 24 (3), 229–237.
- Martinsson, J., Nissling, A., 2011. Nursery area utilization by turbot (*Psetta maxima*) and flounder (*Platichthys flesus*) at Gotland, central Baltic Sea. *Boreal Environ. Res.* 16, 60–70.
- McConnaughey, R.A., Smith, K.R., 2000. Associations between flatfish abundance and surficial sediments in the eastern Bering Sea. *Can. J. Fish. Aquat. Sci.* 57, 2410–2419.
- McCullagh, P., Nelder, J.A., 1989. *Generalized Linear Models*. Chapman & Hall.
- McLusky, D.S., 1967. Some effects of salinity on the survival moulting and growth of *Corophium volutator*. *J. Mar. Biol. Assoc. U. K.* 47 (3), 607–617.
- McLusky, D.S., Elliott, M., 2004. *The Estuarine Ecosystem*. John Wiley and Sons, New York.
- Modin, J., Pihl, L., 1996. Small-scale dispersal of 0-group plaice (*Pleuronectes platessa* L.) and flounder (*Platichthys flesus* L.) in a shallow Swedish bay. *J. Fish Biol.* 49, 1070–1085.
- Mucha, A.P., Vasconcelos, T.S.D., Bordalo, A.A., 2005. Spatial and seasonal variations of the macrobenthic community and metal contamination in the Douro estuary (Portugal). *Mar. Environ. Res.* 60, 531–550.
- Muus, B.J., 1967. The fauna of Danish estuaries and lagoons. Distribution and ecology of dominating species in the shallow reaches of the mesohaline zone. *MeddrDanm Fisk-og Havunders* 5, pp. 7–316.
- Nicolas, D., Le Loc'h, F., Désaunay, Y., Hamon, D., Blanchet, A., Le Pape, O., 2007. Relationships between benthic macrofauna and habitat suitability for juvenile common sole (*Solea solea*, L.) in the Vilaine estuary (Bay of Biscay, France) nursery ground. *Estuar. Coast. Shelf Sci.* 73 (3–4), 639–650.
- Nissling, A., Jacobsson, M., Hallberg, M., 2007. Feeding ecology of juvenile turbot *Scophthalmus maximus* and flounder *Pleuronectes flesus* at Gotland, Central Baltic Sea. *J. Fish Biol.* 70 (6), 1877–1897.
- O'Neill, B., De Raedemaeker, F., Mc Grath, D., Brophy, D., 2011. An experimental investigation of salinity effects on growth, development and condition in the European flounder (*Platichthys flesus* L.). *J. Exp. Mar. Biol. Ecol.* 410, 39–44.
- Phelan, B.A., Manderson, J.P., Stoner, A.W., Bejda, A.J., 2001. Size-related shifts in the habitat associations of young-of-the-year winter flounder (*Pseudopleuronectes americanus*): field observations and laboratory experiments with sediments and prey. *J. Exp. Mar. Biol. Ecol.* 257, 297–315.
- Pihl, L., Cattrijsse, A., Codling, I., Mathieson, D.S., McLusky, D.S., Roberts, C., 2002. Habitat use by fishes in estuaries and other brackish areas. In: Elliott, M., Hemingway, K. (Eds.), *Fishes in Estuaries*. Blackwell Science, Oxford, pp. 10–53.
- Power, M., Attrill, M.J., Thomas, R.M., 2000. Environmental factors and interactions affecting the temporal abundance of juvenile flatfish in the Thames Estuary. *J. Sea Res.* 43 (2), 135–149.
- Ramos, S., Cowen, R.K., Paris, C., Ré, P., Bordalo, A.A., 2006. Environmental forcing and larval fish assemblage dynamics in the Lima River estuary (northwest Portugal). *J. Plankton Res.* 28 (3), 275–286.
- Ramos, S., Ré, P., Bordalo, A., 2009. Environmental control on early life stages of flatfishes in the Lima estuary (NW Portugal). *Estuar. Coast. Shelf Sci.* 83, 252–264.
- Ramos, S., Ré, P., Bordalo, A., 2010. Recruitment of flatfish species to an estuarine nursery habitat (Lima estuary, NW Iberian Peninsula). *J. Sea Res.* 64 (4), 473–486.
- Ricker, W.E., 1975. Computation and interpretation of biological statistics of fish populations. *Bull. Fish. Res. Board Can.* 191, 1–382.
- Russo, T., Pulcini, D., O'Leary, Á., Cataudella, S., Mariani, S., 2008. Relationship between body shape and trophic niche segregation in two closely related sympatric fishes. *J. Fish Biol.* 73, 809–828.
- Scaps, P., 2002. A review of the biology, ecology and potential use of the common ragworm *Hediste diversicolor* (O.F. Müller) (Annelida: Polychaeta). *Hydrobiologia* 470 (1–3), 203–218.
- Schoener, T.W., 1970. Non-synchronous spatial overlap of lizards in patchy habitats. *Ecology* 51, 408–418.
- Selleslagh, J., Amara, R., 2014. Are estuarine fish opportunistic feeders? The case of a low anthropized nursery ground (the Canche Estuary, France). *Estuar. Coasts* <http://dx.doi.org/10.1007/s12237-014-9787-4>.
- Shannon, C.E., Weaver, W., 1949. *The Mathematical Theory of Communication*. University of Illinois Press, Illinois, Urbana, IL (117 pp.).
- Sousa, R., Dias, S., Antunes, C., 2007. Subtidal macrobenthic structure in the lower Lima estuary, NW of Iberian Peninsula. *Ann. Zool. Fenn.* 44, 303–313.
- Strauss, R.E., 1979. Reliability estimates for Ivlev's electivity index, the forage ratio, and a proposed linear index of food selection. *Trans. Am. Fish. Soc.* 108, 344–352.
- Summers, R.W., 1979. Life cycle and population ecology of the flounder *Platichthys flesus* (L.) in the Ythan estuary, Scotland. *J. Nat. Hist.* 13, 703–723.
- Teixeira, C.M., Batista, M.I., Cabral, H.N., 2010. Diet, growth and reproduction of four flatfishes along the Portuguese coast. *Sci. Mar.* 74 (2), 223–233.
- Thiel, R., Potter, I.C., 2001. The ichthyofaunal composition of the Elbe estuary: an analysis in space and time. *Mar. Biol.* 138, 603–616.
- Uzars, D., Ustups, D., Müller-Karulis, B., Urtans, E., 2003. Size structure and feeding ecology of fish communities in the surf zone of the Eastern Baltic. (ICES C.M. 2003/N:06).
- Vasconcelos, R.P., Reis-Santos, P., Fonseca, V., Ruano, M., Tanner, S., Costa, M.J., Cabral, H.N., 2009. Juvenile fish condition in estuarine nurseries along the Portuguese coast. *Estuar. Coast. Shelf Sci.* 82 (1), 128–138.
- Vasconcelos, R.P., Reis-Santos, P., Maia, A., Fonseca, V., França, S., Wouters, N., Costa, M.J., Cabral, H.N., 2010. Nursery use patterns of commercially important marine fish species in estuarine systems along the Portuguese coast. *Estuar. Coast. Shelf Sci.* 86 (4), 613–624.
- Vinagre, C., França, S., Costa, M.J., Cabral, H.N., 2005. Niche overlap between two flatfishes, *Platichthys flesus* (Linnaeus, 1758) and *Solea solea* (Linnaeus, 1758), in a southern European estuary and adjacent coastal waters. *J. Appl. Ichthyol.* 21, 1–7.
- Vinagre, C., Fonseca, V., Cabral, H., Costa, M., 2006. Habitat suitability index models for juvenile soles, *Solea solea* and *S. senegalensis*, in the Tagus estuary: defining variables for species management. *Fish. Res.* 82, 140–149.
- Vinagre, C., Cabral, H., Costa, M.J., 2008. Prey selection by flounder, *Platichthys flesus*, in the Douro estuary, Portugal. *J. Appl. Ichthyol.* 24, 238–243.
- Vinagre, C., Maia, A., Reis-Santos, A.P., Costa, M.J., Cabral, H.N., 2009. Small-scale distribution of *Solea solea* and *Solea senegalensis* juveniles in the Tagus estuary (Portugal). *Estuar. Coast. Shelf Sci.* 81, 296–300.
- Wallace, H., Ramsey, J.S., 1983. Reliability in measuring diet overlap. *Can. J. Fish. Aquat. Sci.* 40, 347–351.
- Walton, O.E., 1979. Active entry of stream benthic macroinvertebrates into the water column. *Hydrobiologia* 74 (2), 129–139.
- Weatherley, N.S., 1989. The diet and growth of 0-group flounder, *Platichthys flesus* (L.), in the river Dee, North Wales. *Hydrobiologia* 178, 193–198.
- Woodland, R.J., Secor, D.H., Fabrizio, M.C., Wilberg, M.J., 2012. Comparing the nursery role of inner continental shelf and estuarine habitats for temperate marine fishes. *Estuar. Coast. Shelf Sci.* 94 (1), 61–73.
- Zar, J.H., 1996. *Biostatistical Analysis*. Third Editions Prentice-Hall International Editions, New Jersey (663 pp.).
- Zuccheta, M., Franco, A., Torricelli, P., Franzoi, P., 2010. Habitat distribution model for European flounder juveniles in the Venice lagoon. *J. Sea Res.* 64, 133–144.