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DOCTORAL THESIS
2017

SETTLEMENT AND RECRUITMENT PROCESSES IN FISH SPECIES OF INTEREST FOR RECREATIONAL FISHERIES

Carlos Díaz Gil

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Doctoral Programme of Marine Ecology

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Carlos Díaz Gil

Thesis Supervisor:	Thesis Supervisor:
Dr. Ignacio A. Catalán Alemany	Dr. Amalia Grau Jofre

Thesis tutor: Dr. Gabriel Moyà Niell

Doctor by the Universitat de les Illes Balears

A mi abuelo Jacinto, por dejarme tener peces en el pozo y en la pila …

… y a mi mexicana porque si tuviéramos pila me dejaría tener peces en ella.

List of publications included in this doctoral thesis

1. Chapter I: Changes in the juvenile fish assemblage of a Mediterranean shallow seagrass nursery area after half century of nearshore trawl prohibition.
2. Chapter II: Using stereoscopic video cameras to evaluate seagrass meadows nursery function in the Mediterranean.
3. Chapter III: Anthropogenic chemical cues can alter the swimming behaviour of juvenile stages of a temperate fish.
4. Chapter IV: The smell of fear induces reversible morphological changes in juvenile gilthead seabreams (*Sparus aurata*).
5. Chapter V: Ocean acidification increases fatty acids levels of larval fish.
6. Chapter VI: Otolith fluctuating asymmetry: a misconception of its biological relevance?
7. Chapter VII: Fishing-related effects on fish life-history revealed by otolith microchemistry.

Table 0.1. Information of the journals where they have been published.

Number	Journal	Impact Factor*	Quartile (area)	Status
1	Ocean and Coastal Management	1.696	28/61. Q2. Oceanography	Under review
2	Marine Biology	2.375	21/104. Q1. Marine & Freshwater Biology	Accepted
3	Marine Environmental Research	2.769	12/104. Q1. Marine & Freshwater Biology	Published
4	Marine Ecology Progress Series	2.361	23/104. Q1. Marine & Freshwater Biology	Under review
5	Biology Letters	2.823	19/86. Q1. Biology	Published
6	ICES Journal of Marine Science	2.626	15/104. Q1. Marine & Freshwater Biology 6/52. Q1. Fisheries	Published
7	Fisheries Research	2.230	9/52. Q1. Fisheries	2nd Under review

**(publish or last year available (2015))*

List of publications not included but related with this doctoral thesis

1. Gouraguine A, Díaz-Gil C, Reñones O, Otegui DS, Palmer M, Hinz H, Catalán IA, Smith DJ, Moranta J (2017) Behavioural response to detection of chemical stimuli of predation, feeding and schooling in a temperate juvenile fish. J Exp Mar Bio Ecol 486:140–147
2. Alós J, Puiggrós A, Díaz-Gil C, Palmer M, Rosselló R, Arlinghaus R (2015) Empirical Evidence for Species-Specific Export of Fish Naïveté from a No-Take Marine Protected Area in a Coastal Recreational Hook and Line Fishery. PLoS One 10:e0135348
3. Alós J, Palmer M, Trías P, Díaz-Gil C, Arlinghaus R (2014) Recreational angling intensity correlates with alteration of vulnerability to fishing in a carnivorous coastal fish species. Can J Fish Aquat Sci 225:1–9

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Abstract

Abstract in English

The processes that regulate the settlement phase in marine fish species, passing from pelagic larvae to juveniles associated with the benthos, are complex and play a key role in the population dynamics. Examples of these processes are the duration of the pelagic larvae stage, metamorphosis, the orientation towards their adult habitat, adaptation to different predatory field or changes in feeding. While these processes are widely studied in coral reef fishes, knowledge of these early life stages is still scarce for temperate regions.

In the Mediterranean Sea, these fish species are subjected to high anthropogenic pressure, either due to pollution, environmental physical alterations or direct exploitation by fishermen. More concretely in the Balearic Islands, Spain, the pressure of recreational fisheries is high enough to be considered as a not negligible stress factor on fish population dynamics and this recreational activity has been steadily increasing for the last years. Furthermore, the recreational fisheries are locally concentrated in areas around towns, normally close to small bays where other environmental stressors are particularly acute.

This thesis evaluates some of the effects of this anthropogenic interaction with the fraction of the fish community that is not directly affected by recreational fishing, i.e. recruits or juveniles, through a series of field collected datasets, laboratory experiments and the use of other indirect indicators of fish fitness such as otoliths.

In the field based chapters, we first describe the sequential use of *Posidonia oceanica* seagrass meadows as nursery habitats by a wide range of juvenile fish, and how this transient community has changed in the last half century. A key example is the increase in abundance of juvenile *Diplodus annularis* in shallow seagrass meadows. We then describe and apply a method based on stereoscopic video-filming to better understand the nursery function of *P. oceanica* meadows. This work generates new potential for further studies including the quantification of nursery function.

Regarding the experimental chapters, we looked at the potential anthropogenic effects on early juvenile dynamics. First, the increment of fatty acid composition of larval fish associated with ocean acidification derived from the humanly induced increment of CO_2 in the atmosphere is suggested. At a more local scale, we show the effect of polluted waters (sunscreen and harbour-influenced water) on the behaviour of juvenile gilthead seabream (*Sparus aurata*). Additionally, we use this last model species for an experiment on the predator-induced phenotypic adaptations. We describe for the first time in a marine species that the odour of predation induced changes in shape, which are reversible.

In the last part of this thesis, we include two chapters evaluating the multiple uses of otoliths (including shape, growth, and microchemical analysis) as indirect indicators of fish life history traits (LHT) among a wide set of experiments and field collected data. Firstly, we proved that the use of otolith fluctuating asymmetry (OFA, as the difference between the pair of bony structures) was not correlated with fish fitness or survival success, contrarily to mainstream belief. Secondly, we found for the first time in the field, that differences in otolith microchemistry among subpopulations may be due to differences in their LHT, which in turn may be affected by recreational fishing pressure.

We believe that this thesis has achieved it mains objective: filling several knowledge gaps about settlement/recruitment processes of coastal fish species, many of them highly relevant for recreational fisheries in the Mediterranean Sea.

Resumen en castellano

Los procesos que regulan la fase de asentamiento en especies de peces marinos, es decir el paso de larvas pelágicas a juveniles asociadas con el bentos, son complejos y juegan un papel clave en la dinámica poblacional. Ejemplos de estos procesos son la duración de la fase larvaria pelágica o la metamorfosis, así como la capacidad de orientación hacia el hábitat adulto, la adaptación a nuevas presiones de depredación o los cambios en la alimentación. Si bien estos procesos están ampliamente estudiados en los peces de arrecifes de coral, el conocimiento de estas etapas tempranas de la vida de los peces es todavía escasa para las regiones templadas.

En el mar Mediterráneo, las especies litorales de peces tienen que soportar una elevada presión antropogénica, ya sea por contaminación, alteraciones del medio ambiente o explotación pesquera directa. Más concretamente en las Islas Baleares, España, la presión de la pesca recreativa es lo suficientemente alta para ser considerada como un factor de estrés nada despreciable en la dinámica de las poblaciones de peces. Esta actividad, por otra parte, ha ido aumentando constantemente en los últimos años. Además, las pesquerías recreativas se concentran localmente en áreas alrededor de las ciudades, normalmente cerca de pequeñas bahías donde estos factores de estrés ambiental son especialmente agudos.

Esta tesis evalúa algunos de los efectos de la interacción antropogénica con la fracción de la comunidad de peces que no está directamente afectada por la pesca, es decir reclutas o juveniles, a través de una serie de conjuntos de datos recogidos en campo, experimentos de laboratorio y el uso de otros indicadores indirectos de la vida de los peces, como pueden ser la forma y microquímica de los otolitos.

En los capítulos basados en estudios de campo, describimos en primer lugar el uso secuencial de las praderas de *Posidonia oceanica* como hábitat de cría por parte de una amplia gama de peces juveniles, y cómo esta comunidad transitoria ha cambiado en el último medio siglo. Un ejemplo clave es el aumento de la abundancia de juveniles de *Diplodus annularis* en las praderas poco profundas. Más adelante describimos y aplicamos un método basado en videos estereoscópicos para comprender mejor la función de las praderas de *P. oceanica*. Este trabajo genera un nuevo potencial para estudios adicionales incluyendo la cuantificación de la función como hábitat de cría.

En cuanto a los capítulos experimentales, se analizaron los posibles efectos antropogénicos sobre los juveniles. En primer lugar, estudiamos el incremento de la composición de ácidos grasos de larvas de peces asociado con la acidificación de los océanos derivada del incremento de CO_2 atmosférico inducido por el hombre. A escala más local, mostramos el efecto de las aguas contaminadas (debido a protectores solares y aguas contaminadas de puertos) sobre el comportamiento de juveniles de dorada (*Sparus aurata*). Adicionalmente, utilizamos también estos juveniles para un experimento sobre las adaptaciones fenotípicas inducidas por depredadores donde describimos por primera vez en una especie marina que los productos químicos asociados con la depredación inducen cambios reversibles de forma.

En la última parte de esta tesis incluimos dos capítulos que evalúan los usos múltiples de los otolitos (incluyendo la forma, el crecimiento y el análisis microquímico) como indicadores indirectos de la historia de vida de los peces usando un amplio conjunto de experimentos y datos recogidos en el campo. En primer lugar, se demostró que el uso de la asimetría fluctuante de otolitos (la diferencia entre el par de estructuras óseas) no se correlacionó con el bienestar de los peces o el éxito de supervivencia, contrariamente a la creencia dominante. En segundo lugar, encontramos por primera vez en el campo que las diferencias en la microquímica de los otolitos entre las subpoblaciones pueden deberse a diferencias en su historia vital que a su vez pueden verse afectadas por la presión de la pesca recreativa.

Creemos que esta tesis ha logrado su principal objetivo: aumentar el conocimiento sobre procesos de asentamiento y reclutamiento de especies costeras de peces, muchas de ellas explotadas principalmente por la pesca recreativa.

Resum en català

Els processos que regulen la fase d'assentament en espècies de peixos marins, és a dir el pas de larves pelàgiques a juvenils associades amb el bentos, són complexos i juguen un paper clau en la dinàmica poblacional. Exemples d'aquests processos són la durada de la fase larvària pelàgica o la metamorfosi, així com la capacitat d'orientació cap a l'hàbitat adult, l'adaptació a noves pressions de depredació o els canvis en l'alimentació. Si bé aquests processos estan àmpliament estudiats en els peixos d'esculls de coral, el coneixement d'aquestes etapes primerenques de la vida dels peixos és encara escassa per a les regions temperades.

En el mar Mediterrani, les espècies litorals de peixos han de suportar una elevada pressió antropogènica, ja sigui per contaminació, alteracions del medi ambient o explotació pesquera directa. Més concretament en les Illes Balears, Espanya, la pressió de la pesca recreativa és prou alta per ser considerada com un factor d'estrès gens menyspreable en la dinàmica de les poblacions de peixos. Aquesta activitat, d'altra banda, ha anat augmentant constantment en els últims anys. A més, les pesqueres recreatives es concentren localment en àrees al voltant de les ciutats, normalment prop de petites badies on aquests factors d'estrès ambiental són especialment aguts.

Aquesta tesi avalua alguns dels efectes de la interacció antropogènica amb la fracció de la comunitat de peixos que no està directament afectada per la pesca, és a dir reclutes o juvenils, a través d'una sèrie de conjunts de dades recollides en camp, experiments de laboratori i l'ús d'altres indicadors indirectes de la vida dels peixos, com poden ser la forma i microquímica dels otòlits.

En els capítols basats en estudis de camp, descrivim en primer lloc l'ús seqüencial de les prades de *Posidonia oceanica* com a hàbitat de cria per part d'una àmplia gamma de peixos juvenils, i com aquesta comunitat transitòria ha canviat en l'últim mig segle. Un exemple clau és l'augment de l'abundància de juvenils de *Diplodus annularis* en les prades poc profundes. Més endavant descrivim i apliquem un mètode basat en vídeos estereoscòpics per comprendre millor la funció de les prades de *P. oceanica*. Aquest treball genera un nou potencial per a estudis addicionals incloent la quantificació de la funció com a hàbitat de cria.

Quant als capítols experimentals, es van analitzar els possibles efectes antropogènics sobre els juvenils. En primer lloc, estudiem l'increment de la composició d'àcids grassos de larves de peixos associat amb l'acidificació dels oceans derivada de l'increment de CO_2 atmosfèric induït per l'home. A escala més local, vam mostrar l'efecte de les aigües contaminades (a causa de protectors solars i aigües contaminades de ports) sobre el comportament de juvenils d'orada (*Sparus aurata*). Addicionalment, utilitzem també aquests juvenils per a un experiment sobre les adaptacions fenotípiques induïdes per depredadors on descrivim per primera vegada en una espècie marina que els productes químics associats amb la depredació indueixen canvis reversibles de forma.

En l'última part d'aquesta tesi incloem dos capítols que avaluen els usos múltiples dels otòlits (incloent-hi la forma, el creixement i l'anàlisi microquímic) com a indicadors indirectes de la història de vida dels peixos usant un ampli conjunt d'experiments i dades recollides en el camp. En primer lloc, es va demostrar que l'ús de l'asimetria fluctuant de otòlits (la diferència entre el parell d'estructures òssies) no es va correlacionar amb el benestar dels peixos o l'èxit de supervivència, contràriament a la creença dominant. En segon lloc, trobem per primera vegada en el camp que les diferències en la microquímica dels otòlits entre les subpoblacions poden deure's a diferències en la seva història vital que al seu torn poden veure's afectades per la pressió de la pesca recreativa.

Creiem que aquesta tesi ha aconseguit el seu principal objectiu: augmentar el coneixement sobre processos d'assentament i reclutament d'espècies costaneres de peixos, moltes d’elles rellevants per a la pesca recreativa al mar Mediterrani.

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General introduction

The settlement and its importance for fish demography

Settlement is the set of processes that regulate the shift from a pelagic phase as planktonic larvae towards a benthonic or demersal phase (Wilson and Meekan 2001; Leis and McCormick 2002; Leis 2006) (Figure 0.1). These processes, apart from the obvious change in habitat, have a high temporal match with the metamorphosis in many species (e.g. the acquisition of more adult-like physiological and morphometric characteristics such as scales, eye-migration in flatfish, etc). Other processes linked to the settlement phase include increased swimming capacity (Leis 2006), shifts in growth rates, development of the sensory system that improve orientation towards new habitats (Atema et al., 2002; Kingsford et al., 2002; Lecchini et al., 2005), shifts in the feeding preferences, and adaptation to increased predation (Sih 1984; Kinoshita et al., 2014). These adaptations may include morphological changes (i.e. phenotypic plasticity (Benard 2004)) as we explore in Chapter V of this thesis, and may be key to reduce mortality at these early life stages.

The settlement typically occurs during few hours (Leis 2006), and tends to have high associated mortalities (Almany and Webster 2005), thus conferring the settlement the capacity to act as a bottleneck for populations (Levin 1994, Nash and Geffen 2012). Therefore, understanding how settlement and associated processes occur at different time and spatial scales and how they are affected by anthropogenic uses of the coastal habitats is crucial for an ultimate management of coastal fisheries, both from the conservationist and from the socio-economical point of view. In this thesis, we try to broad the knowledge about settlement processes in the Mediterranean Sea through a multidisciplinary approach.

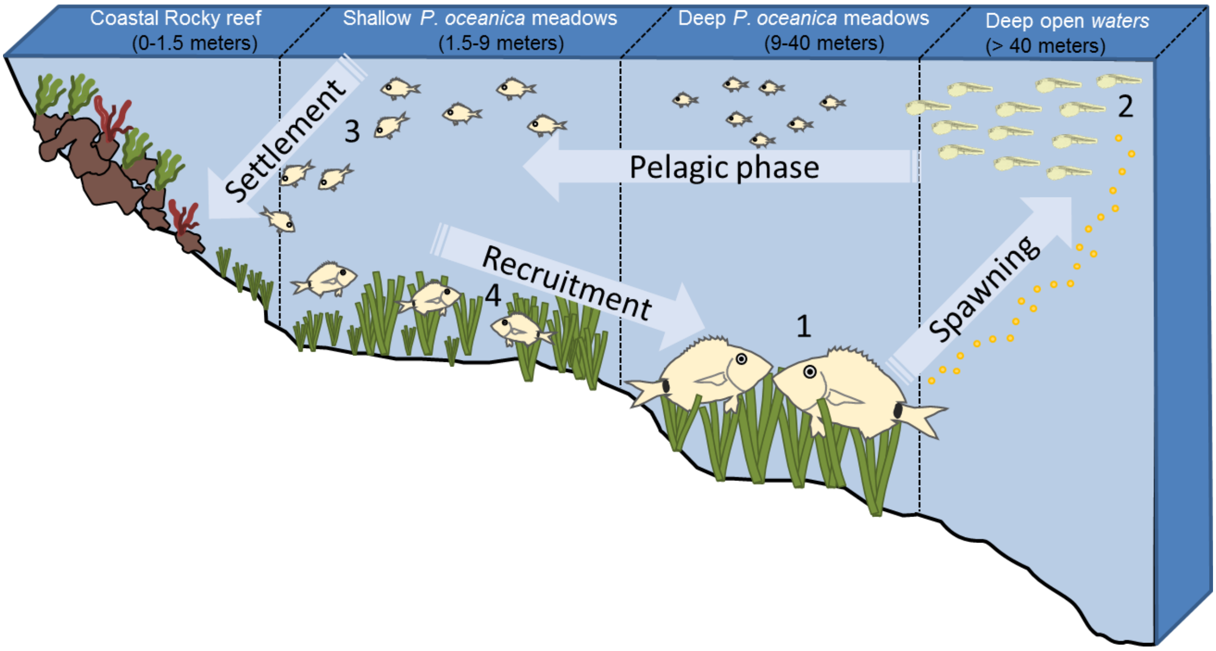


Figure 0.1. Diagram of the life cycle of *Diplodus annularis*. Adults are a typical target species within the Balearic recreational fisheries (1). After mating, the floating eggs and larvae stages (2) are dispersed/retained by currents depending on complex physical-biological factors. Settlement stages (3) find their way back to the shallow *Posidonia oceanica* meadows, where juveniles (4) are mainly found.

Settlement does not occur randomly in space or time: obvious constraints due to reproductive timing and strategies (e.g. Alonso-Fernández et al., 2011), advection/retention and other oceanographic features (Álvarez et al., 2015), larval orientation and navigation (Paris et al., 2013) and patchy predatory environments (Tupper and Boutilier 1997; Kinoshita et al., 2014) partially shape settlement distribution. Further, not all the habitats are suitable for a given species. Settlement habitats such as mangroves, coral reefs, rocky reefs, macrophytes forests or seagrasses meadows often offer shelter structures for the post-larvae but at the same time are the perfect habitat for a wide range of predatory fish that await ambushed. Mortality during the settlement phase (1-2 days) is very high (around 56% in coral reef fishes, Almany and Webster, 2005) and predation is thought to be the main cause of mortality in early life stages (Sih 1984; Kerfoot and Sih 1987).

In the Mediterranean Sea, the main settlement habitats for littoral species differ between species (Garcia-Rubies et al., 1995) even within the same fish family. For example, within *Sparidae*, the white seabream *Diplodus sargus* settle in shallow sand

and pebbles coves, recruiting later to rocky deeper reefs when attain a certain size (Vigliola et al., 1998; Casado 2015) while other sparids such as the annular seabream *Diplodus annularis*, the two banded seabream *Diplodus vulgaris* and the salema *Sarpa salpa* settle mainly on seagrass meadows. These are formed mostly by *Posidonia oceanica* and to a lesser extent by *Cymodocea nodosa* and *Zostera noltii* (Fourqurean et al., 2007), in addition to the macrophyte *Caulerpa prolifera* that also forms meadows in small protected bays (Box Centeno 2008). The key nursery role of structured habitats such as seagrass meadows and rocky reefs has been reviewed several times (Beck et al., 2001; Heck et al., 2003), but the quantitative evaluation of its importance for population dynamics is far from clear (Beck et al., 2001).

The role of *Posidonia oceanica* as a nursery habitat

Seagrass ecosystems are one of the most iconic marine ecosystem around the world (Larkum et al., 2006) and despite the protection measurements they are currently under threat, with worldwide losses between 1879 and 2006 estimated at 3370 km2 (27 km^2 yr^{-1}), which represents 29 % of the maximum area measured (11592 km2) (Waycott et al., 2009). Seagrass meadows are valuable both in ecological and socio-economical terms. The ecological value includes effects on biodiversity (Larkum et al., 2006) or, focusing on the aims of this thesis, offering structured nursery habitats (here defined as the capacity of a given habitat to host higher density of juvenile fish than the surrounding unstructured habitats (Beck et al., 2001)) throughout increasing shelter and food availability (Orth et al., 1984; Larkum et al., 2006). The socio-economic importance involves a key role in carbon sequestration, sediment stability, and buffering the action of waves, which in turn enhances water transparency and protects beaches from erosion (Gacia and Duarte 2001; Boudouresque et al., 2006). Moreover, these habitats are generally related with higher adjacent fisheries production (Unsworth et al., 2014). Seagrass meadows may also have a direct value for traditional and recreational fisheries which are usually permitted over seagrasses meadows as it has been mentioned above for the Balearic Islands.

According to the above, ecological and socio-economical benefits may underlie in the maintenance and preservation of seagrass meadows since they play a fundamental role in the populations of exploited coastal fish of recreational interest through their use as nursery habitats (Jackson et al., 2001). In the Mediterranean, the nursery role of the endemic seagrass *P. oceanica* is paramount as it covers around 23% of the coastal shallow habitats (Pasqualini et al., 1998) with an approximate extension of 2.5 to 5.5 million hectares (Buia et al., 2000). *P. oceanica* belongs to Posidoniaceae which is one of five families of seagrass that are derived from land plants that secondarily recolonised marine habitats, formed by nine species (Larkum et al., 2006). The key ecological role of *P. oceanica* as a nursery area is justified by the general comments above, and has been specifically pointed out in several studies (Orth et al., 1984; Jackson et al., 2001; Larkum et al., 2006). Moreover, the continuous distribution of *P. oceanica,* from very shallow nearshore habitats to deeper areas (from 1 to 43 meters (Marbà et al., 2002)) offer in many places an easy transition from juvenile to adult habitats, which confers advantages for growth, survival and recruitment (Heck et al., 2003; Igulu et al., 2011). Despite its claimed importance, *P. oceanica* is in decline (Marbà et al., 2002) and was included in the Red List of marine threatened species of the Mediterranean, with the meadows defined as priority natural habitats by the Annex I of the EC Directive 92/43/EEC on the Conservation of Natural Habitats and of Wild Fauna and Flora (European Commission 1992).

Figure 0.2. An image taken from the SBRUVs Left Camera's footage used in Chapter II displaying two early juvenile *Diplodus annularis* (marked with a red ellipse).

Despite key nursery role of structured habitats such as seagrass meadows and rocky reefs has been reviewed several times (Beck et al., 2001; Heck et al., 2003), a standard methodology for quantifying its importance on early stages of fish remains elusive. We focus part of this thesis in contributing to this needed knowledge. In this thesis, we purposely avoided the use of scuba diving to evaluate the populations of post settlers in the seagrasses meadows since the smaller individuals are difficult to classify in size classes and even in their specific species. Therefore, we used an epibenthic beam trawl (providing samples in Chapter I of this thesis) and developed stereoscopic baited remote underwater video cameras (SBRUV) to estimate the state of fish assemblages and specifically determine the occurrence of settlement pulses at different spatial and temporal scales (Chapter II). Furthermore, SBRUVs are increasingly used over other sampling techniques as they can be deployed in environments unsuitable for conventional diver-based assessments, detect diver-shy species and provide a usable estimate of the relative abundance of economically important species (Lowry et al., 2011; Gladstone et al., 2012). In addition, the use of this method provides a permanent visual record of the surveys undertaken, allowing for multiple observer analysis, besides being a non-destructive sampling technique (Kelaher et al., 2014; Unsworth et al., 2014) (

Figure 0.2 to see a photograph from one of the video cameras showing two recently settled individuals of *D. annularis*)

Human activities interacting with the settlement process

Approximately half the world's population lives within 100 km of the coast, a figure likely to double by 2025, and the Mediterranean Sea is undergoing a ''basin-wide urbanisation process", with more than two-thirds of the Mediterranean coastline already developed (Benoit and Comeau 2005), including farms, residential housings, commercial harbours and recreational marinas along with other industrial assets (Miller 1993). Globally these processes have led to water pollution and ultimately shifts in species composition (Orams 1997; Zahedi 2008). Moreover, we have to add coastal tourism to the permanent inhabitants and the associated leisure activities which is becoming the largest and most rapidly expanding activity in the world; in the Mediterranean, there is expected to be 264 million foreign coastal visitors by 2030, compared to 98 million in 1995 (Predosanu et al., 2011). For example, the influx of both air and water pollutants can damage of both terrestrial and marine environments due to littering, trampling and increasing coastal inhabitants during the high touristic season (Zahedi 2009). Undoubtedly, unplanned and poorly managed development of tourist-centred infrastructure can damage the natural environment, but the overall understanding of the interaction between tourism and the environment in coastal areas is poor and unfortunately too general and not focusing on how the outcomes of tourism impacts a specific environment or species (Hall 1996).

As it is mentioned before, optimal nursery habitats are generally found where there is an equilibrium between predation and feeding trade-offs (Heck et al., 2003). These areas are usually located at the littoral areas, which in some instances can be severely altered by the above described urbanization processes that may affect the overall survival of a given species or altering the structure of juvenile communities (Munsch et al., 2016).

Consequently, the increment in the seasonal pressure on coastal areas can directly interact with the settlement and post-settlement processes. For example, the reported increment of dissolved pollutants derived from sunscreen used by bathers in touristic areas of Mallorca island (Sánchez-Quiles and Tovar-Sánchez 2015) may interact with the olfactory selection of a propitious nursery habitat, as we evaluate in Chapter III. Briefly, sunscreen products are mainly composed by UV-filters (organic or inorganic), emulsifiers, emollients and silica- or nylon-based moisturising agents (Chen et al., 2012). Organic filters are usually derivate of aromatic molecules for example benzophenone derivatives, salicylates and cinnamates (Giokas et al., 2007), whereas inorganic UV-filters contain metal oxides, titanium dioxide (TiO_2) and zinc oxide (ZnO) specifically. Both types of filters have previously found to be detrimental to aquatic organisms, due to bioaccumulation or contribution to endocrine issues (Kunz et al., 2006a, 2006b, 2006c). In that Chapter, we evaluated not only the effect of sunscreen pollutants on fish orientation, water type selection and behaviour but also if other a priori detrimental habitats due to contamination, such as harbours, have a negative effect on their behaviour.

The extent to which seasonal increases in anthropogenic activity affects and is influenced by environmental, economic and social change remains as a central issue of research on coastal areas (Zahedi 2008; Bulleri and Chapman 2010; Hering et al., 2010). However, we cannot forget that these anthropogenic impacts range further from only local effects to global scale stressors. For example, eutrophication and hypoxia due to increased organic residuals that affect coastal areas, or global warming and the associated ocean acidification due to the increment of CO_2 emissions. Acidification has been related with factors that are key to the survival of fish during settlement, through the effect of fish capacity to orientate, or reducing their abilities in front of predatory threats (Dixson et al., 2010; Simpson et al., 2011; Leduc et al., 2013). These effects can go further and have unaccounted effects on coastal fish, as we explore in Chapter IV, where it is shown that ocean acidification can alter some key biochemical constituents in early life stages of fish. In that Chapter, we performed an ocean acidification experiment on red drum (*Sciaenops ocellatus*) larvae, which is an economically important sciaenid fish species from North America Atlantic coast (Figure 0.3). We were interested in understanding how ocean acidification was reflected on fish early life stages studying their fatty acid composition in search of unpredicted effects of ocean acidification over settlement size fish.

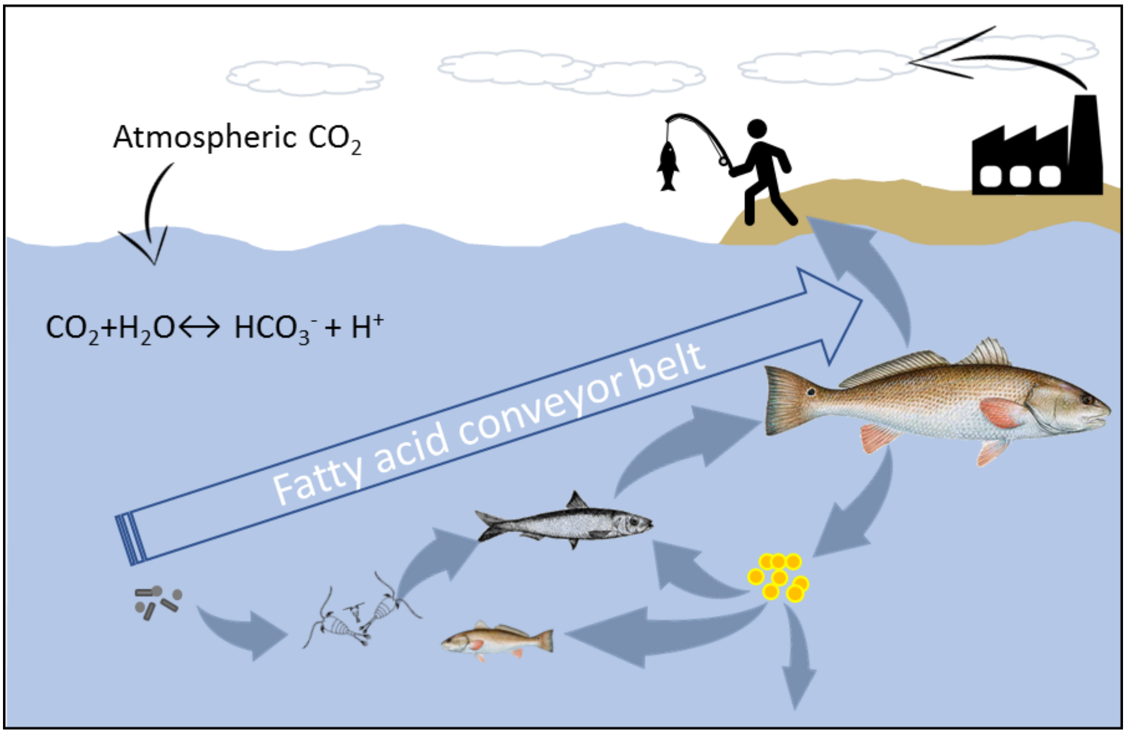


Figure 0.3. Diagram showing two processes, above the ocean acidification, where the atmospheric CO_2, which suffered a rapid increase due to human activities since industrial revolution, dissolves in the ocean. Below the fatty acid conveyor belt, FAs are produced mainly by microalgae, consumed and biomagnified up in the food web until reaching top predators (like red drum in this case, and even humans). In addition, the FAs can return to lower levels of the food web through eggs that are consumed by smaller fish.

Moreover, we explored if their otoliths (earstones which fish use for orientating and hearing) growth and shape were affected by acidification, as had been previously pointed out (Checkley et al., 2009). For this last part, we used fluctuating asymmetry between the two otoliths as indicator which is included in Chapter VI. Furthermore, in that Chapter we widened our analysis in an attempt to understand the usefulness of otolith fluctuating asymmetry for detecting environmental effects on fish traits (growth, behaviour, etc.). For that, we contrasted the effect of different stressors on the otolith fluctuating asymmetry of several species and life stages.

The context of recreational and artisanal fishing on coastal fish populations: an ideal Mediterranean case-study

Among the human activities directly interacting with coastal fish populations we can not forget fishing, especially artisanal small-scale fisheries and recreational fisheries. Although industrial (here defined as non-artisanal) fisheries have an indubitable impact on the life history of the fish species used in this thesis they usually interact with slightly deeper habitats and are forbidden over *P. oceanica* meadows. Here we aimed primarily to better understand the settlement and recruitment processes of littoral fish that tend to be exploited by recreational fisheries and to some extend by artisanal ones. One of our key species, the *Diplodus annularis*, is the typical catch of many recreational fishermen, whose catches are of the same order of magnitude than artisanal catches (Figure 0.4).

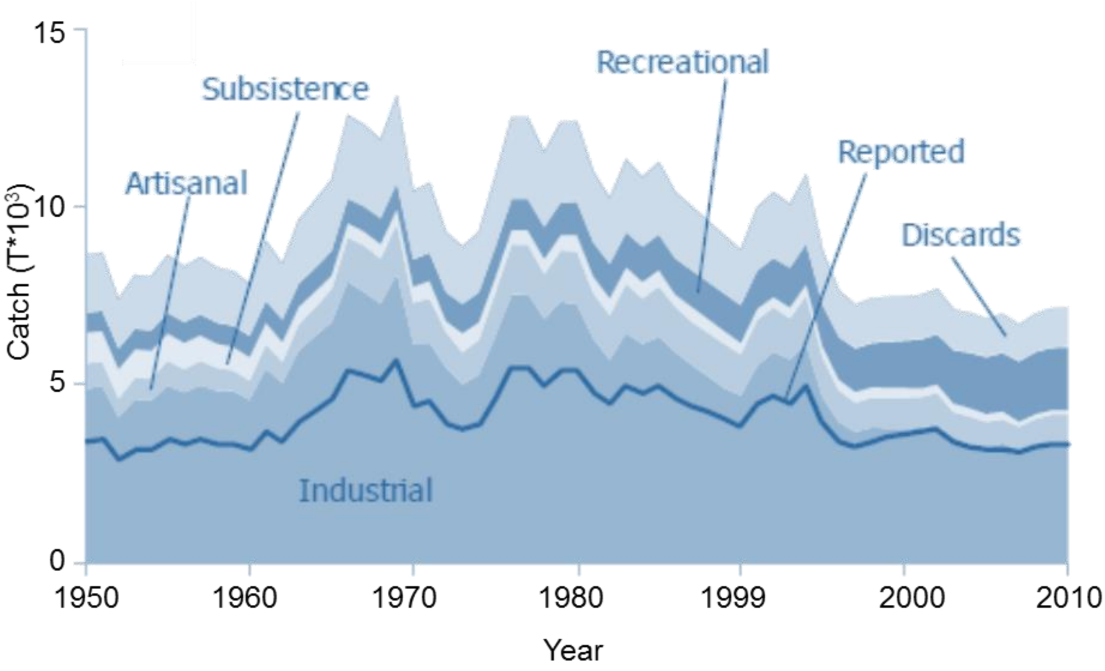


Figure 0.4. Total fisheries catch in the Balealic Islands (1950-2010) by sector compared with official landings (Reported). In the last years Recreational fisheries have significantly increased. Modified from Carreras et al. (2015)

We define recreational fisheries as those people conducting the practice of fishing for sport and leisure with an additional objective of catching fish for personal consumption (Pitcher and Hollingworth 20012; FAO 2016). The recreational capture of marine fish has been practiced for an extended period of time (Pitcher and Hollingworth 2012; Grau 2008) but fishery biologists have long neglected this practice as a relevant source of exploitation (Cardona and Morales-Nin 2013). The activity principally involves angling by hook and line although spearfishing and the use of various nets and traps are also utilised (McPhee et al., 2002). Near shore regions such as estuaries, reefs, flats, mangroves and bays are typically targeted by this fishery, in comparison to larger commercial fisheries that operate further from the shore (Cooke and Cowx 2004). In addition, these near shore zones are often essential habitats for multiple life stages of many different species of fish that use them for spawning, feeding, and as a nursery grounds (e.g. Jackson et al., 2001). The socioecological and economical importance of the recreational fisheries is nowadays being increasingly recognised (Arlinghaus et al., 2016a; Arlinghaus et al., 2016b), and in many places, such as the Mediterranean, it is not uncommon that this practice displays an increasing trend, which contrasts with the declining of traditional fisheries (Grau 2008; Carreras et al., 2015). Globally recreational fisheries have positive social attributes (since is rooted in the traditions) and economically, may represent a high valuable industry (Pitcher and Hollingworth 2012). On the other hand, the negative consequences of recreational fishing, under particular conditions, are increasingly being observed in marine systems through alterations in ecosystem function and quality of habitat as well as the contribution to global fishery declines through harvest mortality (Cooke and Cowx 2004). There is henceforth a growing controversy on the benefits-costs of recreational fisheries that have led to an increasing debate and ecological awareness related with these practices (see for example (Coleman et al., 2004) and the consequent responses (Arlinghaus and Cooke 2005)).

Nevertheless, there are few but well documented instances of declines in fish stocks that are attributed to recreational fisheries (Post et al., 2002). Moreover, these effects are usually not only reflected in a reduced fish abundance and biomass, which is usually uncoupled from catchability (e.g. Alós et al., 2014), but with an alteration in average life history traits such as reduction in asymptotic length, acceleration of reproduction investment or increase in immature growth (Arlinghaus et al., 2009; Jennings et al., 2001; Matsumura et al., 2011). These rapid human-induced changes in a determined set of life history traits has been defined as fisheries-induced evolution (reviewed in Jørgensen et al., 2007). Although many examples of these alterations are attributed to the high fishing mortality associated with commercial fisheries (see examples on cod (*Gadus morhua* (Enberg et al., 2009)), recreational fisheries also have an impact on life history traits of determined species (Alós et al., 2014; Cardona and López, 2007; Chapter VII of this thesis).

Focusing on the Mediterranean, a paradigmatic case-study of the recreational fishing system are the Balearic Islands (NW Mediterranean), where recreational fishing in the shallow coastal area over *P. oceanica* meadows is a long-standing tradition. At least 50 000 fishers (5% of the population) partook in this leisure activity in year 2009 (Cardona and Morales-Nin 2013). The pressure on resources from recreational fishing is equivalent to 25 % of landings of the professional fleet of the Balearic Islands (estimated at 5000 t/year) with an estimated 1209 t/year extracted based on real catches sampled at the quayside (Morales-Nin et al., 2005). The mixed species recreational fishery, known locally as 'roquer', takes place over shallow seagrass, rocky and sandy habitats from shore or with boat angling using hook and line as the most popular method (Alós and Arlinghaus 2013). Up to 54 different species of fish of small body size are targeted by anglers including the Mediterranean Rainbow Wrasse (*Coris julis*), Annular Seabream (*Diplodus annularis*), Painted Comber (*Serranus scriba*) and gilt-head seabream (*Sparus aurata*) among others (Morales-Nin et al., 2005; Grau 2008; Seytre et al., 2013). The local fisheries administration regulations on the fishery include fishing licenses, bag limits (5 kg per day), gear limits (two rods per person with a maximum of three hooks each), closed seasons and minimum legal-size limits for some species (Alós et al., 2009).

Apart from direct regulations on the fishing activities, the creation of marine protected areas (MPAs) is widely used globally for protecting marine ecosystem and the sustainability of future fisheries at all scales (Halpern 2003; Gaines et al., 2010). By 2014 there were around 12000 MPAs in the world (Juffe-Bignoli et al., 2014) a number that is constantly increasing and by year 2016 was covering a total of 16 million km^2 of the global ocean (Spalding et al., 2016). The motivation and efforts in designing and implementing MPAs are of diverse origin and may create conflicts among involved actors (fishermen, conservationist NGOs, scientist and governance at local, national and international level) (Spalding et al., 2016). However, in general the long-term effects of MPAs are welcomed by the society (Halpern and Warner 2002), with extensive ecological and economic benefits including diver based tourism or regulated recreational fisheries (Cooke et al., 2006; Sala et al., 2013). In addition to protecting important habitats and biodiversity, MPAs are also predicted to benefit adjacent fisheries through three mechanisms: a) exportation of pelagic eggs and larvae from restored spawning stocks residing within the MPAs; b) offering protected suitable settlement and nursery habitats to the early life stages of fish and; c) spill over or net migration of adult fishes to unprotected fishing areas (e.g. Alcala et al., 2005; Alós and Arlinghaus, 2013; Coleman et al., 2004; Fujitani et al., 2012; Gell and Roberts, 2003; Villegas-Ríos et al., 2016). Therefore, the protection from extractive pressure would affect all phases of life.

This brief review of the situation of the recreational fisheries in the Balearic Islands, and its interaction with fish ecology, delineates this area as one of the best studied zones in the Mediterranean with regard to: i) the evaluation of the recreational fisheries themselves (e.g. Cabanellas-Reboredo et al., 2014; Grau, 2008; Morales-Nin et al., 2005), ii) the effects of recreational fisheries on populations, including potential evolutionary consequences (Alós et al., 2014a), with detected changes in morphometry (Alós et al., 2014b) and behavioural traits (Alós et al., 2014c; Alós et al., 2015; Alós et al., 2016). Furthermore, the Balearic Islands are the Spanish territory with more Marine Protected Areas, with eight MPAs covering a total of 61000 ha (Govern Illes Balears), plus a protected Maritime-Terrestrial National Park (Cabrera Archipelago) (see Figure 0.5). In all these areas fishing activities, both commercial and recreational, are regulated. Therefore, the Balearic Islands are herein presented as a paradigmatic archipelago to study the interactive effect of fishing pressure vs protection effect on fish population dynamics. These previous works set the stage for research efforts such as this thesis, which is focused mainly on how environmental and anthropogenic impacts interact with settlement and recruitment of coastal fish in the Balearic Islands.

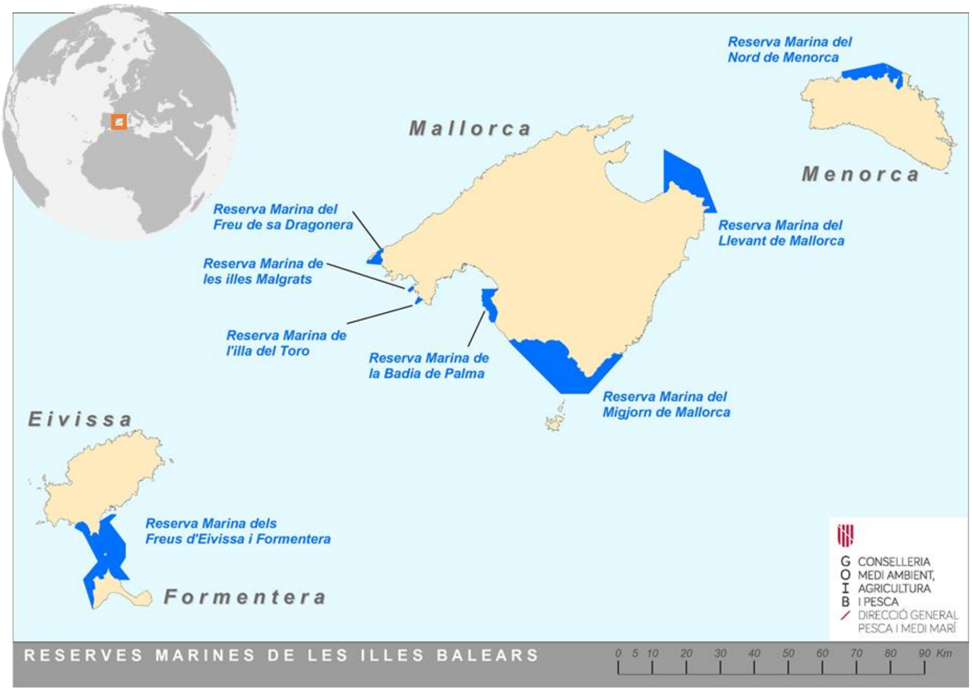


Figure 0.5. The Marine Protected Areas of the Balearic Islands. Modified from the Government of the Balearic Islands webpage (https://www.caib.es/sites/M69/es/las_reservas_marinas_en_las_illes_balears-850)

Methodological developments within this thesis

The detailed methodology for each of the chapters of this thesis is properly described in each of the chapters. However, several of the methodologies used deserve extra attention due to technical innovations and usefulness of the developments.

Stereo baited remote underwater video system (SBRUVS)

In Chapter II a low-cost stereo baited remote underwater video camera system (SBRUV) was designed to properly measure juvenile stages of fish. Although the use of SBRUVs have been increasing during the past few years (refs) they have not been previously used to measure the post settler individuals. We designed a PVC piping structure which held two GoPro Hero 3 cameras that can be attached to any structure (i.e. scaffolds, trawling nets, neustonic skates, floating devices… etc.) or operated manually while taking underwater census. The position of the cameras was optimized to achieve a precise and accurate measurement of recently settled juvenile fish (as small as 15 mm, see

Figure 0.2) with less than 5% error at 3.5 m away from the SBRUV. Both cameras are set in parallel (without angle), at a separation of 200 mm between the objective centres. This very compact design allows for easy alignment (a quick crosscheck of the parallel position of the cameras can be performed on/with any flat surface). However, there is a minimum blind area to the stereoscopic vision (i.e. field of the videos that are not included in both cameras) but this was of only 50 mm apart from the cameras and 90 mm at each lateral (see Figure 0.6)

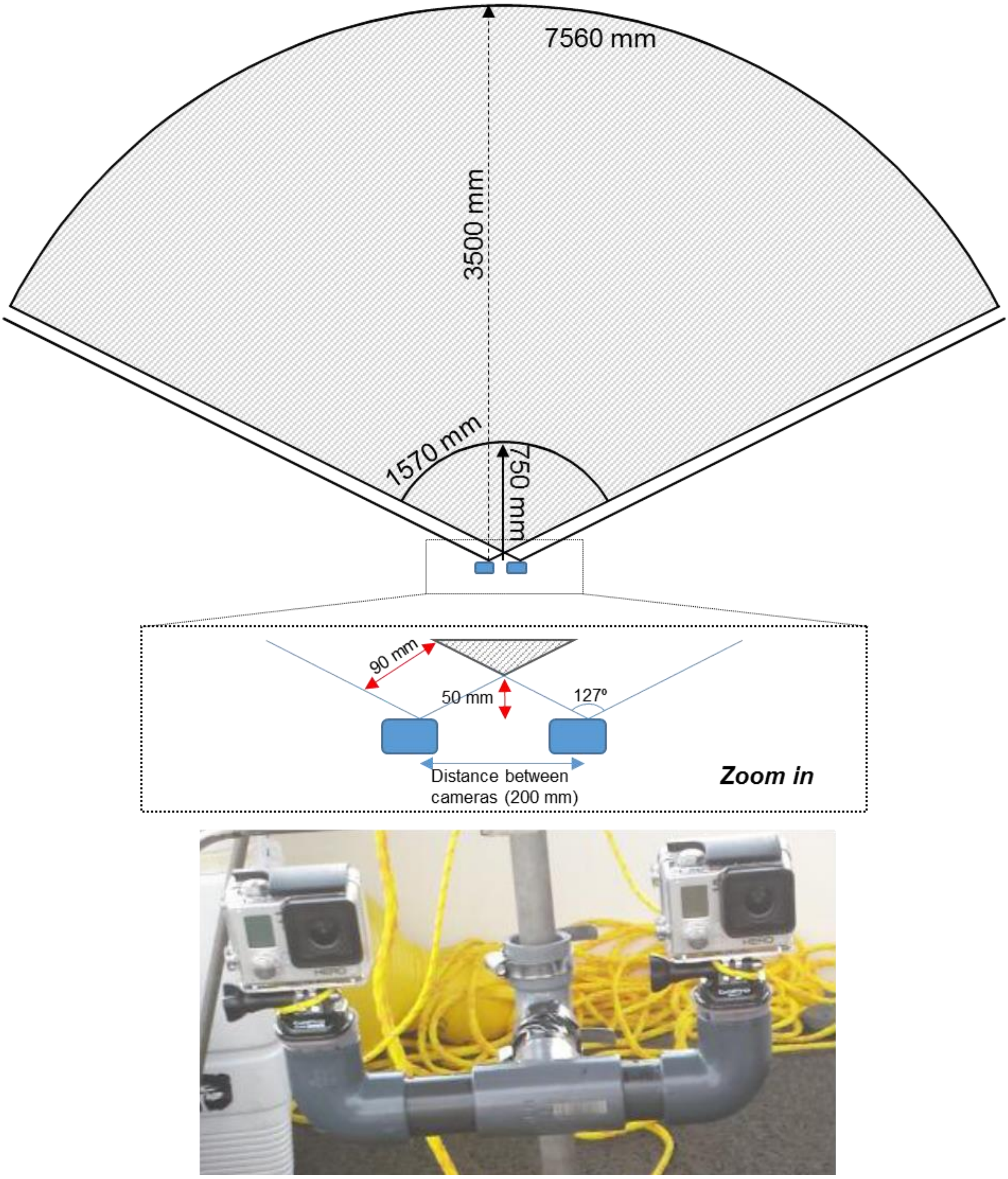
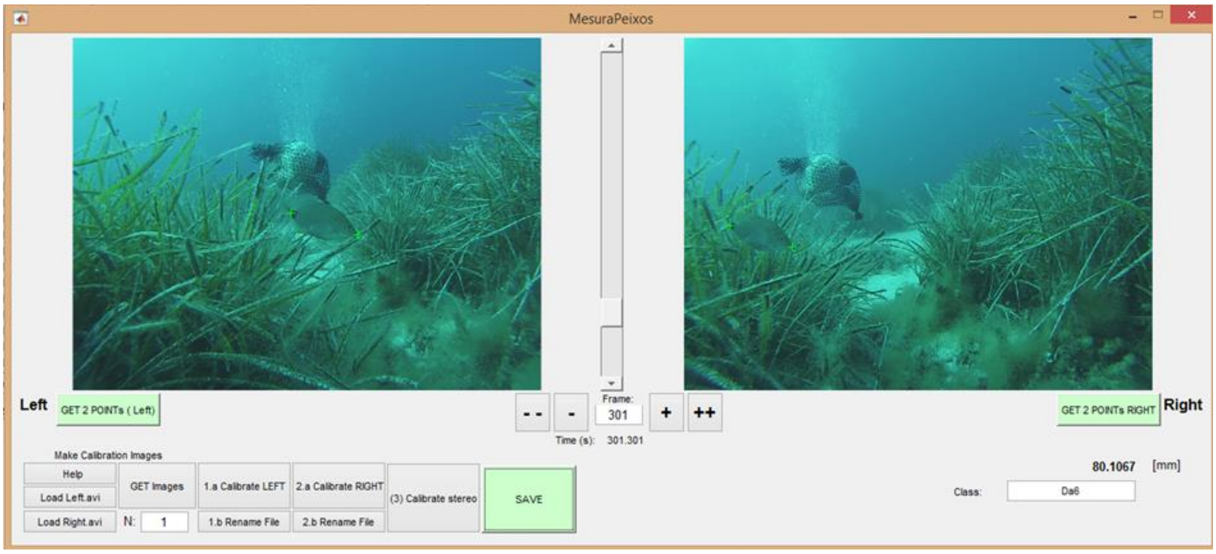


Figure 0.6. Aerial view of the stereoscopic field of view of the SBRUV system with a close-up view and a real picture of the cameras set-up. At 3.5 m from the cameras, the field of view was 7.56 m.

In parallel to the progressive tests of the SBRUV structure, we collaborated with the "Grup de Tractament de Dades i Senyals" from the University of Vic (UVIC-UCC) in the design of a practical User Interface, namely MesuraPeixos (see Figure 0.7), for Matlab 2015 (Mathworks, Natick, MA). Briefly it offers the possibility to visualize two videos simultaneously, calibrate the stereo measurements using a camera calibration toolbox (http://www.vision.caltech.edu/bouguetj/calib_doc/, last accessed 10/02/2017), and finally measure both the distance to the camera and the object size. Even though it is implemented in a pay-per-use software (Matlab), it provides a free alternative that can be further implemented and redistributed in open source programming language (http://opencv.org/, last accessed 10/02/2017).



Frame: 301

Time (s): 301.301

N: 1

80.1067 [mm]

Class: Da6

Figure 0.7. A screenshot of MesuraPeixos, simultaneously showing the same individual of *Diplodus annularis* being measured (80.1067 mm). Features include directly loading of the .mp4 or .avi videos, calibration of left and right cameras independently, stereoscopic calibration (bottom left buttons), and selection of the fish species.

Choice chamber experiments

Choice chambers (henceforth "flumes") have been used for several years to test olfactory/navigational capacities in fish, mostly in coral reef habitats. However, the use of flumes in juvenile temperate species is very scarce. Therefore, we scaled-up a small size choice chamber after the original design from Atema et al., (2002), in order to accommodate juvenile fish (Figure 0.8). Additionally, we improved the functioning of the flume through i) adding water masses quick exchangers (Figure 0.8, 2), ii) automatic pumps to feed the pipes and iii) a PVC-based structure for holding the camera and flowmeters to regulate the flow at both sides of the flume. The basics behind the correct functioning of the flumes is to stablish a sharp separation between two differentiated water masses flowing in parallel, so that the fish can clearly choose between them.

During the thesis, we used the flume in Chapter III, testing whether the anthropogenic chemical cues affected gilt-head juveniles' behaviour.

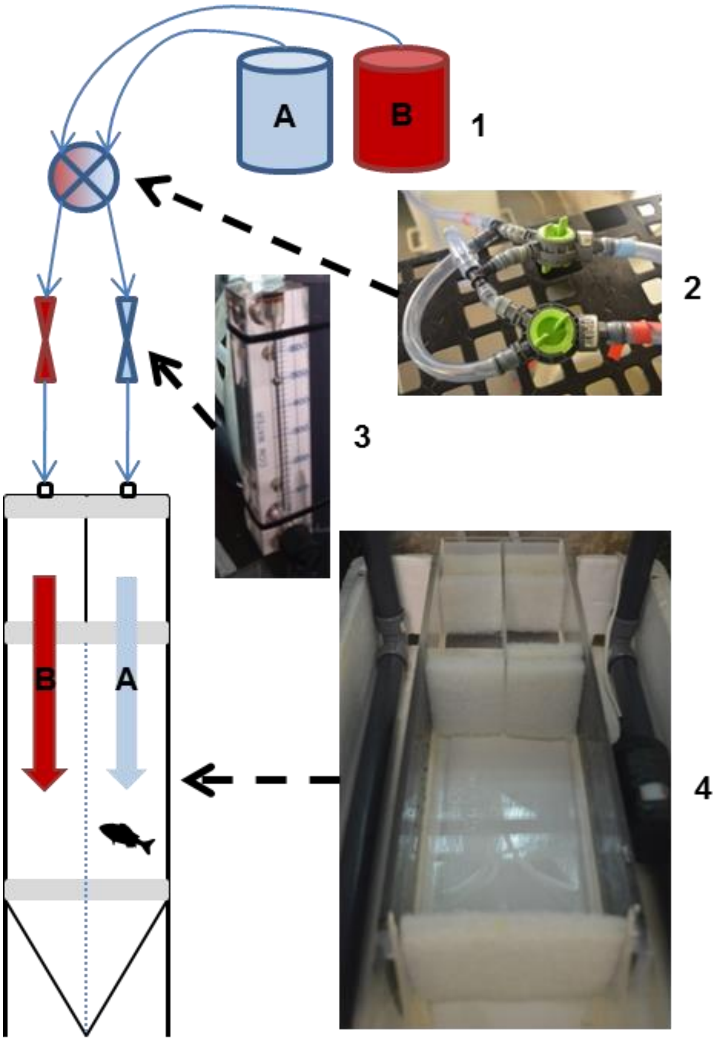
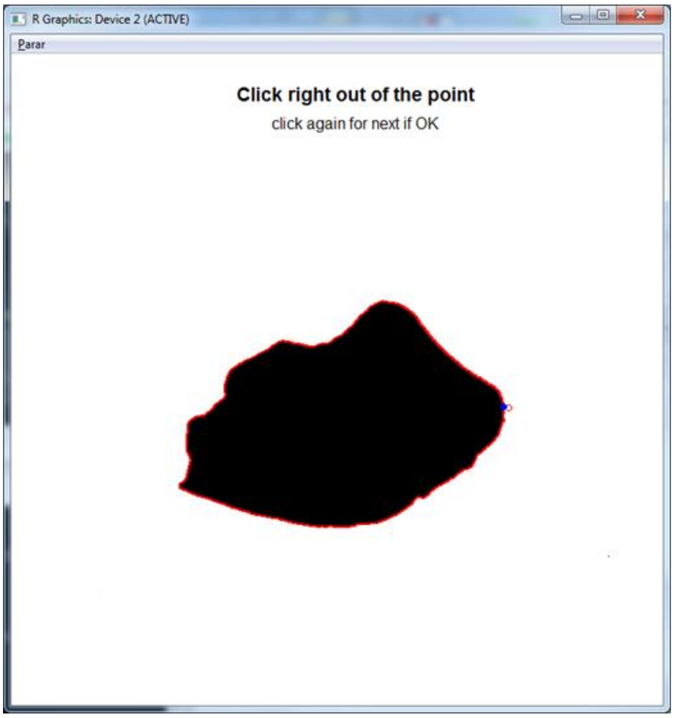


Figure 0.8. Conceptual use of the choice chamber flume accompanied with actual pictures of the components. Water masses A and B (1) were contained in 200 L deposits with aeration, and the system was fed using two aquarium pumps. (2), Water masses side exchanger; (3) Flowmeters set at 500 cm^{3}min^{-1}; (4) Choice chamber arena.

Moreover, we developed a detailed protocol for analysing the behavioural recordings of the tested fish in free available software, including VirtualDub (Lee 2013) for treating the video footage, ImageJ (Schneider et al., 2012) to obtain a proper track of the individual movements and R statistical software (R Development Core Team 2011) for a Bayesian approach to analyse the results (that deserve a detailed description in Chapter III, material and methods). This same methodology has been successfully implemented in another study co-authored by the author of this thesis (Gouraguine et al., 2017).

Otolith shape analysis

For Chapter VI, devoted to the extensive analysis of otolith fluctuating asymmetry, we developed an algorithm for automatic extraction of the outline of otolith pictures in Matlab, which later lead to the development of a complete R routine that embraced all the steps: from automated extraction of the outlines (based on *conte* function originally developed by (Claude 2008), see Figure 0.9) to shape analysis of the otoliths using several multivariate techniques including Fast Fourier analysis, wavelets and Generalized Procrustes Analysis. These functions are freely available under collaborative requests and have been partially used in chapters IV and VI and moreover used a PhD thesis on fish species from the Amazonian Basin (Rodrigues da Costa 2016).



Click right out of the point

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Figure 0.9. A binarized picture of an otolith of *Prochilodus nigricans* a fish species from the Amazonian river basin showing the automatically extracted outline (red) starting at the blue point, R graphical device.

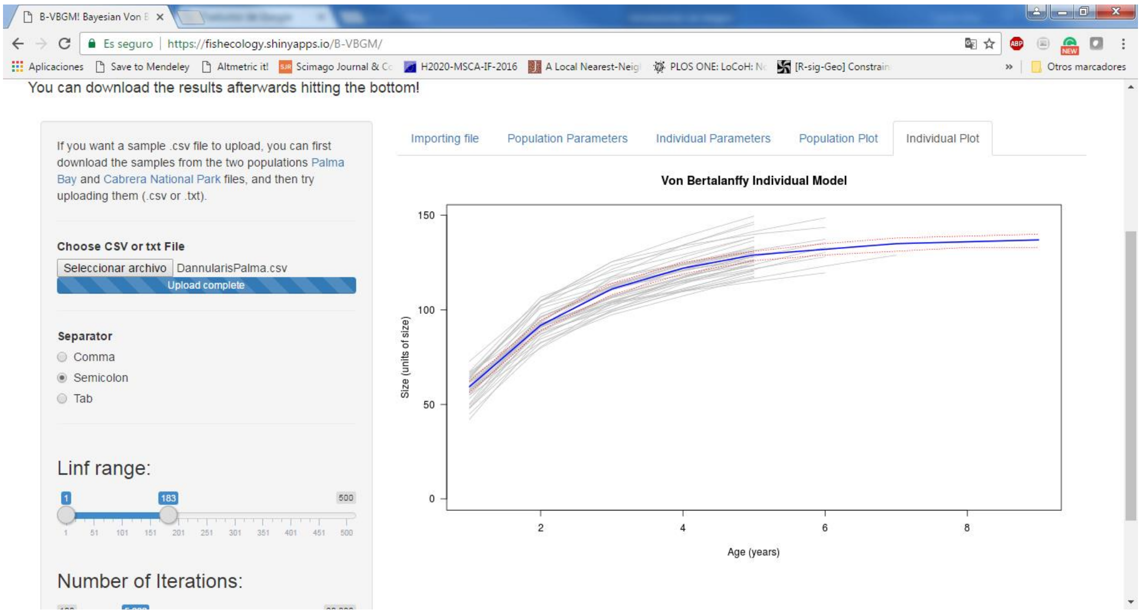
Online von Bertalanffy app

The von Bertalanffy growth model (VBGM) was one of several functions first described in 1938 (von Bertalanffy 1938) and since then the calculation of the VBGM parameters have been fundamental in fisheries science. The standard VBGM is expressed in fish size-at determined age and has been used both at population (one data point per individual) or individual data (many data points back calculated from the otoliths or scales growth.

$$L_0 = L\infty * (1 - e^{-k*(t-t_0)})$$

This equation is based in three parameters: t_0 (the age where the fish would have had zero size), a growth rate (k, which is constant throughout the lifespan of the individual) and an asymptotic length (L_∞).

In the last Chapter of this thesis (VII) we used a Bayesian techniques for fitting the classic von Bertalanffy growth curves for estimating the growth parameters of fish populations and therefore properly estimate other life history traits of interest. Henceforth, we developed a free web app (https://fishecology.shinyapps.io/B-VBGM/) that enables any researcher to fit the von Bertalanffy growth model to their own data (length at age) within the two usual work frames, at population and individual levels (Figure 0.10).



https://fishecology.shinyapps.io/B-VBGM/

You can download the results afterwards hitting the bottom!

If you want a sample .csv file to upload, you can first download the samples from the two populations Palma Bay and Cabrera National Park files, and then try uploading them (.csv or .txt).

Choose CSV or txt File: DannularisPalma.csv

Upload complete

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Figure 0.10. A screenshot of the Bayesian von Bertalanffy growth model (V-VBGM) showing the graphic interface available online for the calculation of the growth parameters at population and individual level including displaying the growth curves. In this case, the individual growth curves (and the mean population growth curve in blue) are shown for *Diplodus annularis* from Palma Bay.

Objectives and structure

The global objective of this thesis is to obtain both qualitative and quantitative information about the settlement processes in coastal fish species of interest for recreational fisheries, and how this process is affected or influenced by anthropogenic uses of the coastal area. The specific sub objectives and underlying hypothesis are listed according to the following structure:

Section I: Settlement processes in Mediterranean seagrass meadows

Chapter I:

In Chapter I we use of a Majorcan traditional fishing gear for bait-collection or “gànguil” to estimate the settlement pulses along a year in shallow *P. oceanica* meadows. The gànguil is a not-shredding beam trawl that do not tear or affect the seagrass meadows structure and efficiently capture early life stages of fish, and has been previously used in Palma Bay in 1960, thus offering us the opportunity of i) analyse the current annual cycle and ii) compare the results between 2013 and 1960.

Objectives: Evaluate the actual estate of juvenile fish assemblages in shallow *P. oceanica* meadows through an annual cycle, and compare it with a similar study conducted half a century ago. Evaluate possible explanations of the differences if any.

Hypotheses: i) The settlement pulses in shallow *P. oceanica* meadows can be described by environmental and reproductive factors. ii) The nursery function (species, abundance, timing) of a shallow *P. oceanica* meadow has been altered in the last 50 years.

Chapter II:

In Chapter II we aimed to study the recruitment to seagrass meadows using stereoscopic cameras as a feasible and more convenient alternative to using “gànguil” compared with traditional methods.

Objectives: i) to develop a low-cost methodology to obtain stereo video, evaluate its accuracy and test them within a field survey. ii) Analyse the post-settlers occurrence over *P. oceanica* meadows along a diel cycle, assessing also the effects of depth and degree of protection (distance to a Marine Protected Area).

Hypotheses: i) Early juveniles' abundance, size structure and diel cycle of *Diplodus annularis* over all the depth distribution of *P. oceanica* can be evaluated using non-invasive methodologies such as stereo baited remote underwater videocameras (SBRUVs). ii) A marine protected area alters at least one of the abovementioned descriptors.

Section II: Experimental insights to early juveniles' behaviour: response to anthropogenic pressure and predatory stimuli.

Chapter III:

The seasonal increase in human pressure in the coastal areas due to leisure activities other than fishing, such as coastal tourism, bathing and yachting entail important environmental stressors in many littoral ecosystems. For example, they may lead to increased dissolved pollutants like sunscreen or fuels/oils from ships and boats, which may disturb the perception of the appropriate nursery habitats for early juvenile fish.

Objectives: evaluate the behavioural reaction of gilt head seabream (*Sparus aurata*) juveniles in front of pollutants like sunscreen or harbour collected water, in comparison with *P. oceanica* chemical stimuli.

Hypothesis: the increment in coastal anthropogenic uses may be affecting how the early juveniles of fish perceive their nursery habitats due to dissolved pollutants.

Chapter IV:

Ocean acidification (OA) affects fish growth, but fish whose larval stages occur in naturally pH fluctuating environments (such as estuaries and coastal lagoons) may have some kind of resilience towards this stressor. We present results from this topic in this

Chapter, analysing biochemical constituents of larval fish that naturally occur in these coastal habitats (i.e. fatty acid composition), and analyse the effects on their otolith shape (Chapter VI).

Objectives: measure the fatty acid profiles of red drum (*Sciaenops ocellatus*) post larvae reared under OA conditions induced by the high levels of anthropogenic CO_2 that is predicted to be dissolved into the oceans by 2300.

Hypothesis: fish larvae and early settlers may suffer metabolic changes due to the effect of elevated CO_2 in the water and this will affect their fatty acid composition.

Chapter V:

Chemical cues from predators induce a range of predator-induced morphological defences (PIMDs) across taxa. However, the mechanisms, direction, reversibility and adaptive value of PIMDs are still poorly studied, especially in marine species.

Objectives: induce measurable PIMDs in juvenile gilt-head seabream (*Sparus aurata*) individuals and check if it is reversible using the smell of a natural predator during a short-time period (30 days) and an equal period without stimuli.

Hypothesis: naturally produced predatory phenotypic plasticity occurs in marine species, and is reversible.

Section III: Otoliths as indirect indicators of life history.

Chapter VI:

Fish otoliths (from the Greek οτο- ear + λιθος, a stone) are the bony structures that can be found in fish ears. They can be used as environmental and growth recorders. In this Chapter, we aimed to test whether the otoliths fluctuating asymmetry (changes in weight or shape of left vs right otoliths) is detected in front of a range of stressors, species and life stages, and whether this information can be used to infer potential recruitment consequences.

Objectives: measure otolith fluctuating asymmetry (OFA; the degree of difference between right and left otolith) in a wide range of different experiments and determine its usefulness as a direct proxy for the fitness of the fish populations.

Hypothesis: if otolith fluctuating asymmetry can be used as proxy of the fitness of the fish population, the proportion of surviving individuals will show smaller degree of OFA than the initial population.

Chapter VII:

In much the same way that tree rings can be counted and analysed to give insight into its past growth and climate conditions in which they have lived, the seasonal growth marks that are deposited in fish otoliths allows us to reconstruct detailed historical life histories (sclerochronological techniques). Furthermore, growth marks can be used to analyse diverse life history traits (LHTs) and fitness across various spatial and temporal scales. In addition, at each “age” the surrounding water chemical composition is recorded in the otolith and can be used to determine for example their natal origin. However, the incorporation of microelements to the otolith may be altered by well differentiated “life styles” that modify expected concentrations.

Objectives: test whether the life history traits (investment in reproduction, growth) of the individuals of a sedentary fish differ between close-by areas under different fishing pressure, and interpret the results within currently existing theoretical frames.

Hypothesis: the dynamics of the incorporation of microelements to the otolith matrix of wild fish is not only related to environmental concentrations but also to the different individual’s traits (growth, maturation, etc), in which case fishing may affect otolith elemental composition.

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Section I: Settlement processes in Mediterranean seagrass meadows

> “What is a scientist after all? It is a curious man looking through a keyhole, the keyhole of nature, trying to know what's going on.”
>
> Jacques-Yves Cousteau

Chapter I: Changes in the juvenile fish assemblage of a Mediterranean shallow seagrass nursery area after half century of nearshore trawl prohibition

This chapter is under review in:

Carlos Díaz-Gil, Amalia Grau, Antonio Maria Grau, Miquel Palmer, Remedios Cabrera, Gabriel Jordà and Ignacio A. Catalán. Submmitted to *Ocean and Coastal Management*

Abstract

The composition of the juvenile fish community over a shallow seagrass-dominated habitat (*Posidonia oceanica*) in a Mediterranean Bay was compared between two concrete years (1960 and 2012-2013). A survey over depths ranging from 2 to 10 m over patched seagrass meadows was conducted during one year (2012-2013) through 72 nocturnal epibenthic tows spread fortnightly in 4 close-by stations. The design resembled in intensity, area, gear and temporal coverage a survey conducted in 1960, right before the prohibition of an intense nearshore trawling activity directed to the collection of crustaceans for bait, which operated over a typical Mediterranean nursery habitat. Although a large proportion of fish species was similar, remarkable and statistically significant differences between years existed: for example, juvenile eel, present in 1960 did not appear in 2013, and one of the most abundant species in 2013, *Diplodus annularis* did not appear in 1960, despite being present in the area as adults at that time. Further, one of the most valuable artisanal species, the black scorpionfish *Scorpaena porcus*, was almost 10 times less abundant in 1960. A finer-scale study of the 2012-2013 survey allowed a formal correlational analysis between species composition and potential environmental drivers through Redundancy Analysis that described well the current pattern of time-dependent recruitment pulses in nearshore meadows from the area through a year, with marked effects of surface temperature and photoperiod. The comparative analysis suggests that some of the differences between years are

unlikely to be due to the environment but that is compatible with the relaxation in a particular type of fishing pressure.

Introduction

Many littoral fish species in temperate areas have a bipartite life cycle where adults live in a small territory or home range (Alós et al., 2014b) but spawn floating eggs that become pelagic larvae for a relatively long period (Raventós et al., 2001). Once these larvae reach the proper size they swim to (and/or sink at) benthonic nearshore habitats, during the settlement phase (Leis and McCormick, 2002). The processes that regulate the success of this settlement phase, in which individuals shift from a pelagic to a nearshore nursery habitats (Beck et al., 2001) are complex and may be critical bottlenecks for shaping recruitment into adult populations (Macpherson and Raventós, 2005; Sponaugle and Grorud-Colvert, 2006; Nash and Geffen, 2012). Nursery habitats vary in complexity and extension depending on the species and topographic characteristics. Optimal nursery habitats are generally found where there is equilibrium between predation and feeding trade-offs (Heck et al., 2003). These areas often result in relatively narrow coastal fringes, which in some instances can be severely altered by anthropogenic actions, that may affect the overall survival of a given species or altering the structure of these transient communities (Munsch et al., 2016).

In the Mediterranean Sea the main settlement habitats for littoral species differ between species (Garcia-Rubies et al., 1995), even within the same fish family. For example, within Sparidae, the white seabream *Diplodus sargus* settle in sand and pebbles coves, recruiting later to rocky deeper reefs when attain a certain size (Casado, 2015; Vigliola et al., 1998) while other sparids such as the annular seabream *Diplodus annularis*, the two banded seabream *Diplodus vulgaris* and the salema *Sarpa salpa* settle mainly on seagrass meadows. These are formed mostly by *Posidonia oceanica* and to a lesser extent by Cymodocea nodosa and Zostera noltii (Fourqurean et al., 2007), in addition to the macrophyte *Caulerpa prolifera* that also forms meadows in small protected bays (Box Centeno, 2008). The key nursery role of structured habitats such as seagrass meadows and rocky reefs has been reviewed several times (Beck et al., 2001;

Heck et al., 2003). In the Mediterranean, the nursery role (here defined as the capacity of a given habitat to host higher juvenile density than the surrounding unstructured habitats, Beck et al., 2001) of the endemic seagrass *P. oceanica* is paramount as it covers around 23% of the coastal shallow habitats (Pasqualini et al., 1998) with an approximate extension of 2.5 to 5.5 million hectares (Buia et al., 2000). In order to guarantee a successful recruitment into adult populations settlers and juveniles must not only find shelter but adequate food, which is available in form of invertebrates and epiphyte algae from the complex and dense canopies of *P. oceanica* (Larkum et al., 2006; Orth et al., 1984). Moreover, the continuous distribution of *P. oceanica* from very shallow nearshore habitats (from 1 to 43 meters (Marbà et al., 2002)) offer in many places an easy transition from juvenile to adult habitats, which confers advantages for growth, survival and recruitment (Heck et al., 2003; Igulu et al., 2011).

Alteration of nursery areas has been the object of numerous studies (Marbà et al., 2002; Ruiz et al., 2015). In the case of *P. oceanica*, meadows are in regression (Diaz-Almela et al., 2007) due to multiple anthropogenic factors from pollution to fishing (Coll et al., 2010). In some Mediterranean areas, such as the Balearic Islands, anthropogenic pressure in littoral areas is elevated not only due to tourism and to an increasing local population, but also to the combined effect of both artisanal and recreational fisheries, which has been notably increasing in the last years (Cardona and Morales-Nin, 2013; Cardona and López, 2007; Morales-Nin et al., 2005). Fishing activity in littoral areas relies in common small-bodied fish throughout the world (Alós and Arlinghaus, 2013; Jacquet and Pauly, 2008). Until the 1970s, in the Balearic Islands, a common practice to obtain lure for recreational and artisanal fishing relied on the use of small epibenthic trawls, called “gànguil” by the locals, over the littoral *P. oceanica* and *C. prolifera*. The obtained bait was composed mainly by small decapoda from the genera Processa, Hippolyte and Palaemon species (Sánchez-Jerez et al., 2000), which were sold as bait.

In the 60`s, Massuti (1965) acknowledged the potential impacts of this non-regulated bait-collection practice on juvenile fish population in Palma Bay (Mallorca), by analysing the juvenile catch within an experimental time-series of night trawling using a “gànguil” during a year (1960). This study did not specifically test impact (e.g. by using control areas or a before-after impact design) but suggested that the littoral area swept every night was large and that most fish caught were juvenile specimens, which provoked the enforcement of existing (but rarely observed) laws banning this coastal trawling (BOE, 1941). In the early 70s the activity declined rapidly after the enforcement of the ban. Previous to the ban enforcement, night-trawling over seagrasses in the nearshore could concentrate up to 200 small boats in Palma Bay (a mid-size Bay of ca 20 km wide, Figure I.1), boats that trawled at low speed over depths of approximately 1-10 meters using rows or small engines. Furthermore, another work from 1966 evaluated the impact of a similar but larger bottom trawl on deeper *P. oceanica* meadows (15-20 meters) (Oliver, 1966), which in combination with Massuti's work in 1965 offer a snapshot of the fish communities inhabiting *P. oceanica* meadows fifty years ago. The main objective of this study is to compare the current composition of juvenile fish over seagrass meadows in the shallow (1-10 meters) fringe of Palma Bay, with the original data from Massuti (1965), following a similar methodology. We hypothesize that, after over 50 year of prohibition of the night trawling in that nursery area, the effects on fish juvenile community should be detected.

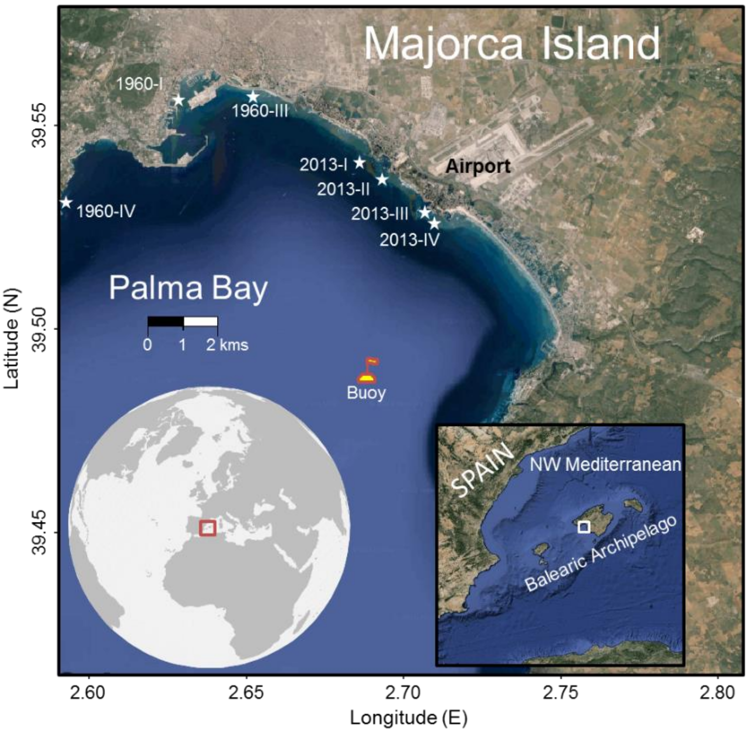

Figure I.1. Centroids of the sampling areas and environmental variables collection spots (Oceanographic buoy). Station II from 1960 was not considered for comparative studies.

Material and methods

Sampling

In his published work in 1965, Massutí described a whole year (1960) sampling of the fish community in shallow (mostly seagrass-covered) nursery areas, using the same epibenthic trawl ("gànguil") as the local fishermen in Palma Bay. Briefly, the "gànguil" is a light-weight epibenthic trawl with an incorporated rolling stainless steel cylinder in the bottom of the mouth that protects the *P. oceanica* leaves from snagging and tearing while operating. To mimic that sample design, we conducted a fortnightly survey during one year (2012-2013) using almost the same gear, over areas close to the ones surveyed by Massutí (Figure I.1). The very same locations could not be sampled due to restrictions to the access and increased anthropization associated to the commercial harbour (see Supplementary figure I.1), so we selected close-by areas with bottom characteristics and depth similar to those surveyed in 1960. Care was taken to include *P. oceanica* meadows sparsely patched with sand and few rocks. One of the stations in 1960 was not included in our analyses due to its proximity to a sporadic freshwater source from a torrent. The remaining sites were otherwise comparable to those in 1960, and covered a stretch of around 2.5 km of littoral zone over *P. oceanica*. To ensure a large sample size for some analyses related to the settlement pulses during 2012-2013, fish were collected after sunset (as in 1960) using two epibenthic bottom trawls "gànguils" as described in Catalán et al., (2014), whereas only one gear was used in 1960. To avoid possible between-surveys differences due to fishing operation skills, the surveys in 2012-2013 were performed with the aid of an experienced traditional fisherman and his boat authorised for scientific sampling. The two gears were towed in parallel covering an elliptical trajectory of 700-1000 m. This sampling methodology is the same used in the traditional bait fishery and has been proved to be adequate to sample juvenile fish with low size ranges close to settlement size (Catalán et al., 2014). After approx. 20 minutes of trawling at approx. 1 knot (Table I.1), samples were sorted on board. Endangered species (i.e. *Sygnatidae*) were immediately released to reduce post-release mortality due to handling for measurements or identification, thus they were not included in the analyses. The additional catch was frozen at -20°C and processed in the laboratory for identification and measure of total length (to the closest mm) and weight (to the nearest mg). For logistic reasons, the data on the Scorpaenid, *Scorpaena porcus* was not available in July, August and September 2012. Therefore, *Scorpaenidae* were not included into the multivariate analyses (see below) and between-year data of *S. porcus* was analysed independently.

Table I.1. Details of the epibenthic trawl surveys in years 1960 and 2012-2013. In year 1960 during April there was no sampling due to problems with the boat.

Sampling characteristics	Year 1960	Year 2012-2013
Mesh size (mm)	5	5
Mouth aperture (cm)	70	80
Length of the net (m)	2.6	3
Number of stations	4	4
Name of the stations and type of habitat	1960-I: Patched *Posidonia* 1960-II: muddy* 1960-III: Patched *Posidonia* + 1960-IV: Patched *Posidonia*	2013-I: Patched (sand) 2013-II: Patched *Posidonia* 2013-III: Patched (small rocks) 2013-IV: Patched *Posidonia*
Depth range (m)	1.8-9	1.5-8
Period covered	January-December 1960	July 2012-June 2013
Number of trawls	76	72**
Mean trawls per month	6.3	6
Speed (knots)	<1	~1
Period	After sunset	After sunset
Duration (mins)	15-20	18-25
Environmental variables	Temperature	SST, Sal., SWH, WEWave, SCurrent, WECurrent, SNCurrent, WindIntensity, WEWind, SNWind, SST$_A$, ModWECurrents, MoonPhase, Photoperiod

* Station 1960-II was discarded for posterior analysis since it was directly situated on the mouth of a sporadic freshwater source (torrent) and over muddy bottoms, hence the fish community was not directly comparable with the rest of sampling sites. **72 tows with 2 devices each, to a total of 144 samples. SST: Sea Surface Temperature; Sal.: Buoy Salinity; SWH:Significant Wave Height; WEWave: West-East Wave component; SCurrent: Surface Current; WECurrent: West-East Current component; SNCurrent: South-North Current Component; WEWind: West-East Wind component; SNWind: South-North Wind component; SST$_A$: Sea Surface Temperature Anomaly; ModWECurrents: Modelled West-East Current component.

In addition, environmental variables were obtained from different sources. Daily surface temperature (SST, ºC) data has been obtained from the CMEMS high resolution L4 reprocessed SST product (marine.copernicus.eu). In particular, the pixel centred at 2.62ºE/39.37ºN has been extracted and considered as representative of the SST in the Palma Bay. SST anomalies (SST$_A$) with respect to the annual cycle have been also computed. Salinity (psu), current intensity (m s^{-1}) and current direction (deg) at 1 m depth were collected from a close by hydrographic buoy operated by SOCIB (www.socib.es) and located at 2.70ºE/39.49ºN (Figure I.1). The buoy also provided significant wave height (m), wave direction (deg), wind intensity (m s-1) and wind direction (deg). The quality controlled buoy data is provided at hourly frequency but in order to get a daily value we have averaged all values between 8.00h and 20.00h each day. The salinity records had large gaps due to malfunctioning of the conductivity sensor. In order to fill those gaps, daily data from the CMEMS Mediterranean reanalysis has been used. The temporal correlation of the modelled and observed salinity at 1 m depth is over 0.65 ($p<0.05$). Additionally, model surface currents and temperature at the buoy location have also been considered.

Statistical analysis

We conducted two types of analyses. In the first approach, the two datasets (i.e. 1960 versus 2012-2013) were compared in terms of the multivariate species composition and relative abundances. Downscaling of the 2012-2013 data was necessary as detailed information from 1960 was lacking (e.g. individual length data and environmental variables). In the second approach, the current (2012-2013) time-series was analyzed in more detail in order to gain an understanding into the settlement/recruitment dynamics in the area and help interpreting potential changes across surveys.

Historical comparison of nursery areas

Both surveys were conducted during a full year, with over 70 tows in each year. All months were covered several times at each station except for April 1960 (Table I.1). In order to compare the 1960 and 2012-2013 datasets, size information was downscaled to ranges of mean size (total length, mm) by month of sampling, which consisted of several trawls (Table I.1). Furthermore, captures were standardized to percentage (of a given species) within station per month. Species with relative abundances <0.5% were considered rare and excluded from the comparative analyses to prevent bias in the multivariate community analysis. Due to similar towing times and speed, and as the exact towing time was lacking for 1960 (Table I.1), we assumed that tows were comparable. Potential differences between years and stations were analyzed using a multivariate linear analysis via Redundancy Analysis (RDA, Borcard et al., 2011). Furthermore, an Indicator Species Analysis (ISA, Cáceres et al., 2010; De Cáceres and Legendre, 2009) was conducted in order to identify the indicator species responsible of the differences between the groups identified through the RDA. In the case of the scorpaenids, their relative abundance per haul was compared between years through ANOVA after transforming the data to meet the implicit assumptions (Zuur et al., 2010), and by selecting the months common to both surveys.

Current dynamics of the nursery area associated to shallow seagrasses

During the 2012-2013 survey, abundance, individual size and weight and environmental variables were recorded. Fish abundances were standardised using the swept area method (Gunderson 1993) and analysed with RDA, first in terms of comparing concurrent trawls in each of the paired hauls, secondly to analyse possible differences among the four locations and finally in terms of the environmental variables. As no differences were found in port and starboard sides tows (RDA, factor *Side* $F_{1,142}$: 0.4215, P< 0.789), the data from both trawls from each haul were combined. The abundance of each species was binned into size classes following the available literature (Crec'hiriou et al., 2015) into "settlers" (<30 mm), "juveniles" (31-60 mm) and "adults" (>61 mm). Species under 0.5% of abundance were considered rare and excluded for posterior analysis. Finally, the abundances were log transformed and distances among sites were computed with Hellinger's distance (Legendre and Gallagher 2001). Therefore, a fish-by-size-range abundance matrix was used as the response and conditioned by the environmental matrix in an RDA model. In addition, for the most abundant species the size distribution per month was plotted to visually asses the detailed size distribution of the species with regard to time over the shallow *Posidonia* meadow.

A total of twenty environmental variables (see Supplementary table I.1 for details) combining those from observations (oceanographic buoy (10), satellite (1), direct measures at each station (1), calculated (2), and numerical models (6)), were first explored with correlation analysis in order to reduce the dimensionality and avoid highly correlated variables. Variables with correlation values > 60% were considered to be redundant and one from each pair was discarded. The selected variables (Supplementary table I.1) were introduced as the explanatory matrix in the RDA model.

In addition, each sampling date was assigned as summer (June-September), autumn (October-November), spring (April-May) and winter (December-March) in order to aid in the visualization of the results.

All statistical analyses were carried out using vegan package (Oksanen et al., 2013) from the R-software (www.r-project.org) (R Development Core Team and Team 2011). Specifically, Hellinger's distances were calculated using the *decostand* function, and the selection of the environmental variables included in the final RDA model was realised with the *ordistep* function that perform a forward stepwise selection of the variables from the null model without explanatory variables to the complete model (including all the variables) based on the P values. (Blanchet et al., 2008).

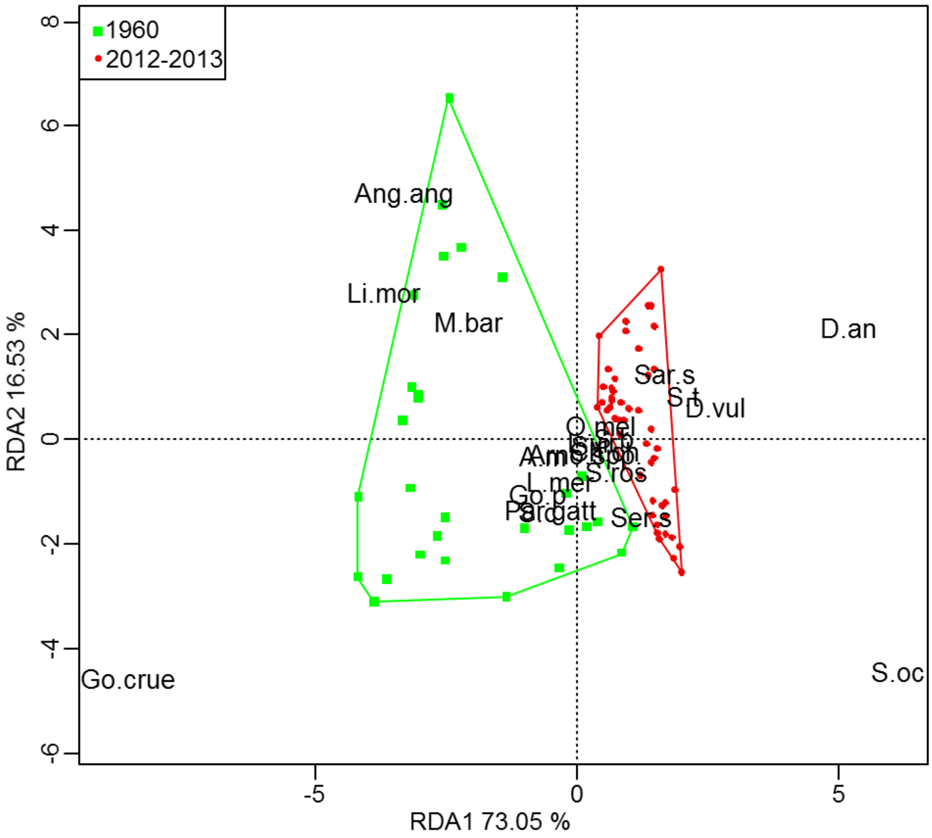

Figure I.2. Ordination plot of between-years RDA results. Symbols represent samples for each of the two years. Fish species names as follows: Ang.ang= *Anguilla anguilla*; A.m= *Aphia minuta*; Arno.spp.= *Argonoglossus* spp.; Ch.ch= *Chromis chromis*; D. an: *Diplodus annularis*; D.vul=*Diplodus vulgaris*; Go.crue= *Gobius cruentatus*; Go.p= *Gobius paganellus*; L. mer: *Labrus merula*; L.vir: *Labrus viridis*; Li.mor: *Lithognathus mormyrus;* O. mel= *Oblada melanura*; M.bar: *Mullus barbatus;* Par.gatt= *Parablennius gattorugine;* Sar.s=*Sarpa salpa;* Ser.s= *Serranus scriba;* ; S.b= *Symphodus bailloni*; S.c= *Symphodus cinereus*; S.ros: *Symphodus roissiali*; S.oc= *Symphodus ocellatus*; S.r= *Symphodus rostratus*; S.t= *Symphodus tinca*).

Table I.2. Complete list of fish species found in shallow *Posidonia* meadows during the sampling season. Families are in bold, standard length (SL) range (Min and Max, of the averages of SL per month) in mm. Species not present in samples of one year are marked with "—". Abundances from year 2012/2013 are divided between two, to make comparison of captures with only one device. Species marked with * were captured mostly in single events in 1960. In addition, *Scorpaena porcus* and Sygnatidae were captured in both surveys.

	Year 1960			Year 2012-2013		
Species	Min	Max SL	n	Min SL	Max SL	n
Anguilidae						
Anguilla anguilla	74	125	**27**	—		
Apogonidae						
Apogon imberbis	—			48.95	70.58	**2**
Atherinidae						
Atherina boyeri	52	63	**8**	39.39	71.69	**2**
Bleniidae						
Lipophrys trigloides	—			27.8	27.8	**1**
Parablennius gattorugine	45	75.2	**26**	84.52	84.52	**1**
Parablennius sanguinolentus	—			54.34	100	**1**
Parablennius tentacularis	76	76	**1**	—		
Bothidae						
Argonoglossus spp.	29.8	29.8	**31**	30	96	**4**
Bothus podas	68	81	**3**	—		
Centracanthidae						
Spicara smaris	55.2	56.2	**9**	—		
Clinidae						
Clinitrachus argentatus	55	55	**1**	39.3	39.3	**1**
Gadidae						
Gaidropsarus mediterraneus	125	125	**2**	34.57	85.325	**5**
Gobiidae						
Aphia minuta	23.5	42.7	**25**	20.85	23.41	**23**
Gobius buchichi	—			26.75	29.88	**1**
Gobius cruentatus	25	57	**344**	36.44	55.28	**3**
Gobius paganellus	52	80	**35**	31.36	106	**20**
Gobiesocidae						
Lepadogaster lepadogaster	30	35	**11**	—		
Labridae						
Coris julis	—			114.6	115.1	**2**
Labrus merula	67	77.4	**12**	153.05	178.02	**1**
Labrus viridis	—			24.87	111.31	**10**
Symphodus bailloni	82	82	**1**	61.53	129.3	**31**
Symphodus cinereus	36.5	85	**46**	29.1	29.1	**1**
Symphodus ocellatus	26.5	75	**155**	27.49	82.08	**443**
Symphodus roissali	—			42.92	90.44	**12**
Symphodus rostratus	65	75	**18**	26.78	121.34	**47**
Symphodus tinca	65	99.2	**7**	24.77	129.21	**89**
Mullidae						
*Mullus barbatus**	47	64.2	**36**	—		
Ophidiidae						
Parophidium vassali	—			43.34	147.64	**11**
Pomacentridae						
Chromis chromis	52.5	86	**7**	43.63	100.01	**19**
Serranidae						
Serranus hepatus	—			33.2	34.6	**12**
Serranus scriba	40	85	**68**	33.9	135.495	**95**
Sparidae						
Diplodus annularis	—			37.89	113.06	**155**
Diplodus puntazo	—			60.65	60.65	**1**
Diplodus sargus	20	63	**3**	—		
Diplodus vulgaris	35	50	**12**	19.6	89.77	**135**
*Lithognathus mormyrus**	29.4	96	**201**	—		
Oblada melanura	—			24.44	97.8	**11**
Sarpa salpa	42	42	**1**	23.64	103.27	**156**

Results

Historical comparison of nursery areas

Both surveys yielded similar mean number of individuals per trawl (16.8 ind. in 1960, 17.7 ind. in 2012-2013, see Supplementary figure I.2 for mean number of individuals per month). Fish composition was relatively similar in the number of species (27 species in 1960 vs 31 species in 2012-2013, see Table I.2 for complete list of species captured, and size distribution) and size ranges overlapped (Supplementary figure I.3), ensuring reasonably unbiased datasets for comparison between years. Moreover, the sea surface temperature from both years (the only environmental variable quantified in 1960) were reasonably similar despite being taken with a Negretti & Zambra thermometer in 1960 and from satellite measurements in 2012-2013 (see Supplementary figure I.4).

Table I.3. Indicator species analysis (ISA) results. IndVal$_{ij}$ is the indicator value for the species in parts per unit. P values are based on 999 permutations. Significant indicator fish species for each sampling period are shown. Species marked with * are typically found in sandy bottoms.

	Species	IndVal$_{ij}$	P
Year 1960	*Gobius cruentatus*	0.92	0.005
	Parablennius gattorugine	0.554	0.005
	Anguilla anguilla	0.525	0.005
	*Lithognathus mormyrus**	0.525	0.005
	Symphodus cinereus	0.509	0.005
	*Mullus barbatus**	0.455	0.01
	Labrus merula	0.39	0.03
Year 2012-2013	*Diplodus annularis*	0.913	0.005
	Symphodus tinca	0.879	0.005
	Symphodus ocellatus	0.859	0.005
	Serranus scriba	0.815	0.005
	Symphodus bailloni	0.706	0.005
	Symphodus rostratus	0.682	0.015
	Diplodus vulgaris	0.665	0.01
	Chromis chromis	0.581	0.01
	Sarpa salpa	0.553	0.02
	Labrus viridis	0.479	0.015
	Oblada melanura	0.456	0.035
	Symphodus roissali	0.456	0.015

The comparison of the relative abundances from both years using RDA showed a significant effect of both *Year* ($F_{1,70}$: 17.99, P< 0.001) and *Station* ($F_{5,70}$: 1.69, P=0.016) explaining a R^2 of 27.42% of the total variance. RDA Axis 1 separated the samples from *Year* with a 73.05% of the explained variance, by far much stronger effect than *Station* effect (RDA 2, 16,53%) (Figure I.2), and henceforth these two differentiated groups were selected for the ISA, which showed a clear shift in the species composition from year

1960 to year 2012-2013 (Table I.3). Selected indicator species can be unique to one of the year (such as *Anguilla anguilla or Mullus barbatus* in 1960 or *Diplodus annularis* in 2012-2013) or on the other hand be present in both years, but more abundant in one of them (as *Gobius cruentatus* in 1960 or *Symphodus ocellatus* in 2012-2013). Moreover 20 species were captured in both sampling periods although some in very low abundances (*Diplodus vulgaris* and *Sarpa salpa* in 1960) (Table I.2). Comparison of the *Scorpaena porcus* abundances, for coincident months, showed a significant increment in relative abundance in year 2012-2013 (ANOVA $F_{1,14}$=11.65, P=0.004**) (Figure I.3).

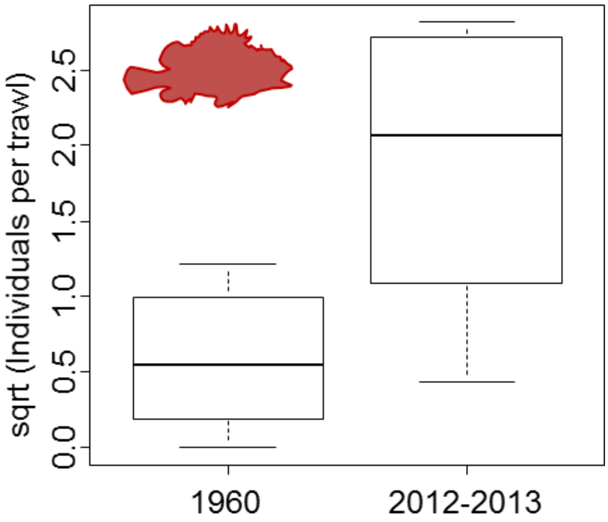

Figure I.3. Individuals of *Scorpaena porcus* per gànguil trawl in both surveys. Only common months are included (i.e. April 1960 + July, August and September 2012 are excluded).

Dynamics of the nursery area in 2012-2013

As no differences in the fish communities between the two tows per station trawl were observed (RDA, factor *Side* $F_{1,142}$: 0.4215, P< 0.789), we pooled the catches per trawl in order to maximize the representativeness of the size-species matrix. From the 20 available environmental variables, 14 were selected (Supplementary table I.1) for the RDA model (R^2 = 32.76%) which found six of them to be significant (Table I.4) by forward stepwise selection. Selected variables in terms of decreasing explained variability were SST (°C), photoperiod (h), dominant currents in the area (i.e. Dominant component of surface current (West-East component)) from the oceanographic buoy (cm s^{-1}), temperature anomaly (SST_A°C) and salinity) (Figure I.4). Clearly, season-related variables as SST, photoperiod and SST_A basically explained the ordination of species-size composition and particularly the settlement of species (Figure I.4), with additional significant influence of surface salinity and the direction of the surface currents (West-East component).

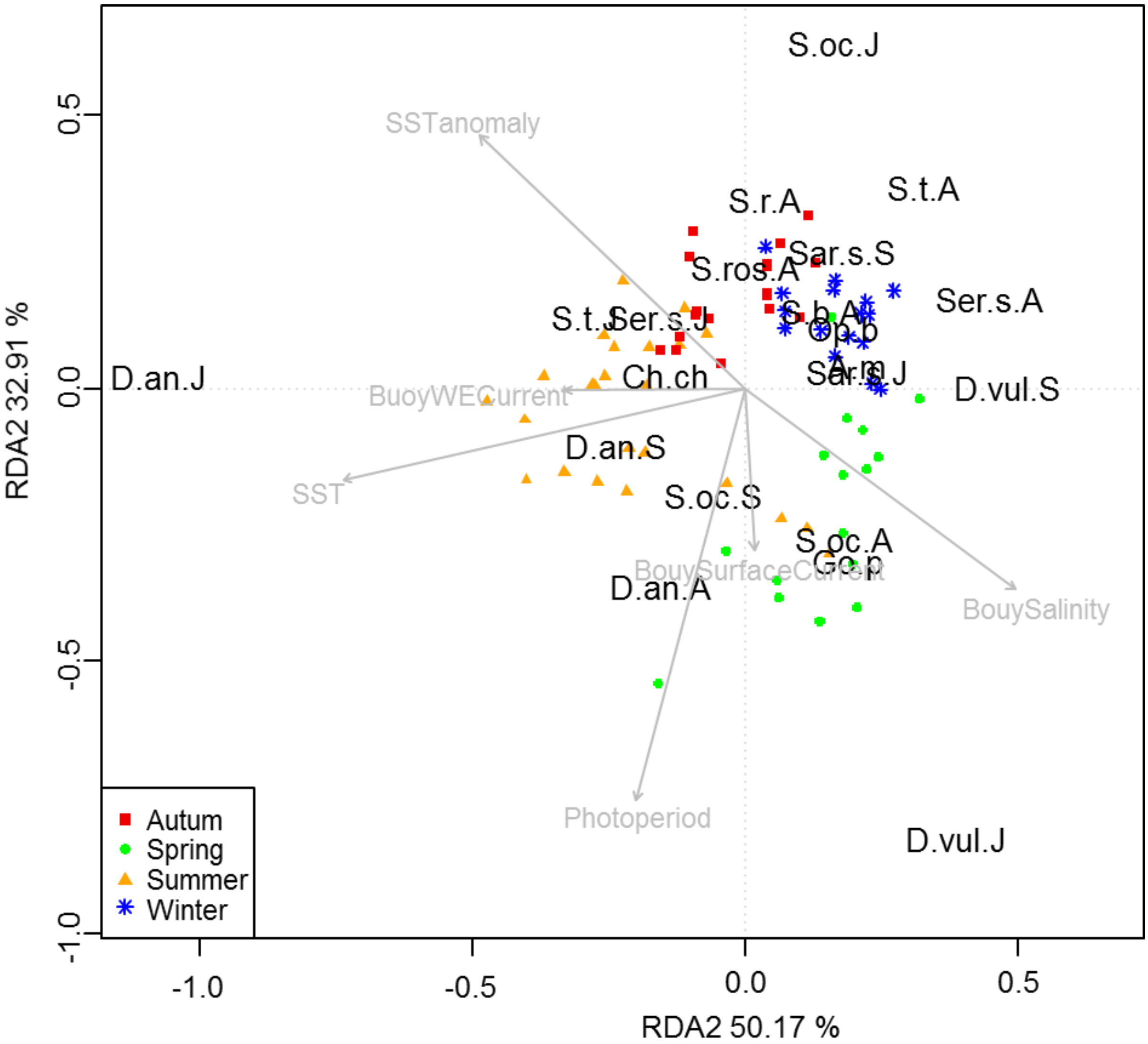

Figure I.4. Ordination plot of RDA results for year 2012-2013. Axes depict the variance of the model explained. Grey arrows signal the strength and direction of the different environmental variables selected in the RDA model. Symbols represent samples divided by season of sampling. Fish species names are contracted, final letter showing the life stage: S for Settler, J for Juvenile, A for Adult. A.m= *Aphia minuta*; Ch.ch= *Chromis chromis*; D. an: *Diplodus annularis*; D.vul=*Diplodus vulgaris*; Go.p= *Gobius paganellus*; Op.b= *Ophidium barbatum*; Sar.s=*Sarpa salpa*; Ser.s= *Serranus scriba*; S.b= *Symphodus bailloni*; S.ros: *Symphodus roissiali*; S.oc=*Symphodus ocellatus*; S.r= *Symphodus rostratus*; S.t= *Symphodus tinca*).

The detailed size distribution of the four more abundant species through time (Figure I.5) showed that the use of *P. oceanica* shallow habitats shifted between several species sequentially along the year: *Diplodus annularis* settlers appeared in July (~2.5 cm) and grew over the summer season; *Symphodus ocellatus* juveniles ~4 were detected in the *Posidonia* shallow beds and stayed over autumn and winter months; *Sarpa salpa* settlers appeared in January till March, similarly to *Diplodus vulgaris,* and settled down in late winter, growing over the spring season.

Table I.4. Results of redundancy analysis (RDA) by variables and constrained axis computed for the fish community composition by size classes (see Figure I.4).

		Eigenvalue	Proportion explained	Variance	F	P	
Variables	SST			0.069	14.525	0.001	***
	Photoperiod			0.050	10.484	0.001	***
	BuoyWECurrent			0.015	3.093	0.002	**
	BuoySurfaceCurrent			0.009	1.915	0.033	*
	SSTAnomaly			0.010	2.018	0.021	*
	BuoySalinity			0.010	2.109	0.022	*
	Residual			0.298			
Axis	RDA1	0.0783	0.5017	0.078	16.281	0.001	***
	RDA2	0.0514	0.3290	0.051	10.678	0.001	***
	RDA3	0.0163	0.1048	0.016	3.403	0.001	***
	RDA4	0.0100	0.0643	0.010	1.093	0.016	*
	Residual			0.2984			

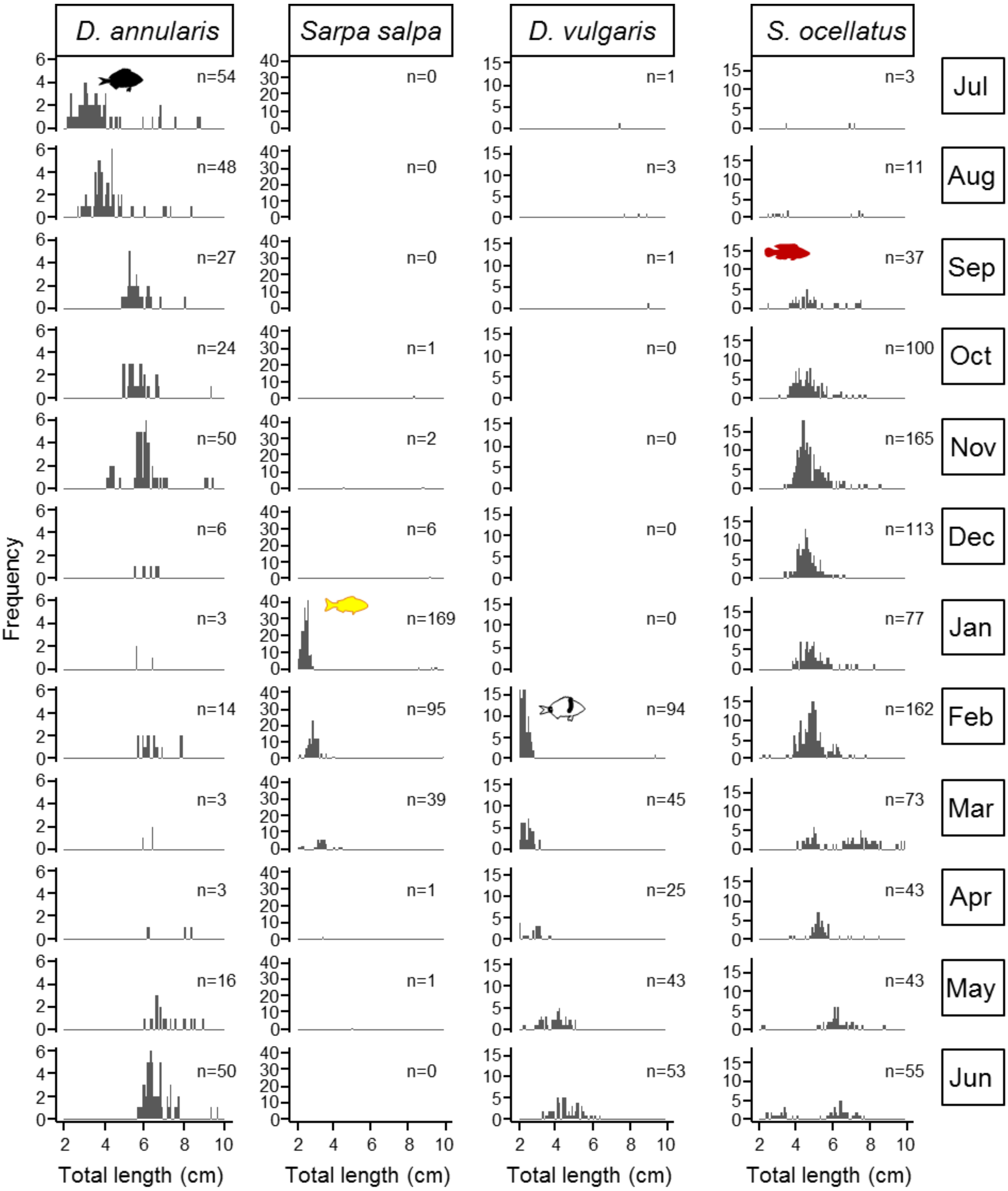

Figure I.5. Settlement pulses of the four most abundant species. *Diplodus annularis*, *Sarpa salpa*, *D. vulgaris* and *Symphodus ocellatus* along the whole sampling period (1 year). The number of individuals per sampling month and species is shown.

Discussion

Seagrasses and macrophytes meadows are key nursery habitats in the Mediterranean Sea and fish communities associated with them are well described by numerous previous studies, usually using scuba diving (Harmelin-Vivien and Francour 1992; Vigliola et al., 1998; Guidetti 2000; Guidetti and Bussotti 2000; Félix-Hackradt et al., 2013). In the Balearic archipelago, there have been several works describing the spatial variation of the fish assemblages within deeper *P. oceanica* meadows (Oliver 1966; Moranta et al., 2006), or along relatively short periods of time, for example bimonthly over a year (Reñones et al., 1995; Deudero et al., 2008). These previous works were based on deeper meadows and used bigger mesh size that our study. The early works by Massutí (1965) that enabled the present comparative study plus the work by Oliver (1966) using a bigger "gànguil" (similar to the one used by Deudero et al., (2008) and Moranta et al., (2006)), in deeper meadows allow us to sketch a picture of the state of the fish assemblages half a century ago and to perform a long term comparison of the fish communities associated to the *P. oceanica* meadows in the Balearic archipelago nowadays.

The first description of the use of the small "gànguil" to capture crustaceans for bait dates back to 1897 in a description of the Balearic Islands uses and traditions by the German naturalist Ludwig Salvator archduke of Austria, although larger "gànguils" were used at least one century before that (Sañez Reguart 1792). The use of this traditional fishing trawl had been common until the first half of the XX century, and despite being officially forbidden since 1941 (due to the small mesh size used (*Spanish Oficial Bulletin (BOE)*, 1941)) still in 1960 this art was commonly used. Massutí (1965) described the fishing pressure to be around 330 to 1100 trawls per night of Saturdays and Sundays. These figures are based on an average of 5.5 trawls (paired tows) per night and an observed minimum of 60 boats operating during the night, with potential peaks of 200 boats only in Palma Bay. Assuming a conservative mouth size of 70 cms, a mean speed of 1 knot and, a mean duration of 30 minutes per haul a range between 0.4 and 1.4 km^2 were swept each night. Because the target bait and the gear restricted the operations to shallow depths between approximately 1 and 10 m, the trawling pressure represented a 10.8% of this shallow fringe covered by patched seagrass, trawled at least twice per week. In that paper, Massutí related "it is important to highlight the damage caused by fishing enthusiasts on the development of the species that inhabit these habitats in their post-larval phase". Despite the prohibition from year 1941 it was not until his observations in 1960 that the use of this fishing gear effectively declined due to the reinforcement of the Spanish laws (this type of gears was regulated sequentially in 1962, 1973 and 1975 (BOE 1962; BOE 1973; BOE 1975)) and local enforcement.

The detailed analysis of 2012-2013 survey allows us to further investigate potential explanations for the observed historical change. Our results from 2012-2013 showed that settlers and small juveniles of many species use the shallow *P. oceanica* meadows as settlement habitats along the different seasons of a given year. The arrival of early settlers of the different species was triggered by different environmental variables that modulate the fish community along the year. For example, higher sea surface temperature during summer is apparently related with *D. annularis* settlement but these relationships cannot be assessed by only one year of sampling. Nevertheless, the RDA results showed a differential use by many species' settlers of this habitat along the different seasons during the year, coinciding with previous works in the Mediterranean (Garcia-Rubies et al., 1995). These shallow *P. oceanica* meadows are spatially delineated by sand areas or rocky reefs in the proximities of the coastal line and usually deeper seagrasses habitats. The rocky reefs are well characterized by abundance of macrophytes such as Fucales, mainly of the genus *Cystoseira* that offer also a good settlement niche (Ballesteros et al., 1998; Cheminée et al., 2013). Our ISA results selected several species that are considered as indicator of the period 2012-2013, including the four species showing clear settlement pulses: *D. annularis, D. vulgaris, S. sarpa and S. ocellatus*. The shallowest area is characterized by the high abundance of settlers of some species like *S. ocellatus* during summer that we found later in autumn in the shallow *P. oceanica* meadows in our samples. Conversely, the adults of *S. ocellatus* are very abundant in the deeper *P. oceanica* meadows; apparently this Labrid species uses the seagrasses shallow habitats as a feeding grounds during their juvenile stage (4-6 cm) (Gouraguine et al., 2016) before moving to deeper habitats later in the season. On the other hand, the Sparids *D. annularis, D. vulgaris* and *S. sarp*a recruited in summer, winter and early spring sequentially, showing a seasonal shift in the use of this nursery habitat (Figure I.6 for a conceptual diagram).

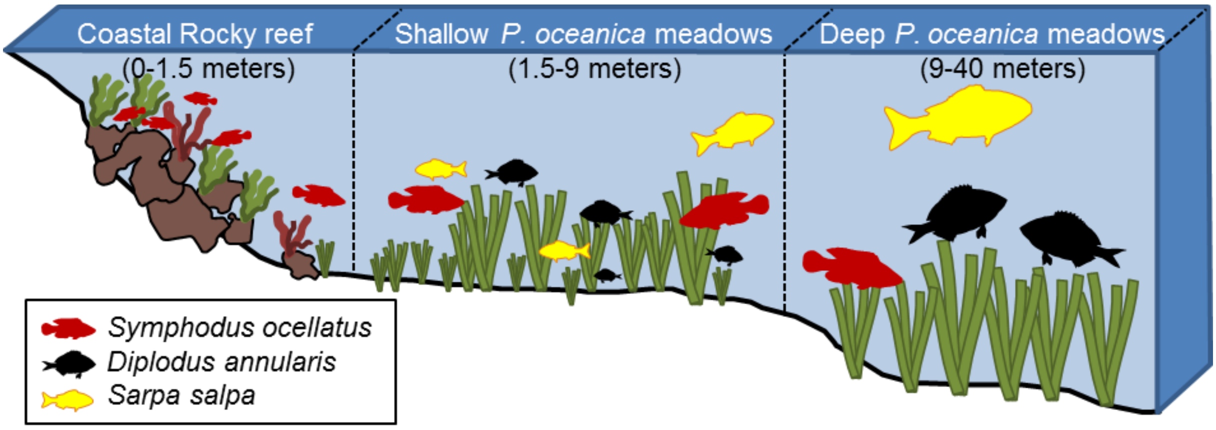

Figure I.6. Conceptual diagram of the shallow habitat utilization by the three most common species in the current samples. *S. ocellatus* settle at smaller sizes in the immediate nearshore rocky reef during summer, migrating afterwards (autumn) towards the shallow *Posidonia* meadows. *D. annularis* settle in the shallow *P. oceanica* meadows in summer and migrate to deeper adult habitats in winter, while *S. sarpa* and *D. vulgaris* (see Figure I.5) settle down in winter over the shallow *P. oceanica* habitats which use for 2-4 months before recruiting to deeper habitats.

Despite capturing approximately, the same number of individuals per trawl in 1960 and 2012-2013 (16.8 vs 17.7 ind./trawl), fish composition changed significantly. When comparing the total species composition (27 species for 1960 and 31 in 2012-2013) some species have totally disappeared in year 2012-2013, such as *Anguilla anguilla*, probably due to a degradation of the freshwater output habitat nowadays totally anthropized and not only locally (Feunteun 2002). However, one of the most striking changes between both surveys is the absence of *D. annularis* in the samples from 1960. This species is one of the most abundant Sparid species in Palma Bay nowadays and, within the Bay, their adult phases were highly abundant in 1964-1965 in deeper *P. oceanica* habitats (Oliver 1966) and also in the early 1990s (Reñones et al., 1995). Although we cannot discard the possible effect of climate change as a driver in species shift, the similar temperature cycles between 1960 and 2012-2013 showed herein suggest that other causes might explain the differences between juvenile fish assemblages in shallow seagrasses after 52 years, which we condense in two hypotheses:

i) We may not be comparing exactly the very same habitat. We acknowledge that the spatial variability in the nursery areas can be large at relative small scales, especially when affected by manmade structures (Bulleri and Chapman 2010; Cheong et al., 2013; Munsch et al., 2016). However, we believe that the risk of this is low, as we controlled for the effect of depth, bottom type, time period, and tows were integrated over hundreds of meters along a stretch of few km. Further, our data show that species typical from *P. oceanica* (e.g. *Symphodus rostratus* and *Serranus scriba, Scorpaena porcus,* and Sygnatids although the latter species were not included in the analyses) were frequent in both years. Although we acknowledge that habitat conservation status (density of *P. oceanica* shoots, quality of water) probably has changed (Ruiz et al., 2015), these effects would be confounded with fishing ones. However, this would not explain the lack of (nowadays) extremely common, in space and over extended periods, juvenile fish such as *D. annularis*, whose adults were present in the area at greater depths (Oliver 1966). Some species in 1960 might be suspect of pertaining to a different habitat; for example, one indicator species in 1960, *Lythognatus mormyrus*, is typical from sandy bottoms although it can dwell seagrass beds (Garcia-Rubies et al., 1995). However, its capture was temporally and spatially infrequent, because 91% of all individuals (183) were captured in a single station. Thus, this capture may be a signal of fortuitous over-sand trawl and/or simply a catch of a sparse juvenile school. Moreover, the absence of this species in the following months reinforces this as an anecdotic capture. In addition, the appearance of three individuals of *D. sargus* in 1960, whose juveniles tend to inhabit the shoreline (Casado 2015) may be explained if some tows were extremely shallow; however, we do not believe that the bulk of the trawled area was in a different depth range, not only according to their description of the methods but also because the range of sizes of the common species overlapped.

ii) Fishing effort in the 60’s was so strong and concentrated on the shallow seagrass nursery habitats that the abundance of the nowadays very abundant fish species was kept low. This can explain the fact that in the 60s the lower thropic fish species as Gobiidae and Blenidae were more abundant. A striking feature potentially related with fishing pressure is the lack of specimens of *D. annularis* in 1960, whereas in 2012-2013 it was one of the most common species, and the juveniles appeared through extended periods of time in our study and in previous within Palma Bay (Oliver 1966; Deudero et al., 2008). If fishing pressure lowered the abundance of these recruits at early stages (e.g. at the beginning of the settlement period), low juvenile abundance would be expected in the data. This fraction, which should have existed in order to maintain the adult populations observed at deeper areas by Oliver (1966), might be found at either deeper *P. oceanica* meadows or at slightly different habitat configurations. Furthermore, since the distance from harbour was a major driver of where to go fishing, especially for those boats without motor in the first half of the XX century, the nursery seagrasses meadows close to Palma harbour (and other minor marinas) were possibly over exploited at juvenile stages, being the adult populations maintained with individuals from close by populations (Alós et al., 2014a). *D. annularis* is not the only case: the virtual absence from 1960 samples of other species that use this nursery habitat for short periods of time during the post settlement as *D. vulgaris* or *S. salpa* (only 12 and 1 individuals in 1960 respectively), and, moreover, the lower abundance of species that had, and actually have, a local economic value like *Serranus scriba* or *Scorpaena porcus* in 1960 reinforce the hypothesis of the intense fishing pressure on this nursery habitats.

We contend that the historical difference in juvenile fish between the 60's and the current survey might obey to the decrease in the trawling pressure in shallow areas, although we cannot discard that other concurrent effects were operating, including pressure on the adults' populations, anthropization of the nearshore habitats and/or water quality. Since many of the species nowadays common are of interest for artisanal and recreational fisheries nowadays (e.g. *D. annuaris, D. vulgaris, S. porcus* or *S. scriba*), their increase in the samples might be thought as positive for this sector and, assuming that the hypothesis of the release of fishing pressure is correct, the increase in the surveillance to reinforce the prohibition of the use of "gànguils" to capture shrimps in early 1970s can be regarded as a correct management measure with respect to the nursery function of shallow seagrass habitat in the Balearic Islands.

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Chapter II: Using stereoscopic video cameras to evaluate seagrass meadows nursery function in the Mediterranean

This chapter has been accepted:

Carlos Díaz-Gil, Sarah Louise Smee, Lucy Cotgrove, Guillermo Follana-Berná, Hilmar Hinz, Pere Marti-Puig, Amalia Grau, Miquel Palmer and Ignacio A. Catalán. *Marine Biology*

Abstract

Seagrasses provide an important ecological value as nursery habitats, hosting higher juvenile densities than their surrounding less-structured habitats by offering shelter and food to early stages of fish. However, the quantitative evaluations of this nursery function remain elusive. Surveys assessing abundances of juvenile fish over seagrass meadows have been largely conducted by diving visual censuses, where typically only a shallow depth range is adequately covered. Within this study, we developed a low-cost stereo baited video camera (SBRUV) with high precision and accuracy able to deliver length frequency distributions, including the smallest juvenile fraction. The capacity of the SBRUV system increased the synopticity with respect to typical surveys. We tested the system over seagrass meadows of *Posidonia oceanica* in a Mediterranean bay, investigating the relationship of juvenile abundance and size of the sparid *Diplodus annularis* with depth, time of day and protection status (i.e. inside and outside of a Marine Protected Area, MPA). We found significant effects of the depth on the length of the older size classes fraction (larger sizes at deeper stations) and an effect of time of the day on the abundance (less abundant during the evenings), and an opposite pattern was observed for early juvenile's abundances. The MPA protection had no effect in *D. annularis* population structure. Interestingly, the relative abundance of *D. annularis* early juveniles was comparable at all depths, from 2 to 20 m, which suggests a potentially higher nursery value of *P. oceanica* meadows than earlier thought.

Introduction

Optimum nursery habitats can be conceptualized as areas where predation is minimal, due to either less predators or offering shelter, and feeding opportunities are favourable (Heck et al., 2003). These areas are often present in relatively narrow coastal fringes, for example mangroves or marshes (Barbier et al., 2011). The key nursery role of structured habitats such as seagrass meadows and rocky reefs has been reviewed several times highlighting the capacity of these habitats to host higher juvenile density than the surrounding unstructured habitats (Beck et al., 2001; Heck et al., 2003). In general, there seems to be a consensus that seagrasses are key to providing habitat to feed and/or hide for both permanent and temporary residents, including early developmental stages (Giakoumi and Kokkoris, 2013; Jackson et al., 2001). As a consequence, seagrass meadows have been linked to higher/intermediate biodiversity values and fisheries production (Unsworth et al., 2014). Worldwide seagrass ecosystems are under threat with a loss between 1879 and 2006 estimated at 3370 km^2 (27 km^2 yr^{-1}), which represents 29% of the maximum area measured (11592 km^2) (Waycott et al., 2009). An example of this habitat in European waters is *Posidonia oceanica*, a marine phanerogam endemic to the Mediterranean forming the largest seagrass meadows in that Sea where it covers an area of approximately 2.5-5 million ha (Boudouresque et al., 2006; Pergent et al., 1995) from close to the surface down to a depth of 40 m (Marbà et al., 1996). The nursery value of *P. oceanica* to fish populations seems to be clearly more important than less vegetated areas (e.g. Bell and Harmelin-Vivien 1982; Francour 1997; Guidetti 2000).

One typical drawback in the evaluation of nursery function is that it is difficult to obtain quantitative measurements of how juvenile use those habitats, and what percentage of the adult population is replenished by juveniles coming from those specific habitats (Beck et al., 2001). Among the reasons in failing to obtain those data, there is the fact that it is often difficult to obtain fish size structure and abundance data from these habitats along their whole bathymetrical distribution. Partly, in the case of *P. oceanica* this is due to its status of protected species (is included in the Red List of marine threatened species of the Mediterranean with the meadows defined as priority natural habitats by the Annex I of the EC Directive 92/43/EEC on the Conservation of Natural

Habitats and of Wild Fauna and Flora (European Commission, 1992)). This prevents conventional extractive survey methods and requires non-invasive, robust and cheap methods. In fact, estimating the habitat use of a given species/life stage requires complex experimental designs because spatial/temporal usage is highly multifactorial (e.g. (García-Charton et al., 2004)) and the scales of variation may scape to conventional design (e.g. daily, storm-dependent). Therefore, surveying methods that increase the synopticity should be preferred, in terms of improving the recognition of both the spatial (meters, kilometres…) and temporal scales (days, weeks, months…) at which the settlement processes occur..

Stereo baited remote underwater video (SBRUV) is increasingly used over other sampling techniques as they can be deployed in environments unsuitable for conventional diver-based assessments (e.g. deep habitats), detect diver-shy species and provide a valuable estimate of the relative abundance by size classes of economically important species (Gladstone et al., 2012; Lowry et al., 2012). The use of this method provides a permanent visual record of the surveys undertaken which allows for multiple observer analysis in addition to being a non-destructive sampling technique (Kelaher et al., 2014; Unsworth et al., 2014). Further, bias in length estimate due to observer are largely avoided (Harvey et al., 2003; Letessier et al., 2015; Starr et al., 2016). Several previous works review the use of cameras for estimating abundances and compare the effectiveness of this sampling methodology against direct capture through fishing (Gardner and Struthers, 2013; Parker et al., 2016) or sampling with SCUBA surveys (Harvey et al., 2004), observing in most cases differences in estimates of abundance from the distinct methodologies. The use of SBRUVs has been advocated for in the last few years as a wider range of depths can be surveyed (Starr et al., 2016; Stobart et al., 2015), with larger data samples obtained due to increased duration of battery life and storage devices, maintaining accuracy of estimates and providing a lower survey cost (Letessier et al., 2015).

The objective of the present work was to test the capacity of a low-cost sturdy stereo-baited camera system to conduct a survey of fish size-structures, with focus on early juveniles, in dense meadows of *P. oceanica* that are difficult to sample. We tested the capacity of the system to provide spatial (distance from a Marine Protected Area centre), depth and diel information on fish size structures in a quasi-synoptic survey design.

Material and methods

Study area and survey design

The selected study area, Palma Bay, is a marine temperate bay dominated by seagrass meadows. Located on the southern part of Mallorca Island, NW Mediterranean (39.4800˚N, 2.6400˚E), the Bay is 31 km wide with a maximum depth of 50 m. In this area, anthropic pressure is high due to the surrounding region supporting 44.8% of the population of Mallorca as well as being one of the main destinations for tourism in the Balearic Islands (March et al., 2013). The coastal population and the high concentration of marinas contribute to this bay being one of the most recreationally fished areas in Spain (Morales-Nin et al., 2005). Conservation methods within the area include the Palma Bay Marine Reserve (PBMR), a marine protected area (MPA) located in the eastern part of the bay (Figure II.1). The MPA extends from the coastline to the 30 m isobath and creates a protected area for fish that inhabit the *P. oceanica* seagrass. Since 1982, all fishing activities within the MPA have been regulated although this was not enforced until 1999. The MPA is comprised of two areas with differing levels of protection: (1) the integral reserve (~2 km^2), where all fishing activities and anchoring are strictly prohibited and (2) the buffer zone (~24 km^2), where artisanal fishing is allowed under certain restrictions and recreational fishing is banned three workdays a week. In addition, a minimum hook size (7 mm gape) is required and fishing tournaments are banned (Alós and Arlinghaus, 2013).

A survey was designed to obtain information on the size structure of one of the most common demersal species dependent on *P. oceanica*, and exploited by anglers; the sparid annular seabream (*Diplodus annularis*), with regard to depth, distance to the centre of PBMR and time of day (to control for diel behavioural changes related to size ranges). The survey was carried out over the course of 3 days in June 2015 (21st, 22nd and 24th) matching the settlement peak of the species (Alonso-Fernández et al., 2011). Three identical SBRUV structures (see below) were deployed at 54 randomly distributed sampling points on bottom ecosystem dominated by *P. oceanica*, at a minimum separation of 200 m away from each other and within the following factorial design: i) equal distribution in three depth categories (shallow: 2 m, medium: 10 m and deep: 20 m, 18 videos at each; ii) spread within three different MPA protection zones (integral, buffer and no protection, 18 videos each) (Figure II.1); and iii) deployed during dawn, day and evening (from 5 a.m. to 9 h, 9-17 and 17-21h local time; 18, 20 and 16 videos respectively).

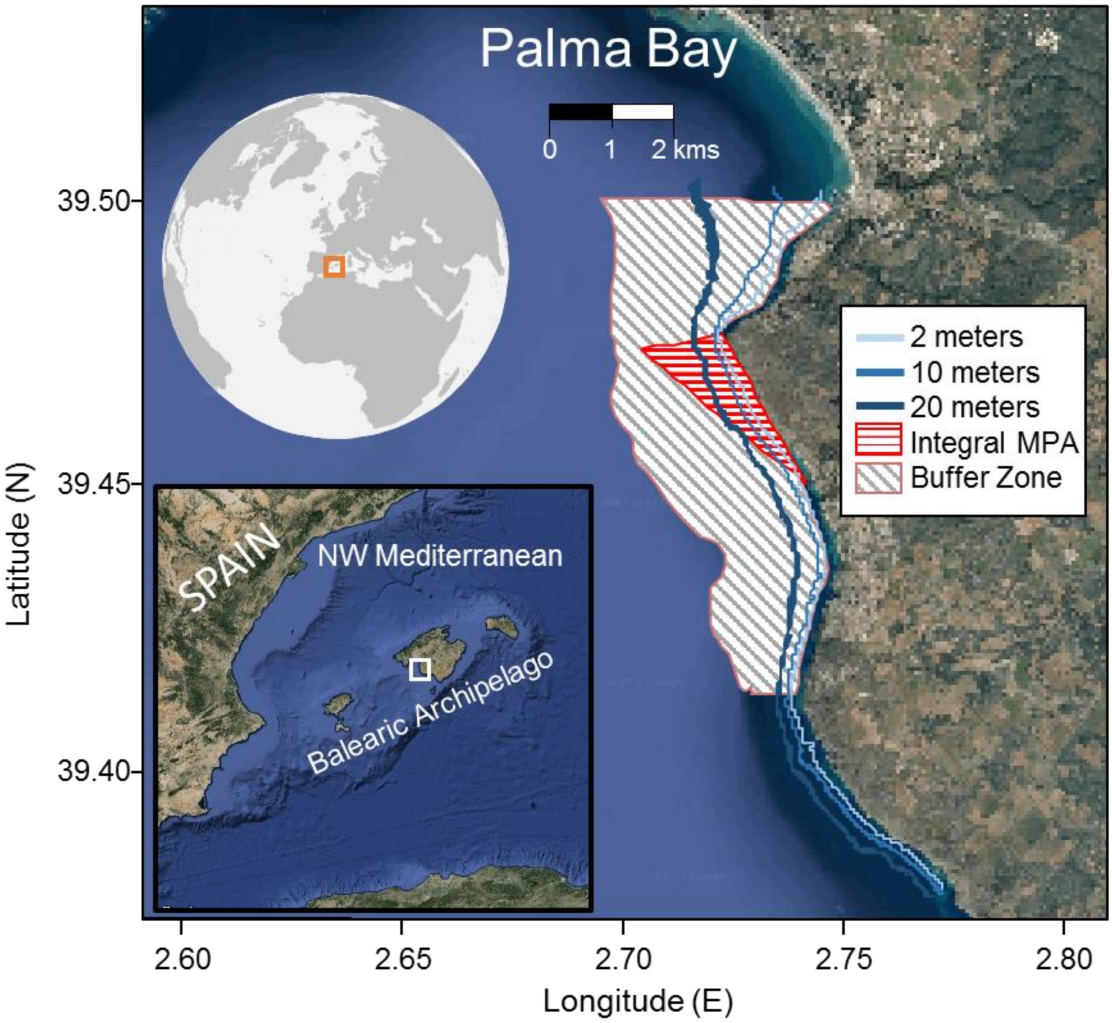

Figure II.1. The selected study area, the Palma Bay, that features the Palma Bay Marine Reserve (PBMR), comprising both the Integral and Buffer Zones. The different depth strata fringes in which the cameras were deployed are depicted following the coastline. Base maps from Google, Imagery© 2007, TerraMetrics.

SBRUV development

The structure of each SBRUV consisted of PVC piping which held two GoPro Hero 3 camera housings attached to a stainless-steel scaffold, equipped with a 1.5 m steel pole to hold the bait (Figure II.2, A). The position of the cameras was determined by previous trials with differing inter-camera distances and angles that converged in an easy to use design that allowed for accurate estimates of fish size. Both cameras were placed straight on the same plane (without angle) at a separation of 200 mm between the objectives and centred from the middle of the steel bait pole. This compact design allowed for fast alignment of the cameras on board while surveying and produced a small blind area to the stereoscopic measurements, with a minimum stereo focal zone of 50 mm apart from the cameras (see Figure II.2, B). With this configuration, an accurate measurement of recently settled juvenile fish (as small as 15 mm) was achieved, and the blind area of the stereoscopic visual field was reduced, losing only 90 mm at each side (Figure II.2, B; for a complete description of the stereoscopic visual field see Figure 0.6 or Supplementary figure II.1). The height of the cameras on the SBRUV system was also tested to record directly above the average length of *P. oceanica* leaves at 600 mm (Marbà et al., 1996). The bait chosen for the SBRUV followed Stobart et al., (2007) and consisted of a mesh bag containing a small buoy, 1-2 chopped fresh sardines and an effervescent spherical pellet of approximately 200 g. The combined use of citric acid and bicarbonate in the bait pellet, reacts with seawater causing an effervescence that dissolves to release small bait particles that are especially attractive to fish, including very small juveniles as we observed (see Supplementary figure II.3). The optimal distance of the bait from the stereo-system was set at 750 mm, and a height of 600 mm from the seafloor, to center the bait in the stereoscopic camera field.

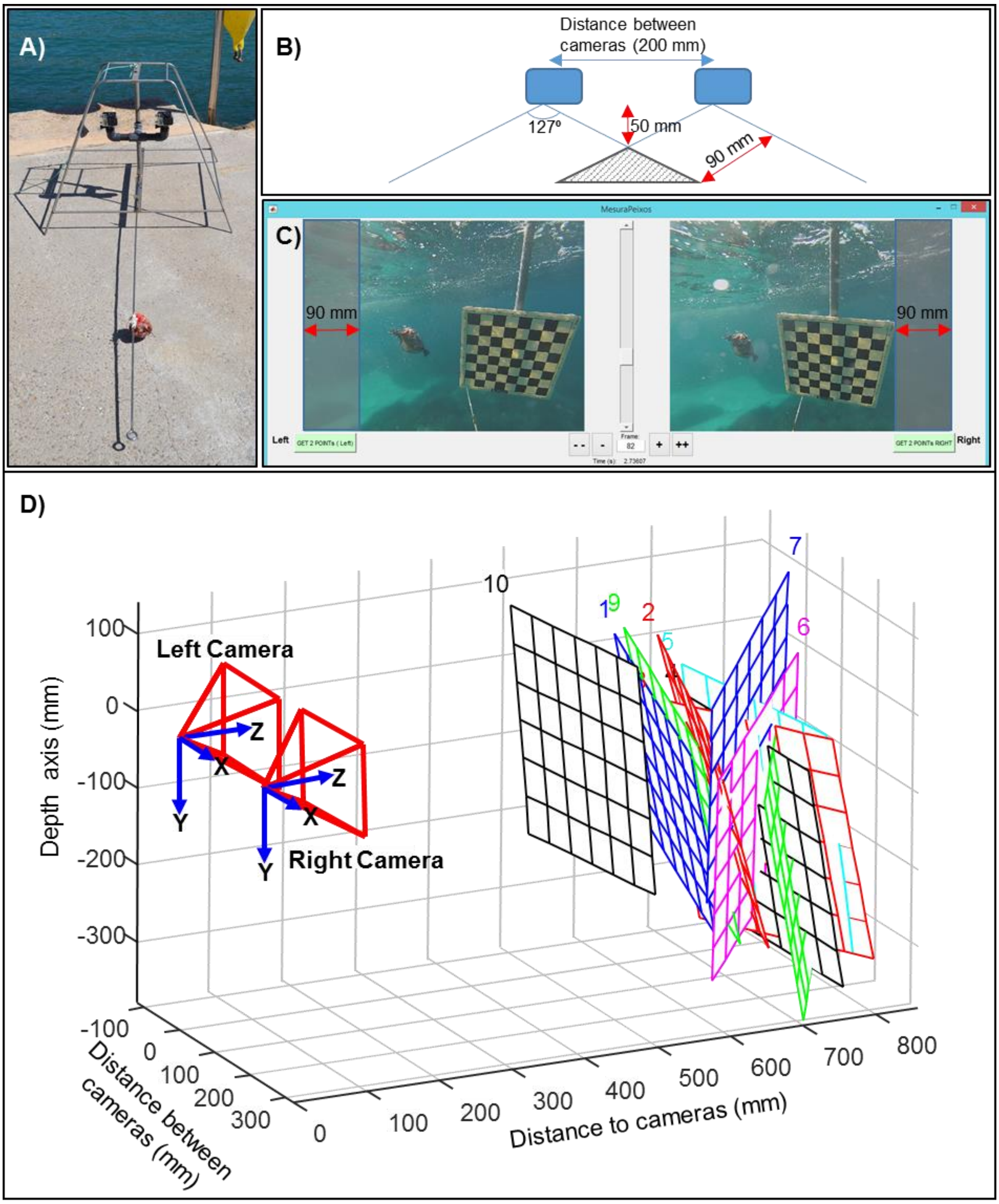

Figure II.2. A) Photograph of the SBRUV system structure, including the bait pole; B) Aerial close up diagram of the position of the video cameras; the shaded zone depicts the beginning of the stereoscopic measurement area. The double pointed arrows indicate blind stereoscopic areas: very close to the cameras (50 mm) and the fringe not covered by both cameras (90 mm at each side); C) Snapshot of the calibration process using a checkerboard, notice the fringes of video (shaded) that cannot be used for stereo measurements. D) Output of the calibration process displaying ten differentially positioned pictures of the checkerboard plate.

Video recording, length measurements and statistical analysis

The use of a SBRUV in order to obtain length measurements requires a calibration procedure. The plate calibration technique (Williams et al., 2010) was chosen for this purpose. A planar checkerboard with a printed pattern (8 x 8 black and white 50x50 mm squares) was waterproofed and secured to a pvc tube to allow for underwater calibration prior to each SBRUVs deployment, right below the sea surface (see Figure II.2, C). The process of calibration involved presenting the plate in the field of view of both cameras while it was moved both horizontally and vertically to allow several images of different orientations to be taken. In the laboratory, each video pair was individually calibrated using a stereo camera calibration toolbox for Matlab 2015, (Mathworks, Natick, 2015) (http://www.vision.caltech.edu/bouguetj/calib_doc/, last accessed 11/01/2017) (Figure II.2, D). The calibration intrinsic parameters were used to correct the videos for optical distortion resulting from the “fish eye” effect of the camera lenses. In addition, prior to calibration, a flash light was shot in front of the SBRUV to provide a synchronisation frame for later alignment of video footage. Calibration, synchronization and measurement of the selected individuals was performed using a customized user interface in Matlab. Prior to the survey, several tests were conducted using artificial fish of known sizes inside an experimental tank for quantifying i) the accuracy of the measurement (here defined as mean absolute error, MAE=$\frac{1}{n}\sum|observed - true|$) and ii) the precision, calculated as the variability around the measurement due to observer selection of the snout and tail points of the fish, for deriving both the fish length and the distance to the camera.

During the field survey, each SBRUV was set to record an initial short footage of the calibration set (checkerboard) right below the surface, and were subsequently deployed to the benthos where they recorded continuously during 30 minutes. This period was pre-defined in order to reach the minimum duration at which the MaxN (defined as the maximum number of each species recorded at any frame of the video footage, Cappo et al., (2004) is reached in this ecosystem (i.e. 20 minutes, Stobart et al., (2007)), at 29 fps in 1280 x 960 pixels definition. After at least 30 minutes of effective recording, each SBRUV was retrieved, the bait replenished and the system redeployed at the next sample point. The videos were inspected using only one camera (usually Left) to assess if they were recorded properly and over relatively homogeneous *P. oceanica* meadows. Out of 54 pairs of videos, 16 were discarded as they were over sand, tipped over, or in the worst case one of the cameras was covered by *P. oceanica* leaves not allowing stereo measurements (see Supplementary figure II.3). In the remaining 38 videos, the whole video footage of the whole fish community was examined. For all of the species, the MaxN was noted for intervals of 2.5 minutes.

Although all species were counted, the length measurements were extracted only for *D. annularis* as it was the most abundant and our objective species. The frame at MaxN for each 2.5 minute period for *D. annularis* were used to rapidly find these individuals in the Matlab user interface and measure them. The exact frame could be moved backward or forward to localize the most suitable fish orientation to be measured. According to Alonso-Fernández et al., (2011) spawning and therefore settlement of *D. annularis* occurs very concentrated in time, and one clear modal class assigned to this process could be easily detected from the length distribution histograms; a separation of the newly settled individuals or “early juveniles” (0+) from the older individuals (hereafter “residents”) was achieved using normal mixture modelling (NMM, Fraley and Raftery 2002) for classification and density estimation within the R library *mclust* after taking logarithms of the lengths. This procedure estimates the mean length and density of the main normally-distributed size classes, after classifying individuals as early juveniles coming from the settlement of that year, and residents (from previous years) from each video size distribution (see Figure II.3 for a schematic view of the processing of length measurements). Finally, the parameter values from each distribution were used in the comparison between the different factors comprised within the survey multifactorial design: depth of the seagrass meadow (shallow, middle and deep), MPA protection status (Integral, Buffer or NotMPA) and time of day (Dawn, Day, Evening). Full-factorial analysis (with interaction) was not possible due to the lack of videos, e.g. combining factors as Depth and Time of day had between 3 and 7 videos per possible combination. Natural logarithm or BoxCox transformations (Box and Cox, 1964) to meet the assumption of ANOVA were conducted when necessary, and non-parametric Kruskal-Wallis test was performed when parametric assumptions were not met. In addition, extra continuous variables were tested against the length distributions, such as the linear distance to the centre of the MPA or the solar irradiance at the depth where the cameras were recording, according to: $I = I_0 e^{-kz}$; where I_0 is the incident solar radiation at the surface (data extracted from a nearby hydrographic buoy operated by SOCIB (www.socib.es) and located at 2.70ºE/39.49ºN), *K* is the attenuation coefficient for the Bay of Palma (0.06, Basterretxea pers. comm.) and *z* is depth (m). All statistical analyses were performed using R software (R Development Core Team, 2011))

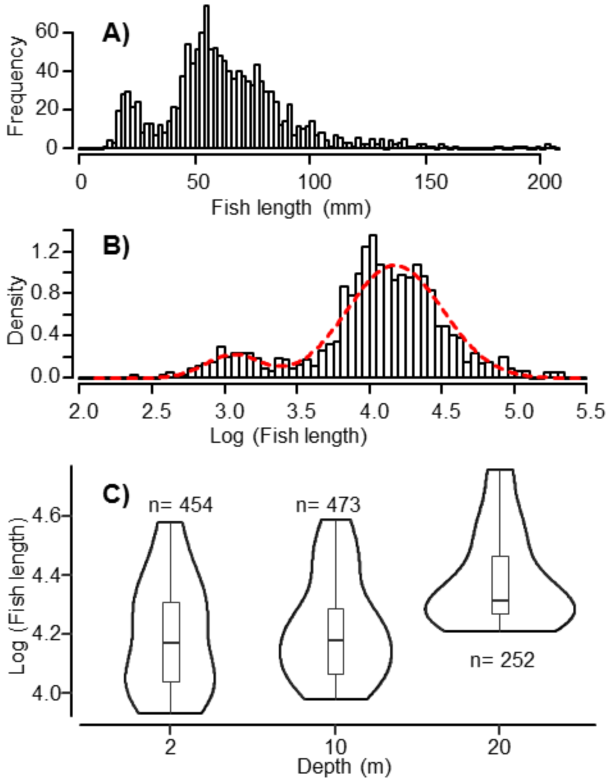

Figure II.3. Processing fish length data. A) Histogram of raw dataset of all fish length distribution, at least two cohorts are clearly differentiated. B) Natural logarithm transformation of A) to obtain a combination of two Gaussian distributions used to calculate abundance (n) and mean length (±SD) for both distributions (early juveniles and residents). C) Both, abundance and lengths can be used to analyze differences according to factors, in this case depth of the seagrass meadows.

Results

SBRUV accuracy and precision

The test for accuracy and precision of the SBRUV measurements yielded satisfactory results for the purpose of the study (Figure II.4). The measurements had less than 5 mm error as far as 4 m for both residents and early juvenile (post-settlers) individuals. Over this distance, location of the snout and tail pixels of the fish were difficult to be located on the image with good precision, hence 3.5 m was selected as the maximum distance to measure individuals in the field. The precision or repeatability of the measurements had a standard deviation of less than 10 mm at 4 meters.

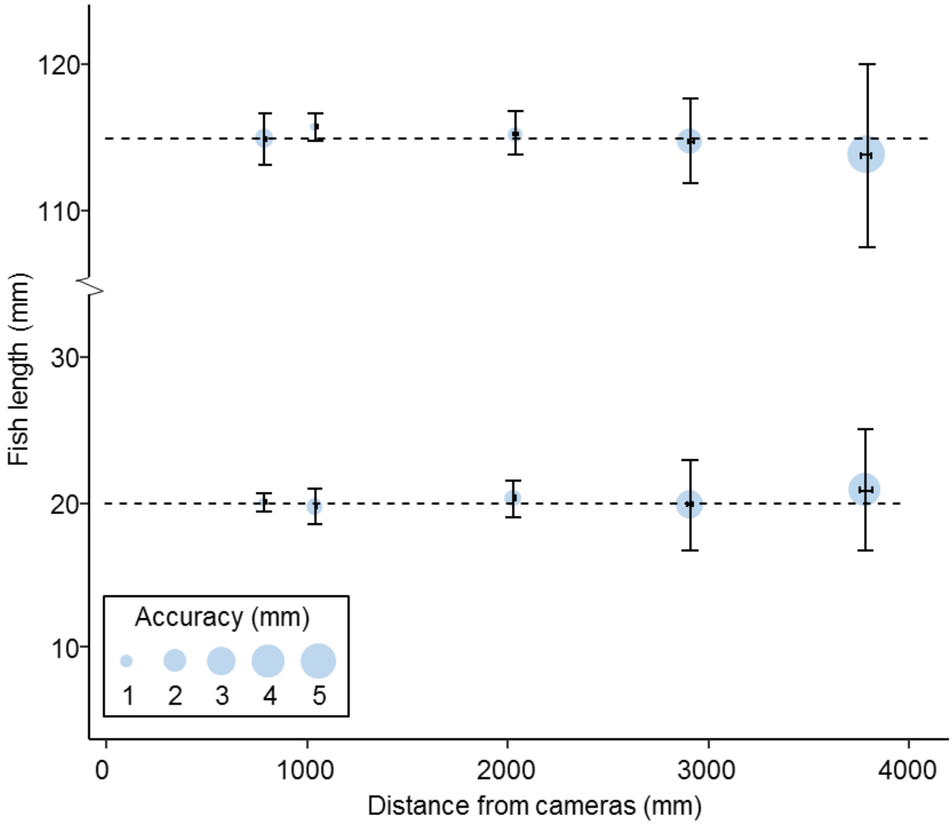

Figure II.4. Repeated measurements (n=30 in each spot) of two known size plastic fish (20 and 115 mm, dashed lines) at different distances from the SBRUV system. Circles represent the accuracy of the measurement with respect to the real size of the object, error bars (SD) represent the precision or repeatability of the measurements in both fish length (vertical) and distance of the fish to the cameras (horizontal).

MaxN, observed and measured individuals.

The frame or frames around the sequence of MaxN for each 2.5 minute period for *D. annularis* were used to measure the individuals. Not all individuals in the scene could be seen by both cameras (influence of some seagrass leaves, blind area etc.). Nevertheless, the number of measured individuals correlated well with the observed number of individuals along the video (i.e. the sequence of MaxN for each 2.5 minute period, R^2 = 0.93) and with the MaxN of *D. annularis* along the video (R^2 = 0.89) (Figure II.5). Thus, the length measurements were conducted representatively along the abundance axis.

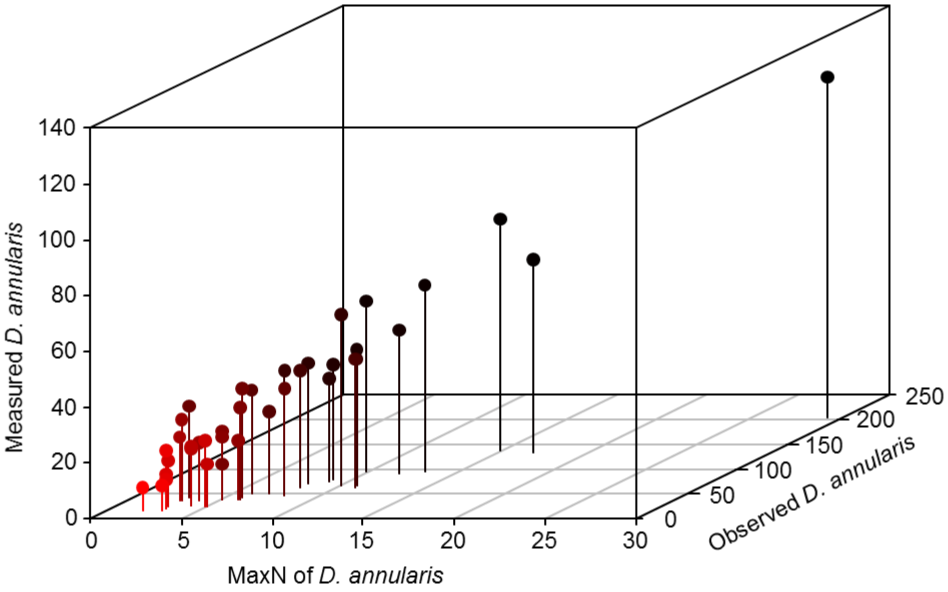

Figure II.5. 3D representation of the correlation of measured individuals of *D. annularis* per video (n=38) in relation with the observed number of *D. annularis* along the whole video clip in left camera (R^2 = 0.93) and the maximum number of *D. annularis* present together in a single frame along the video (MaxN) (R^2 = 0.89). The red coloured dots are closer to the origin of coordinates for all variables.

Relationship of D. annularis size classes with depth, time of day and protection level

A total of 1347 individuals of *D. annularis* were measured, and the lengths distributions were classified into early juveniles (<35 mm, total N=168 individuals, mean n per video= 4.5, average size= 23.10 ± 3.44 mm) and resident individuals (>35 mm, total N= 1179, mean n per video= 31.86, average length= 72.50 ± 14.87 mm) using the finite Gaussian mixture modelling for each video. The continuous variables (linear distance to MPA center and solar irradiance at the bottom) were also tested against the size distributions, but no significant effects were found. Both abundance and size distributions of early juveniles and residents were contrasted against the factors “Depth category”, “Time of day” and “Protection Level” separately: Depth category had a significant effect over the resident fraction of *D. annularis* with larger individuals at deeper stations; Time of day had a significant effect on the abundance of residents (lower abundances during the evening) contrasting with an opposite tendency on the early juvenile individuals with higher number during the evenings. Protection level had no effect on any of the tested responses (see Table II.1; Supplementary figure II.2 for an exploratory set of boxplots).

Table II.1. *Diplodus annularis* early juveniles and residents’ abundances and size distribution within the different factors of the field survey. Depth category (2, 10 and 20 m); Time of day (Dawn, Day, Evening); and Protection Level (Integral, Buffer or NotMPA). † denote marginal significance; * significant effect.

		Factors		
Stage	Variable	Depth category	Time of day	Protection level
Early juveniles	Abundance	Kruskal-Wallis $X^2_{df:2}$ = 3.2799, P = 0.194	Kruskal-Wallis $X^2_{df:2}$ = 5.7303, P = 0.05697 †	Kruskal-Wallis $X^2_{df:2}$= 1.007, P = 0.6044
	Length	BoxCox transformed (λ=-2.919192). ANOVA; $F_{1,16}$: 0.0082; P = 0.9289	BoxCox transformed (λ=-2.59596). ANOVA $F_{1,16}$: 3.0538; P = 0.099	Log transformed. ANOVA, $F_{1,16}$: 1.6558; P = 0.2165
Residents	Abundance	Log transformed. ANOVA, $F_{1,35}$: 2.9017; P= 0.09735	Log transformed. ANOVA, $F_{1,35}$: 7.3539; P= 0.0103 *	Log transformed. ANOVA, $F_{1,35}$: 0.4251; P= 0.5187
	Length	Log transformed. ANOVA, $F_{1,35}$: 6.3775; P = 0.0164*	Log transformed. ANOVA, $F_{1,35}$: 0.1447; P = 0.706	Log transformed. ANOVA, $F_{1,35}$: 0.1788; P= 0.675

Discussion

The methods for analysing fish settlement over coastal areas have been focused on the capture of pre-settlers using light traps (Félix-Hackradt et al., 2013b; Grorud-Colvert and Sponaugle, 2009), epibenthic bottom trawls (Catalán et al., 2014), direct observation through post-larvae attractors (Ammann, 2004; Tavernetti et al., 2009), and snorkelling or scuba diving transects (e.g. Garcia-Rubies et al., 1995; Casado 2015). Each method has pros and cons but, within the non-invasive methods (e.g. SCUBA transects), confidence in the previous experience of the observer is paramount and this can indeed be its major pitfall. For example, while annotating the size of a determined individual the sampler's attention is momentarily pulled away from the surrounding environment to focus on recording the information and hence a bias is expected (Assis et al., 2013). This can be particularly problematic in the typical case of many mobile juvenile fish in the field of vision. With cameras, this is avoided as it gives the opportunity to follow a chosen individual and take multiple measurements of it. Furthermore, in species where their settlement habitat may extend further than the limits for usual SCUBA visual censuses, the use of SBRUVs opens new opportunities to map the ecological value of these nursery habitats

Our results showed high accuracy (less than 5 mm mean absolute error at 3.5 meters away from the camera) and precision for both distance from the fish to the cameras and fish length (less than a ± 5 mm SD at 3.5 meters' distance). Therefore, we contend that this design is adequate to derive size information from individuals around settlement, of fish species whose abundance is relatively high and whose early stages may be difficult to assess by divers (Colton and Swearer, 2010). We also contend that the design enables intensive use and in areas that, due to their depth would require either a high number of divers or a higher survey time, without losing synopticity. To this respect, we were able to sample different times of day, different depths and different areas in three days, using two people from a small boat. In our case, the numbers of measured individuals can be extrapolated to the total number of individuals in the area, since both measurements were highly correlated. The strength of this work also resides in the demonstration that relatively cheap techniques could be used to assess recruitment patterns over protected areas, obtaining high-quality measurements independent of the observer.

The spatio-temporal patchiness of settlement processes remains as the main pitfall when evaluating settlement success (Félix-Hackradt, 2012; Félix-Hackradt et al., 2013a; Planes et al., 1998). In as much as this patchiness is not properly conceptualized, it will be the combination of several tools that will provide a hint of the realization of settlement (Catalán et al., 2014). Although nursery habitats are usually constrained to shallow habitats there may be deeper habitats that have an underestimated nursery value. Moreover, settlement occurs in small schools of post-larvae all along the coast, constrained by species' capabilities to orient (Kingsford et al., 2002), microhabitat availability (Harmelin-Vivien et al., 1995), predatory fields (Kinoshita et al., 2014), stochastic processes associated to eggs and larval transport (Alós et al., 2014) etc. Overarching this spatial variability, there is an inter-annual variability due to maternal stock size and length of the reproductive season, all factors configuring complex reproductive systems that ultimately drive fluctuations in recruitment (Lowerre-Barbieri et al., 2016). To this respect, our most ecologically interesting results is that *D. annularis* juveniles around settlement (around 1.5 cm) were found at 20 m depth, at densities comparable to those at shallower depths, although the reported settlement habitat for this species was thought to be/ have previously been reported to be between 5-8 m (Harmelin-Vivien et al., 1995). The current survey did not sample down to the maximum observed seagrass meadows in the area (ca 35 m), however, using this technique opens a possibility to re-define the nursery value of *P. oceanica* meadows for this species by for example designing a survey more concentrated on the effect of depth across a smaller area.

We did not find any significant difference in *D. annularis* abundance and size distributions (both for early juveniles and residents) related with the protective effect of the MPA. However, we showed that the average length of *D. annularis* is larger for resident individuals at deeper stations, and that abundances are higher during early morning and midday in comparison to evening. On the other hand, the early juveniles appeared in higher abundances during the evenings. The interpretation of these patterns would be rather speculative and goes beyond the scope of this work but opens further questions on the potential cross effects of the predatory fields (abundance of piscivourus fish for instance) with diel cycles and their interaction with small juveniles (e.g. Kinoshita et al., 2014). In any case, the use of SBRUVs, which preserves the information on other fauna, enables further investigation of these questions.

Although there is current controversy about the comparative use of cameras, single or stereoscopic, versus visual census (reviewed by Murphy and Jenkins 2010; and a practical example by Colton and Swearer 2010), it appears that the estimate of recently settled individuals using cameras has not received much attention until now. Key topics to be dealt with in the future are the necessary number of devices employed according to the type of survey/species, the use or not of bait for attracting the fish and if the chosen bait is suitable for the studied species. In our case, we followed a previously proven as effective recipe for the fish community of interest (Stobart et al., 2007) but this is a matter for detailed preliminary research before any survey is designed. Furthermore, bait plume dispersal due to water currents is increasingly driving researchers' attention since the estimated relative abundance is directly related to the extension of the bait odour (Bassett and Montgomery, 2011). Henceforth we encourage the development of theoretical models that estimate the necessary number of sampling devices (cameras in this case) to estimate the true abundance of fish (i.e. absolute and not relative) in a determined area (Campos-Candela et al., 2017 submitted) and to further expand this knowledge to include the attractive effect of the bait plume.

Although the sampling survey may be improved, the present work was mainly designed to show the potential of the SBRUV system to analyse settlement patterns in future surveys, an objective that had been largely accomplished. However, the sampling effort needs to be adequate to solve any particular ecological question as for example compare two different nursery habitats. In general, the SBRUV design developed in this work is economically affordable and moreover it can be used widely both temporally during the settlement season, and spatially at multiple sites which may help to assess the ecological quality of the nursery habitats and quantitative evaluate the importance of the settlement phase for fish dynamics.

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Section II: Experimental insights to early juveniles' behaviour: response to anthropogenic pressure and predatory stimuli

"When we read about Creation in
Genesis, we run the risk of imagining
God was a magician, with a magic wand
able to do everything. But that is not so"

Pope Francis

Chapter III: Anthropogenic chemical cues can alter the swimming behaviour of juvenile stages of a temperate fish

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Abstract

Human pressure on coastal areas is affecting essential ecosystems including fish nursery habitats. Among these anthropogenic uses, the seasonal increment in the pressure due to leisure activities such as coastal tourism and yachting is an important environmental stressor in many coastal zones. These pressures may elicit understudied impacts due to, for example, sunscreens or other seasonal pollutants. The island of Majorca, northwest Mediterranean Sea, experiences one of the highest number of tourist visits per capita in the world, thus the surrounding coastal habitat is subject to high anthropogenic seasonal stress. Studies on early stages of fishes have observed responses to coastal chemical cues for the location or avoidance of habitats. However, the potential interferences of human impacts on these signals are largely unknown. A choice chamber was used to determine water type preference and behaviour in naïve settled juvenile gilt-head sea bream (*Sparus aurata)*, a temperate species of commercial interest. Fish were tested individually for behavioural changes with respect to water types from potential beneficial habitats, such as seawater with extract of the endemic seagrass *Posidonia oceanica,* anthropogenically influenced habitats such as water extracted from a commercial and recreational harbour and seawater mixed with sunscreen at concentrations observed in coastal waters. Using a Bayesian approach, we investigated a) water type preference; b) mean speed; and c) variance in the movement (as an indicator of burst swimming activity, or “sprint” behaviour) as behavioural descriptors with respect to water type. Fish spent similar percentage of time in treatment and control water types. However, movement descriptors showed that fish in sunscreen water moved slower (98.43% probability of being slower) and performed fewer sprints (90.1% probability of having less burst in speed) compared to control water. Less evident increases in sprints were observed in harbour water (73.56% more sprints), and seagrass (79.03% more) in comparison to control water. When seagrass water was tested against harbour water, the latter elicited a higher number of sprints (91.66% increase). We show that juvenile gilt-head seabream are able to react to a selection of naturally occurring chemically different odourscapes, including the increasingly important presence of sunscreen products.

Introduction

Approximately half the world’s population lives within 100 km of the coast, a figure likely to double by 2025, the Mediterranean Sea is undergoing a ‘‘basin-wide urbanisation process”, with more than two-thirds of the Mediterranean coastline already developed (Benoit and Comeau, 2005). Moreover, coastal tourism and leisure activities are becoming the largest and most rapidly expanding activity in the world; in the Mediterranean, there is expected to be 264 million foreign coastal visitors by 2030, compared to 98 million in 1995 (UNWTO, 2011). Consequently, this further increases the seasonal pressure on coastal areas that already suffer from a high degree of urbanisation, such as farms, residential premises, harbours, marinas and other industrial assets (Miller, 1993). The urbanisation of the coastal zone leads to water pollution and shifts in species composition (Orams, 1997; Zahedi, 2008). The extent to which seasonal increases in anthropogenic activity affects and is influenced by environmental, economic and social change in coastal areas remains one of the central issues of research on coastal tourism (Bulleri and Chapman, 2010). Furthermore, sustainability is a leading concept influencing tourism research, focusing on the environmental dimensions of tourism in coastal and marine areas (Hall and Lew, 1998; Hering et al., 2010; Zahedi, 2008).

One relevant line of ecological research in the last decades has been devoted to understanding fish orientation and how nursery habitats, and their associated cues, are related to fisheries replenishment. Finding the way to nursery habitats may be a process of varying complexity; pelagic larvae can use a suite of acoustic, olfactory and visual sensory capabilities to detect, orient towards, and discriminate between potential settlement sites (e.g. particularly, tropical reef fish, see Leis (2006)). Fish larvae have also been known to orientate themselves using temperature, geotaxis and even celestial navigation (Leis et al., 2014; Paris et al., 2013). Once settled, the ability for fish to sense both beneficial and detrimental environments is critical to survival. Visual cues are crucial for exploration, orientation, foraging, reproduction and anti-predator behaviours (Guthrie and Muntz, 1986). However, fish are required to rely on other senses when visual stimuli are unavailable or reduced. Auditory cues from habitats, dominated by the clicks and chirps of resident crustaceans and fish, are also important in guiding directional behaviour and habitat preferences (Radford et al., 2011; Simpson et al., 2010). Chemical cues that allow for discrimination between habitat types, stem from a variety of compounds including nutrients, amino acids, proteins, lipids and other products of decomposition that are likely to have a unique chemical signature, for example mannitol from algae, lignins from seagrasses and terrestrial plants, and anthropogenic pollutants (e.g. Dixson et al., 2008; Havel and Fuiman, 2015; Kingsford and Gray, 1996). Additionally, fish are able to respond to a variety of chemical signals such as those from coral tissue and conspecifics (Lecchini et al., 2005a, 2005b), or symbiotic partners (Arvedlund et al., 1999), and consequently locate a critical nursery habitat. An aversion to disadvantageous conditions, such as pollution, or a selection for optimal conditions as seen in attraction to refuge, and prey cues, will enhance an individual’s chances of reaching sexual maturity (reviewed by Kingsford et al., 2002).

While many previous studies have been successful in determining the use of olfactory cues in tropical fish larvae (e.g. Dixson et al., 2008; Gerlach et al., 2007, among others), there is a distinct lack of literature regarding temperate species (Radford et al., 2012). Additionally, juvenile response to chemical cues is under-researched in comparison to larval phases despite the importance of juvenile survival in contributing to adult populations (e.g. Nash and Geffen, 2012). The aim of this study is to advance knowledge in the use of chemical cues in a temperate post-settled coastal juvenile fish and evaluate the effects of different water types on behaviour as an indicator of habitat choice. Gilt-head seabream (*Sparus aurata* (Linnaeus, 1758): Sparidae) was the selected temperate species since it is widespread in Europe and it has an important economic role in both commercial and recreational fisheries, as well as in aquaculture (Brown et al., 2005). In addition, *S. aurata* uses littoral zones as nursery habitats, including coastal lagoons (Arias, 1976), and it is frequently detected both over seagrass meadows and highly anthropized areas (Francour, 1997).

A recent series of studies have drawn attention to the effects of sunscreen products on marine organisms. Tovar-Sánchez et al., (2013) investigated sunscreen effects on phytoplankton around beaches of Majorca (Balearic Islands, NW Mediterranean), inferring adverse effects on growth of marine phytoplankton at certain (observed) concentrations. However, the effect of this pollutant on fish behaviour has to be ascertained. In Majorca Island, juvenile *S. aurata* are found inside bays and beaches frequently polluted by sunscreen products, although the effect of these chemicals is largely unknown. In addition, Sánchez-Quiles and Tovar-Sánchez (2015) showed an adverse effect of sunscreen pollutants on the seagrass *Posidonia oceanica*, which is crucial as nursery habitat for several sparids (Francour, 1997; Harmelin-Vivien et al., 1995). *P. oceanica* is endemic to the Mediterranean Sea and plays an important role in sediment stabilization, primary productivity, the provision of a physically complex habitat for fish and invertebrates and, offering shelter for juvenile fish species to avoid predation and locate suitable prey (Orth et al., 1984). It is, therefore, plausible that *P. oceanica* meadows elicit chemical cues that play a role in habitat selection by juvenile fish. Finally, the large amount of marinas and harbours in Majorca Island (30 harbours which, together with wavebreakers, occupy 17% of its 725 km coastline), with summer peak activities that have tripled since 1997 (Ibestat 2016 at http://www.ibestat.es, last accessed October 10, 2016), are likely to change the chemical environment of many coastal areas used by this juvenile species.

In this study we analyse the behavioural response of juvenile gilt-head seabream to several types of chemical cues. By using a flume-based design based in previous works (e.g. Atema et al., 2002; Gerlach et al., 2007), we adopt a Bayesian approach to the analysis of fish movement that enables the study of behavioural responses to water type; these responses can be compared by replicating water conditions found in less human-impacted habitats such as *P. oceanica* meadows, and polluted ones such as harbours and visitors-dense beaches.

Materials and methods

Experimental design and trials

The choice chamber (henceforth “flume”) design was scaled up from Atema et al., (2002) to account for the size of post-settled juveniles (Figure III.1). The flume design has been found successfully elicit a response when testing for chemical cues based on vegetation, conspecifics or other symbiotic organisms, both in freshwater, seawater and in a wide variety of species (e.g. Fabian et al., 2007; James et al., 2008). Previous studies using larval fish obtained from a natural environment showed that individuals exhibited a preference for the water type most similar to the habitat in which they were found (Gerlach et al., 2007). On the other hand, studies conducted using various tropical reef fish species determined that naïve individuals, raised in aquaculture, were less likely to display a previously distinguished preference for habitat choice (Munday et al., 2009; Radford et al., 2012). In the present study, 110-day-old naïve juvenile gilt-head seabream (*Sparus aurata* (Linnaeus, 1758): Sparidae) coming from a reduced group of broodstock of Mediterranean strain were obtained from an aquaculture station (Aquicultura Balear, CULMAREX, Balearic Islands, Spain) where they were hatched and raised at a controlled constant temperature of 20°C and salinity of 37.0. They were transported to the Laboratory of Marine Research and Aquaculture (LIMIA) from the Balearic Government (Port d’Andratx, Balearic Islands, Spain), for the experimental trials. All individuals were acclimatised in control seawater (T=22°C ± 0.5°C, salinity = 36.8 ± 0.01 through the experimental period) for two weeks prior to performing the tests and therefore individuals were naïve to any distinct chemical cues that could affect the choice preferences. All procedures were carried out in strict accordance with the recommendations from Directive 2010/63/UE, adhering to Spanish law (RD53/ 2013, BOE n. 34 February 8th 2013).

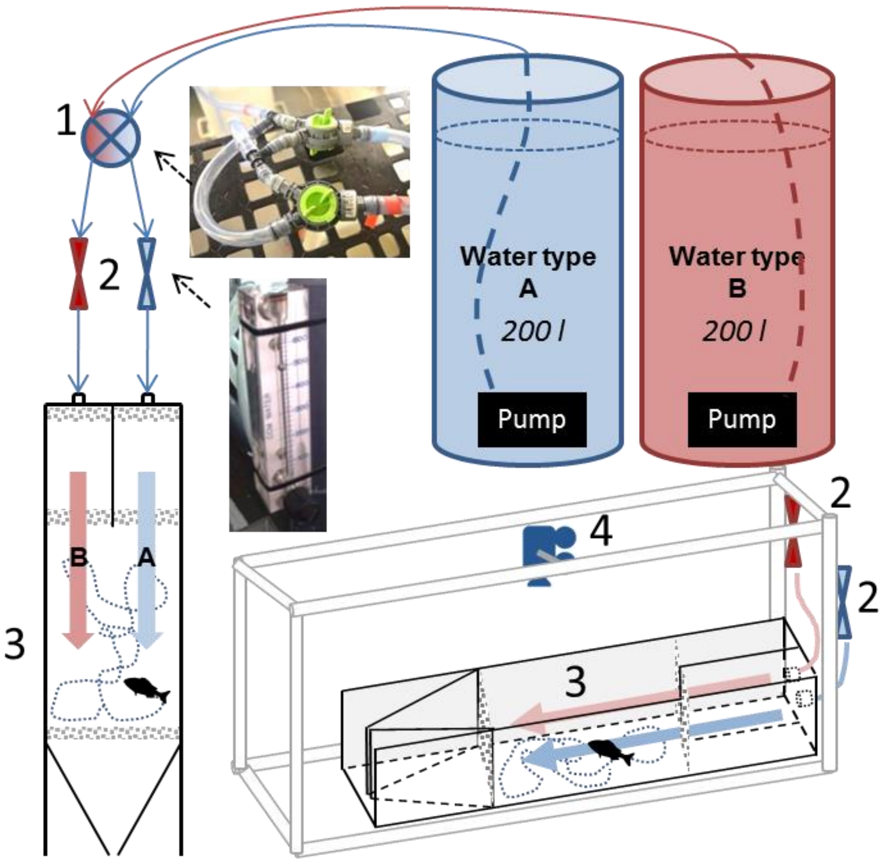

Figure III.1. Conceptual use of the flume accompanied with actual pictures of the components. Water types A and B were contained in 200 L deposits with aeration, and the system was fed using two aquaria pumps. (1), Water type side exchanger; (2) Flowmeters set at 500 cm^{3}min^{-1}; (3) Choice chamber arena (2D and 3D views); (4) Video setting.

The flume was designed to allow a single fish to swim between two water types. Each flume channel was continuously fed with 500 cm^{3}min^{-1} achieved by equipping the structure with flowmeters (Omega Engineering) attached to aquaria pumps placed in two 200 L tanks of water, containing the different water types being tested. Inert artificial sponges were placed at the entrance of the water channels, acting as collimators, providing the laminar flow required to generate a stable sharp boundary between water masses (see below). Similar sponges were also placed at the rear of the flume, creating a rectangular arena (16 x 25 cm size and 5 cm of water column) to record fish movements. To ensure a sharp separation between each pair of water masses being tested, a dye test was performed every day at the beginning of the trials and every 10 individuals: an inert tracer was injected directly in the flow conducts before the flowmeter regulation using a syringe injection rubber plug (see supplementary video at http://www.fishecology.es/photo-video-gallery/ last accessed October 10, 2016). Fish were placed in the centre of the arena and exposed to five minutes of water flow, followed by a switch in the side (left or right) of water type with a further five minutes of analysis to control for a possible unaccounted preference (bias) for a given side. To prevent any reaction to shadows, as seen in McCartt et al., (1997), the flume structure was covered with a thick mesh and the room was left undisturbed throughout each test. Each test video was recorded with a commercial camera (GoPro Hero 3) that was attached centred on top of the structure parallel to the flume. In addition, a luminous signal, hidden from sight of the fish, was cued to the beginning of each test and the moment of water change, facilitating the posterior video analysis (Figure III.1). To account for acclimatisation to the flume, one minute was omitted from the start of the first five-minute period and again at the occurrence of water type change (second period). Once tested, fish were euthanized by electrical shock followed by immersion in a lethal dose of the anaesthetic tricaine methanesulphonate (MS-222). After euthanasia, length (TL, to the nearest 0.1 mm) and weight (to the nearest 0.01 g) were measured. Following this protocol five different trials were performed, each consisting of 16-29 fish measured (although initial aim was 30, some fish not moving at all or not crossing both water types had to be discarded). The treatments were as follows:

Control vs control seawater

Current seawater used in LIMIA (sand filtered and UV sterilized) was used as the control throughout comparisons and as a base for testing chemical cues, hereby known as 'control water'. The control vs control experiment (n = 16) was performed in order to detect (and incorporate into statistical models if needed) any bias due to the structural design. The tested water types were both 22.5 (± 0.1) °C and salinity of 36.8.

Seagrass vs control seawater

Previous studies utilised a variety of methods to replicate the smell of vegetation when analysing habitat preference (Havel and Fuiman, 2015; Radford et al., 2012). Dixson et al., (2008) created water "smelling" of marine and terrestrial vegetation by soaking organic matter in seawater for at least two hours before the experiment occurred. We used this approach to produce treatment water emulating seagrass bed's potential chemical cues around the island of Majorca, by soaking 25 g L^{-1} (wet weight, equivalent to 5.25 g dry weight) shoots of *P. oceanica* in control water for 12h with strong aeration to promote mixing. The sample size was n=21.

Harbour *vs* control seawater

We selected subsurface (1 m) water from Port d'Andratx, a traditional fishing and touristic village located in a small semi enclosed bay around the mouth of a creek with a seasonal discharge of freshwater that was historically a suitable nursery habitat for *S. aurata*. In the last 75 years the village has grown disproportionally and the population and boating activity (over 500 moorings) trebles in the summer months (Government of the Balearic Islands, Council Tourism). The effective sample size was n=25.

Sunscreen *vs* control seawater

The concentration of sunscreen used in our experiment mirrored the average acute toxicity concentration for the diatom *Chaetoceros gracilis* of (0.125 ± 0.071 g L^{-1}) (Tovar-Sánchez et al., 2013) which is similar to environmental relevant concentrations found in water samples during the summer (Sendra et al., 2016 in press). The sunscreen was mixed with control water and vigorously aerated throughout the experiment to reduce the possibility of insoluble particles floating on the surface and to maintain a similar concentration throughout the mixture. The brand of sunscreen used in this trial (a solar spray) was specifically determined by Tovar-Sánchez et al., (2013) as the most harmful to phytoplankton as it induced the highest level of toxicity in organisms. For legal reasons the specifics may not be disclosed and will hereby be known solely as 'sunscreen'. The effective sample size was n = 26.

Harbour *vs* seagrass seawater

Finally, as *P. oceanica* meadows are one of the most abundant nursery habitats around the island of Majorca, naïve juvenile *S. aurata* behaviour was tested in the flume containing harbour water and water treated with *P. oceanica* of the same concentrations as above. The effective sample size was n = 29.

Video and statistical analysis

We developed a protocol for behavioural analyses using exclusively publicly available tools. First VirtualDub (v.1.10.4) was utilised to trim down each video to the exact initiation of the trial using the first frame of the luminous cue. The second luminous cue during the trial (around five minutes later) was used to calculate the second period of five minutes. One image per second of footage was obtained from each video for further analysis using the computer software ImageJ (Schneider et al., 2012). Arena coordinates were set for each video before tracking the paths of each individual, ensuring an exact match of the arena and fish motion despite possible camera movements caused by substituting the storage card or batteries between the trials. Mtrack2 plugin of ImageJ was used to follow the fish throughout the video. Tracks of fish and arena coordinates were subsequently used in a customized script of R software (www.r-project.org, 2015) to i) visually assess the quality of the data and ii) obtain the metrics required throughout the analysis, accounting for change in water type side and acclimation time.

Bayesian methods were used for calculating both proportion of time spent in each side of the flume (preference of water type) and behavioural response of each individual in each of the water types to provide a consistent analytical scheme. Concerning the proportion of time spent in each side, input data was the count of frames an individual fish *i* was at a given side (C_i) in a given number of snapshots (N_i). The count C_i was assumed to come from a binomial distribution:

$$C_i \sim \text{Binomial}\,(p_{\text{i}}, N_i)$$

Between-fish variability was accounted for assuming that the (logit-transformed) probability for a given fish (p_i) was normally distributed around the average value (P_j) of the treatment *j* (each experiment):

$$\text{Logit}\,(p_i) \sim \text{Normal}\,(\text{Logit}(P_j), \sigma)$$

Flat priors (normally distributed priors with zero variance and 10^{-6} tolerance) were assumed for Logit (P_j). In order to ensure temporal independence between successive observations, the same analysis was carried out taking one frame per second and one frame each five seconds.

Behavioural response was quantified by means of the mean speed and its variance within each water type. Mean speed was expected to vary due to the presence of chemical cues that individual fish could detect, meanwhile the variance of the speed was expected to inform on the existence of an increasing number of bursts in speed or accelerated speed movements (*sprints*) as a response to entering a given treatment water. Preliminary histograms of speed distribution of the same fish in the same type of water strongly suggested a gamma distribution. Accordingly, the speed at a given moment (t) of the fish *i* ($S_{t,i}$) when it is inside a given water type (either at control or treatment *water*) is given by:

$$S_{t,i,water} \sim \text{Gamma}\,(shape_{i,water}, rate_{i,water})$$

where *shape* and *rate* are the two parameters of the gamma distribution. The mean and variance of the distribution are related with *shape* and *rate* by closed expressions:

$$\text{shape}_{\text{i,water}} = (\text{mean}_{\text{i,water}} / \text{sd}_{\text{i,water}})^2$$

$$\text{rate}_{\text{i,water}} = \text{mean}_{\text{i,water}} / \text{sd}_{\text{i,water}}^{\;2}$$

Means ($\text{mean}_{i,j,water}$) and standard deviation ($\text{sd}_{i,j,water}$) of each fish (*i*) were assumed to be normally distributed around a treatment (*j*) average ($MEAN_{j,water}$ and $SD_{j,water}$) for dealing with fish specific responses to the same treatment.

$$mean_{i,j,water} \sim \text{Normal}\,(MEAN_{j,water}, \sigma M_{j,water})$$

$$sd_{i,j,water} \sim \text{Normal}\,(SD_{j,water}, \sigma SD_{j,water})$$

Flat priors were assumed for $MEAN_{j,water}$ and $SD_{j,water}$. Finally, in order to facilitate between-treatments comparisons, treatment-related effects were expressed as control minus treatment differences (see Figure III.2, a and b; for a schematic view of the work flow):

$$\Delta\text{MEAN}_\text{j} = \text{MEAN}_\text{j,control} - \text{MEAN}_\text{j,treatment}$$

$$\Delta\text{SD}_\text{j} = \text{SD}_\text{j,control} - \text{SD}_\text{j,treatment}$$

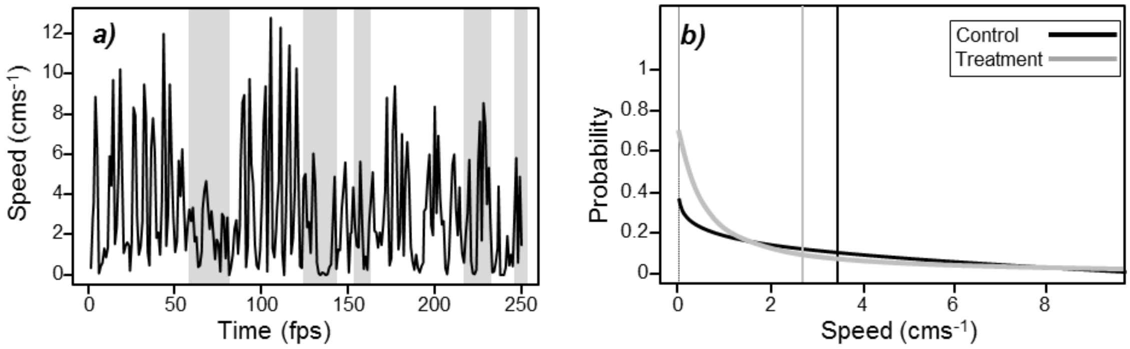

Figure III.2. Movement analysis: a) Example (real data) time series of speed variation for a given individual. Shaded in grey are the periods where the fish was inside the "treatment" water. b) Gamma distributions of the movements of the same example individual from inside the control water (black line) and the treatment water (grey line). Vertical lines depict the mean speed for both water types.

Results

Juvenile *S. aurata* did not exhibit a clear preference or avoidance for any of the water types tested in terms of time spent in each water during flume trials (Table III.1). The sensitivity analysis performed in order to prevent errors due to autocorrelation of the position in the immediate following second and the actual position (performed every 1 and 5 seconds), showed very similar results (Table III.1), and the percentage of time spent in each water type in the different trials was not differentiable from a random distribution in any of the experiments.

Table III.1. Bayesian Credibility Intervals (BCI) (median and 95%) for posteriors distributions of the probabilities of being in either one water types or the other. A median value (50%) of exactly 0.5 corresponds with no preference for being in any of the water types; median values >0.5 indicate slight preference for the Control water (or *P. oceanica* in the last experiment).

		1 second			*5 seconds*		
		Bayesian Credibility Intervals			*Bayesian Credibility Intervals*		
	n	2.5%	50%	97.5%	2.5%	50%	97.5%
Control vs Control	16	0.407	0.485	0.567	0.405	0.485	0.563
Harbour vs Control	25	0.486	0.552	0.615	0.48	0.548	0.611
P. oceanica vs Control	21	0.47	0.541	0.609	0.473	0.543	0.614
Sunscreen vs Control	26	0.433	0.496	0.559	0.429	0.492	0.556
Harbour vs *P .oceanica*	29	0.466	0.526	0.584	0.465	0.524	0.582

Despite this clear lack of preference for any of the water types, other components of fish movement showed heterogeneous results between treatments. The credibility intervals of the difference in speeds and variances (expected to increase if several bursts occur) showed that sunscreen treatment caused slower average movements with a probability of 0.98 (Table III.2, Figure III.2, a) and less bursts in speed (i.e. differences in variance) with a probability of 0.90 (Table III.2, Figure III.2, b). On the other hand, the harbour and *P. oceanica* treatments caused an increase in the amount of these sprints with a probability of 0.74 and 0.79 respectively (Figure III.2, b), but a null variation in mean speed (Figure III.2, a). The last experiment, comparing *P. oceanica* vs harbour water, had a similar mean speed in both water types but remarkably more sprints (P = 0.92) in the Harbour water (Table III.2, Figure III.2, b). However, the latter results should not be compared with the other experiments as they did not use the same control water as a baseline.

Table III.2. Bayesian Credibility Intervals (BCI) (median and 95%) for posteriors distributions of the probabilities of having differences in speed between water types. A BCI including fairly the value 0 means no difference in the speed distributions. An * indicates a 95% probability of difference. See Figure III.3 for a graphical representation of the probability distributions of these differences in both speed and variance of the speed for the five first experiments that use control water as baseline.

		Differences in means				**Differences in variance**			
		Bayesian Credibility Intervals				*Bayesian Credibility Intervals*			
	n	2.50%	50%	97.50%	*Probability*	2.50%	50%	97.50%	*Probability*
Control vs Control	16	-0.863	-0.11	0.465	0.36 > 0	-0.286	0.015	0.319	0.55 > 0
Harbour vs Control	25	-1.343	-0.082	0.662	0.42 > 0	-0.371	0.149	0.549	0.74 > 0
P. oceanica vs Control	21	-1.633	-0.084	0.722	0.43 > 0	-0.385	0.21	0.711	0.79 > 0
Sunscreen vs Control*	26	-2.152	-0.739	-0.064	0.98 < 0	-0.811	-0.305	0.165	0.90 < 0
Harbour vs *P. oceanica*	29	-0.509	0.064	0.515	0.6 > 0	-0.076	0.16	0.385	0.92 > 0

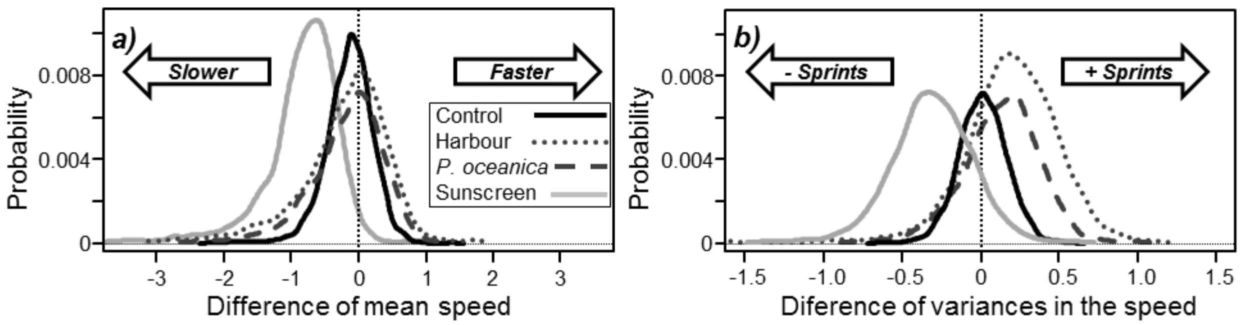

Figure III.3. a) Probability distributions of the results from comparing the differences between the means of the speed in each water type including all the individuals in the four experiments. b) Probability distributions of the difference in the variance contained within the movements (sprints or burst in speed) for all the individuals in the four experiments.

Discussion

The present results prove that i) behavioural responses can be detected within typical flume designs only if appropriate statistical analyses are performed and ii) fish reactions to chemical cues can differ in direction. We found that juvenile *S. aurata* displayed no preference for a determined water type in comparison to control water with regard to the length of time spent in each treatment water type. However other behavioural responses of the naïve individuals differed when exposed to harbour, seagrass and sunscreen treatments. Differences in both mean speed and the variance (SD) in individual fish movement provided a proxy for the behavioural response of juvenile *S. aurata* for each of the water types tested. The reaction of the juveniles varied from slight increments in the sprints (i.e. a higher variance in the speed or an increase in the number of faster movements) in the harbour and seagrass treatments to the opposite reaction: a reduction both in mean speed and sprints in sunscreen treated water.

Changes in speed as described above can be interpreted as positive or negative responses towards a stimulus and therefore interpreting the observed differences in movement patterns with respect to their ecological function and relevance remains challenging. For instance, faster movements within a given water mass can be the consequence of perceiving a risk (e.g. predator fields may be more associated to particular water masses) or excitement due to presence of food. This was the case of, for example, Harbour waters and *P. oceanica*, which both elicited higher number of burst swimming episodes. Equally, slower movements of a fish may be interpreted as a response to stress with the extreme end point being motionlessness or may signify that the animal is calm and therefore less stressed. To conclusively evaluate the root causes of these responses more sophisticated experimentation would be required that also monitor the physiological state of the animal. As such a conclusive interpretation of the presented results is not possible. Nevertheless, it is shown that juvenile *S. aurata* do respond to chemical cues, and that coastal leisure activities induce behavioural responses and therefore we attempt a cautious interpretation focusing on the most plausible explanations for the observed differences in movement patterns.

Despite the importance of *P. oceanica* meadows as one of the main nursery habitats in the Balearic Islands, juvenile *S. aurata* displayed a weak reaction to the chemical cue of this seagrass since only differed with slightly more sprints observed during the trials while in *P. oceanica*. In the face of existing information, one likely explanation is that *P. oceanica* treatment is a combination of several chemical cues that inform of a beneficial habitat, thus potentially activating the fish exploratory behaviour linked to foraging. Kolkovski et al., (1997) explored the behavioural response of *S. aurata* larvae to prey chemical cues and determined that foraging success is heavily influenced by olfactory stimuli and the attraction of larvae to secretions by prey organisms. Many previous studies have explored the role of food provision in the attraction of fish to specific habitats such as the quantities of dissolved amino acids that commonly provide fish with chemical cues to food (Ishida and Kobayashi, 1992; Wright et al., 2005). This ability may be innate in juvenile *S. aurata* as they displayed higher activity while in *P. oceanica* water*,* and seagrass is likely to emanate cues from benthic/epiphytic prey and algae, despite being removed from natural habitat (Dixson et al., 2008).

The use of naturally protected bays, coastal lagoons and estuaries for establishing ports, harbours and marinas directly interfere with their role as nursery habitats, which are essential for *S. aurata* to progress from the juvenile stage (Arias, 1976). Furthermore Sánchez-Lamadrid (2004) examined patterns of reared *S. aurata* distribution after release, and observed that juveniles avoided areas with high levels of organic pollution from nearby settlements. Marine port activities degrade water quality due to a variety of sources including the excretion of antifoulants from painted boat hulls, wastewater and oil contamination from boats, freshwater runoff from the development of harbour property, and dredging. These factors are usually harmful to marine life, ecosystems and human health (Herz and Davis, 2002). However, our study showed that *S. aurata* did not actively avoid the water extracted from Port d'Andratx, but displayed an increase in burst swimming episodes within this water. The reasons behind this reaction is unknown, yet harbours not only generate pollution cues but are often rich in inorganic nutrient inputs that stimulate primary production in the oligotrophic waters of the Mediterranean Sea, representing a significant and previously overlooked nutrient source for nearshore marine environments (Vaulot et al., 1996). The latter might imply that if pollution levels are tolerable, these areas might be seen as refuges for growth: recent works have shown that harbours in the Mediterranean Sea may be suitable nursery areas for certain sparids (Bouchoucha et al., 2016) and dusky groupers (Mercader et al., 2016). Moreover, when compared with control water, the harbour collected water had a similar effect as the seagrass water (increase in the sprints). Interestingly, when seagrass and harbour water were jointly offered as a choice (Experiment V), the number of sprints increased when exposed to harbour water. Whether this reaction to harbour water has a potential adaptive value should be clarified through further experimentation that allow for the inclusion of both visual and chemical cues.

When people bath in the sea with sunscreen lotion, sunscreen compounds have been demonstrated to release organic or inorganic UV-filters such as benzophenones and titanium dioxide nanoparticles into the surrounding water (Tovar-Sánchez et al., 2013). How these sunscreen compounds affect fish and other marine fauna has thus far not been researched in great detail, despite the yearly expansion of the sunscreen market (Sánchez-Quiles and Tovar-Sánchez, 2015). Past research has thus far mostly focused on bioaccumulation of organic UV filters in a model fish species e.g. Zebra fish, *Danio rerio* (Blüthgen et al., 2012; Gago-Ferrero et al., 2013), or concentrations in different parts of the ecosystem (Balmer et al., 2005). However, the behavioural effect of sunscreen on fish species is not as widely researched (but see Hannan et al., 2015) and therefore the reported changes of slowing swimming speeds to sunscreen concentrations mirroring those found in small enclosed bays with a high number of beachgoers is a new finding. The value of our results cannot be interpreted in terms of consequences for fish populations or nursery value, but show clearly that observed sunscreen concentrations do induce behavioural reactions.

We offer a rigorous methodological approach to analyse behavioural responses, which is not only based on the calculation of percentage occupancy at arbitrary times and that does not fall into the pitfalls of these experiments pointed out in a recent review (Jutfelt et al., 2016 in press), and furthermore, demonstrated that behavioural responses may not be detected with conventional analyses. Variation in manoeuvre speed during flume tests, an aspect that has not been explored by other studies (e.g. Atema et al., 2002;

Gerlach et al., 2007, but see Gouraguine et al., 2016 in press), needs to be taken in account since response to contrasting water types go beyond differences in occupancy time. While we provided tentative explanations for observed variance in mean speeds more research will be needed to consolidate these interpretations. Henceforth, we advocate that behavioural responses to different chemical cues of habitats, at different life stages, need to be further explored in temperate fishes in order to expand the knowledge of how essential habitats are affected by human activities. Pre-conceived ideas on effects of anthropogenic pollution on fish ecology may not hold if combined cues and life-stage adaptations are explored on a species-specific basis. Further work should be directed to combined experiments of chemical, visual and sound cues at different life stages, and to evaluate these effects in the nature.

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Chapter IV: The smell of fear induces reversible morphological changes in juvenile gilthead seabreams (*Sparus aurata*)

This chapter is under review in:

Carlos Díaz-Gil, Josep Alós, Pablo Arechavala-Lopez, Miquel Palmer, Inmaculada Riera-Batle, Amalia Grau, Ignacio A. Catalán. Submmitted to *Marine Ecology Progress Series.*

Abstract

Chemical cues from predators induce a range of predator-induced morphological defences (PIMDs) across taxa. However, the mechanisms, consistency, direction and adaptive value of PIMDs are still poorly studied, especially in marine species. We tested if predatory cues can induce changes in the body shape of the marine fish species *Sparus aurata* by exposing naïve juveniles to the olfactory stimulus of a natural fish predator. We tested our hypothesis using a nested replicated before-after-control-impact experiment, including recovery (potential reversibility) after the cessation of the predator stimulus. Differences in the size-independent body shape were explored using landmark-based geometric morphometrics and analysed through multivariate morpho-spaces. Results demonstrated that individuals exposed to a predatory cue presented deeper bodies and longer caudal regions, in agreement with previous empirical evidence in freshwater and following theoretical adaptive expectations. However, these plastic responses were reversible after withdrawal of the stimulus and individuals return to average body heights suggesting an energetic trade-off of maintaining deeper bodies. The mechanisms, consequences and ecological advantages of PIMDs observed in our study for marine juvenile fish are discussed.

Introduction

Phenotypic plasticity in morphological traits is a defence mechanism that preys across different taxa use to increase resilience to predation (reviewed in Benard, 2004 and Bourdeau and Johansson, 2012). In fish species, there are many examples of predator-induced morphological defences (PIMDs) in response to the fear induced by predators. Reported cases of PIMDs have been mainly focused on the increased body depth of freshwater species such as crucian carp (*Carassius carassius*) (e.g., Brönmark and Miner, 1992; Brönmark and Pettersson, 1994; Chivers et al., 2007a) and Eurasian perch (*Perca fluvialitis*) (Eklöv and Jonsson 2007). The ecological and adaptive values of PIMDs have been interpreted in terms of decreasing predation success by selecting for higher individuals to avoid the predator's maximum mouth gap (Bourdeau and Johansson 2012). Other works have demonstrated enhanced body components of the external morphology in order to protect from predation such as the size of the defensive spines in the pumpkinseed sunfish (*Lepomis gibbosus*) (Januszkiewicz and Robinson 2007) or the increment in the number of bony lateral plates and spines in the three-spined stickleback (*Gasterosteus aculeatus*) (Ab Ghani et al., 2016). Moreover, these PIMDs may have some less expected morphological consequences, such as those demonstrated in juveniles of a coral reef damselfish (*Pomacentrus amboinensis*), which presented an increment in the ratio between the eye and false eye or eyespot supposedly to confuse predators (Lönnstedt et al., 2013). Therefore, there is substantial evidence of phenotypic plasticity in morphological traits trough different PIMDs expressed in the presence of predators.

PIMDs can be caused either by the piscivorous predators signals (Nilsson et al., 1995) but also the conspecific alarm pheromones (Magurran et al., 1996; Stabell and Lwin 1997) released into the water when a predator attacks and tears off the skin of the prey (Chivers et al., 2007b; Chivers et al., 2007a) among other stressors. Moreover, morphological changes in fish can be also produced by environmental stressors such as hypoxia events (Sollid et al., 2003; Scarabotti et al., 2011) or human actions over the fish populations through selecting certain morphotypes while fishing (Jørgensen et al., 2007; Alós et al., 2014) or through aquaculture practices (i.e. selection of inbreeding lines for a better production and stocking conditions). However, many basic questions remain to be solved on the PIMDs, not only the mechanisms of action and adaptive value but also other relevant issues such as their reversibility (Bourdeau and Johansson 2012).

In addition, the study of PIMDs in marine fish species has been traditionally underappreciated and most of the literature is restricted to freshwater species. Improving our understanding on PIMDs in the marine realm may have profound consequences for better understanding long-standing problems, spanning from a better knowledge on the predator-prey dynamics (Preisser et al., 2005) which can help disentangling complex reproductive systems (Lowerre-Barbieri et al., 2016), to evolutionary reasons for fish plasticity (West-Eberhard 2005) or even potential application of human-induced PIMDs to restocking of fish in the wild (Gil et al., 2015).

In this study, we tried to fill the following two gaps regarding PIMDs: the poor knowledge of PIMDs in marine taxa, and the reversibility of such PIMDs. As a case-study, we focus on juveniles of a temperate fish species of interest for fisheries and aquaculture, the gilthead sea bream (*Sparus aurata*).

Material and methods

Experimental design and treatment preparation

A total of 500 juveniles of *S. aurata* of 55 days post-hatching (dph) were obtained from a wild-type broodstock from a local aquaculture station (Aquicultura Balear S.A.U., CULMAREX Group, Balearic Islands, Spain). Fish were transported to the laboratory in specific transport tanks with controlled temperature and oxygen (transport duration: less than 1 h). Individuals were then acclimatised in controlled seawater conditions (T = 20 °C ± 0.5 °C, salinity = 36.8 ± 0.01, natural photoperiod) in six 100 L tanks with open water recirculation, containing 70 individuals each. After two weeks, the experiment begun by randomly defining three tanks as control and the other three as treatment tanks with no difference in fish size distribution. The rest of the individuals were kept in a similar tank for other procedures.

The predatory cue water for the treatment group was obtained combining the odour of conspecifics exposed to predation and the essence of the conspecifics skin. The combination of both chemical cues has been demonstrated to maximise the strength of predator-induced changes in previous experiments on freshwater species (Stabell and Lwin 1997). The odour of conspecific exposed to predators was obtained using 20 individuals of wild black scorpionfish (*Scorpaena porcus*) (mean total length 10 ± 2 cm), catalogued as a piscivorous predator on *S. aurata* at seagrass meadows (*Posidonia oceanica*) (Deudero et al., 2008). Wild *S. porcus* specimens were captured using a not shredding experimental beam trawl designed to fish over seagrass meadows (Catalán et al., 2014). *S. porcus* individuals were maintained in captivity in an oxygen-saturated open flow aquarium (80 L) and were fed daily with 5 *S. aurata*. After ten days, the water flow was turned off and 20 *S. aurata* juveniles were introduced into the aquarium. About 24 h later and ensuring that *S. porcus* had fed over all the sea bream, a total of 12 L was extracted from the aquarium, filtered and used for the mixture of predator odour preparing 150 mL individualised doses. To extract the odour of conspecifics the skin of 20 euthanised *S. aurata* (approx. 10 g) were dissected with a scalpel, homogenised in 3 L of seawater and finally filtered. Then, 50 mL aliquots were incorporated to the 150 mL of the water with conspecific and predator water described above and frozen at -20ºC in 200 mL plastic bags (a total of 60 individual doses). Other 60 ice cubes were simultaneously made with seawater to be used as controls.

The experiment was divided into two phases (months): during the first month, the water flow of each tank was turned off at 10:00 AM daily. One of the ice cubes of 200 mL (control or predator water) was incorporated into the six tanks every working day while the water flow remained off. After 4 hours, the water flow was turned on again to ~4 L H^{-1}. This represented a total of 20 doses per tank during the first month. During the second month, the fish were kept under equal conditions without adding any olfactory cue (ice cubes). Fish were fed equally with commercial food pellets for sea bream juveniles (1.5 mm, Perla MP, Skretting Spain S.A.) during all the experiment at the recommended doses for this life stage. All procedures were carried out in strict accordance with the recommendations from Directive 2010/63/UE, adhering to Spanish law (RD53/ 2013, BOE n. 34 February 8th 2013) approved by the CEEA-UIB (Animal

Experimentation Ethical Committee from the University of the Balearic Islands, n. CEEA 01-11-13) ensuring good practises as reduction of the number of individuals and general fish welfare.

Statistics and morphology analysis

According to a before-after-control-impact design, including recovery after the cessation of the impact (predator stimulus), the six tanks were sampled 3 times: firstly, at the beginning of the experiment (starting up without any treatment); secondly one month after being exposed to the olfactory cue (3 tanks under control, 3 tanks under treatment); and finally, at the end of the second month after being the last month without cue exposure. At each sampling time, 10 fish per tank were randomly sampled (total of 30 control individuals and 30 treated individuals each sampling time) and euthanised using an overdose of anaesthetic MS-222. All the morphometric analyses were conducted separately for each sampling time. Fish were measured (total length to the nearest mm) and weighed (eviscerated weight to the nearest mg) after taking a standardised photograph (see below). Fish total length, eviscerated weight and Fulton condition index ($K=100\cdot Weight/Length^3$) were compared along the experiment using Linear mixed-effect model (LMM) with Satterthwaite approximations to degrees of freedom using *lmer* function from *lme4* package (Bates et al., 2015) in R software (R Development Core Team and Team 2011), to include the random effects of the different tanks. In all the cases the assumptions of the model were checked and data natural log-transformed if necessary and, prior to the analyses, a few outliers were removed to select proper size groups within each sampling date (Zuur et al., 2010)

In addition, standardised photographs were taken on every sampled fish for geometric morphometric analyses (Nikon D3100, from the exact same fixed height and including a ruler for proper calibration in all the cases). The body shape of each individual was analysed using the landmark-based method (Rohlf and Marcus 1993). A total of 10 homologous landmarks were selected based on a previous study with this species (Verhaegen et al., 2007) (see Figure IV.1). The *xy* coordinates of these landmarks of each individual were acquired from a lateral (left side) photograph of the fish using the tpsDig2 software (Rohlf 2004). The raw coordinates were superimposed using General Procrustes Alignment (GPA) as implemented in the function *gpagen* from the *geomorph* library in R (Adams and Otárola-Castillo 2013). Afterwards a multivariate linear regression via Procrustes distances ANOVA (Goodall 1991) was performed on the superimposed coordinates used as shape descriptors using a residual randomization permutation procedure to evaluate the observed sum-of-squared of Procrustes distances (Collyer et al., 2015). We specifically used *procD.allometry* function which accounts for the confounding effect of the fish size (included as the natural logarithm of the centroid size) on the body shape (allometry) (Adams et al., 2013; Adams and Otárola-Castillo 2013). This allowed us to visualise and remove the effect of the different sizes on the body shape, a widespread property of organisms (Adams et al., 2013), and focus on the effect of the treatment. Finally, the mean size-adjusted residuals (i.e. allometry-free shapes) of the body shapes from the Procrustes ANOVA were derived for the control and treatment mean individuals and represented showing the vectors displacements between the reference (control) and the target specimens (treatment). These vectors were magnified in all the cases x20 to aid the visualisation of the encountered differences.

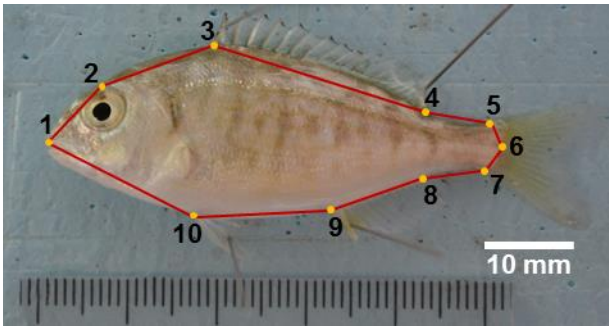

Figure IV.1. Standardised photograph of a juvenile *S. aurata* (100 dph old) displaying the resultant body-shape line (red line) from the landmark configuration: 1) The anterodorsal tip of the premaxillary; 2) The posterior border of the skull; 3) The base of the first spine of the dorsal fin; 4) The base of the last soft ray of the dorsal fin; 5) The dorsal onset of the caudal fin; 6) The mid lateral posterior edge of the peduncle; 7) The ventral onset of the caudal fin; 8) The base of the last ray of the anal fin 9 The base of the of the first ray of the anal fin and; 10) The base of the pelvic fin.

Results

Prior to the analyses, a few size outliers were removed to select proper size groups. At the first sampling time, selected length (TL) range varied between 20 and 40 mm (N= 56, 28 control and 28 treatment); at the second time TL ranged 40 to 60 mm (N= 53, 27 control and 26 treatment); and at the third sampling time TL ranged from 50 to 70 mm (N= 49, 26 control and 23 treatment). *S. aurata* juveniles presented similar growth patterns, in terms of length, weight and Fulton´s K along the experiment (Table IV.1, Figure IV.2), and no differences were detected due to either the treatment (non-significant effect of the treatment and the interaction between treatment and date of sampling) or the experimental tanks within-treatment (Table IV.1, Figure IV.2).

Table IV.1. Results of the linear mixed-effect models for Total length, Weight (log transformed) and Fulton condition index (K). Interaction model fitted using treatment (Control/Treatment) and sampling date (1st, 2nd, and 3rd) as fixed effects. Est.: estimate value; S.E. Standard error of the estimate; Pr(>|t|); p-values via Kenward-Roger approximation. In bold the significant p-values. The six different tanks were used as random effects of the model. $\tau_{00,\,tank}$ is the between tanks variance and σ^2 is the within each tank variance (Residuals).

	Total length (mm)			*Log (Weight)*			*Fulton's K*		
	Est.	*S.E.*	*Pr(>|t|)*	*Est.*	*S.E.*	*Pr(>|t|)*	*Est.*	*S.E.*	*Pr(>|t|)*
Fixed Effects									
(Intercept)	18.7	1.402	**<.001**	-2.214	0.121	**<.001**	-0.774	0.086	**<.001**
Treatment	0.227	1.972	0.909	0.053	0.17	0.756	0.056	0.121	0.644
Sampling date	14.281	0.669	**<.001**	1.12	0.058	**<.001**	0.795	0.041	**<.001**
Treatment:Date	-0.503	0.931	0.591	-0.043	0.08	0.589	-0.037	0.057	0.523
Random Effects									
$\tau_{00,\,tank}$		<0.001			<0.001			<0.001	
σ^2		22.656			0.168			0.085	
N_{tank}		6			6			6	
R^2		0.852			0.826			0.557	

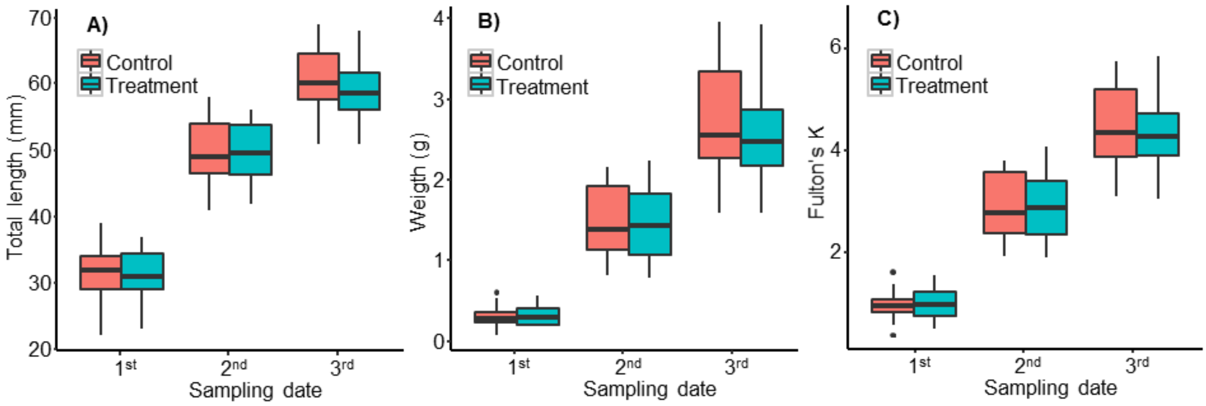

Figure IV.2. Boxplots of *S. aurata* lengths (A), eviscerated weight (B) and Fulton's K index (C) during the experiment. Boxes represent the lower and upper quartiles (25 and 75%) of the values, the horizontal line the median (50%) and the vertical lines the minimum and maximum.

The overall body shape was highly correlated with the fish size at the three sampling times, i.e. allometry was present, according to the Procrustes ANOVA on the superimposed landmark

coordinates (Table IV.2). There was a significant effect on the body shape after one month of being exposed to the olfactory treatment, reflected mainly in the dorsoventral and tail regions Table IV.2,

Figure IV.3). Thus, the size-standardized shape of exposed fish became deeper and the peduncle tip entrained a larger portion into the tail. At the end of the experiment the fish shape returned to its natural state (Table IV.2,

Figure IV.3).

Table IV.2. Results of the Procrustes ANOVA including the allometry effect on the body shape. A residual randomization permutation procedure is utilised.

Sampling	*Samples*	*Variable*	*SS*	*Rsq*	*F*	*Pr(>F)*
1st	*C= 26* *T= 26*	ln(Csize)	0.005878	0.16315	10.495	0.002**
		Treatment	0.000465	0.012918	0.831	0.566
		Residuals	0.029687			
2nd	*C= 27* *T= 26*	ln(Csize)	0.0027148	0.099932	5.7509	0.002**
		Treatment	0.0008484	0.031228	1.7971	0.046*
		Residuals	0.0236031			
3rd	*C= 26* *T= 23*	ln(Csize)	0.0008409	0.050429	2.4822	0.01*
		Treatment	0.0002508	0.015039	0.7402	0.686
		Residuals	0.0155838			

Discussion

Phenotypic plasticity in life-history, behavioural, morphological and physiological traits of prey exposed to predatory cues have been widely reported across taxa (Bourdeau and Johansson 2012). In this study, we did not find any effect of predatory and conspecific alarm cues on the growth-related life-history traits of juvenile *S. aurata* along the two months' duration of the study (from 70 to 130 dph) as revealed by the equal range of total lengths, weights and/or condition index values. We observed the common ontogenetic change (allometry) in the overall body shape consisting in a progressive widening of the body depth along juvenile stages of *S. aurata* (Russo et al., 2007). After removing allometry effects, fish exposed to predatory and conspecific alarm cues displayed deeper bodies and elongated peduncles after only one month. Moreover, this phenotypical induced change in body shape disappeared within just one more month of recovery from the treatment and individuals from both groups became indistinguishable. Therefore, we provide strong evidence for PIMDs in *S. aurata* juveniles reinforcing our hypothesis of reversible PIMDs in a marine organism.

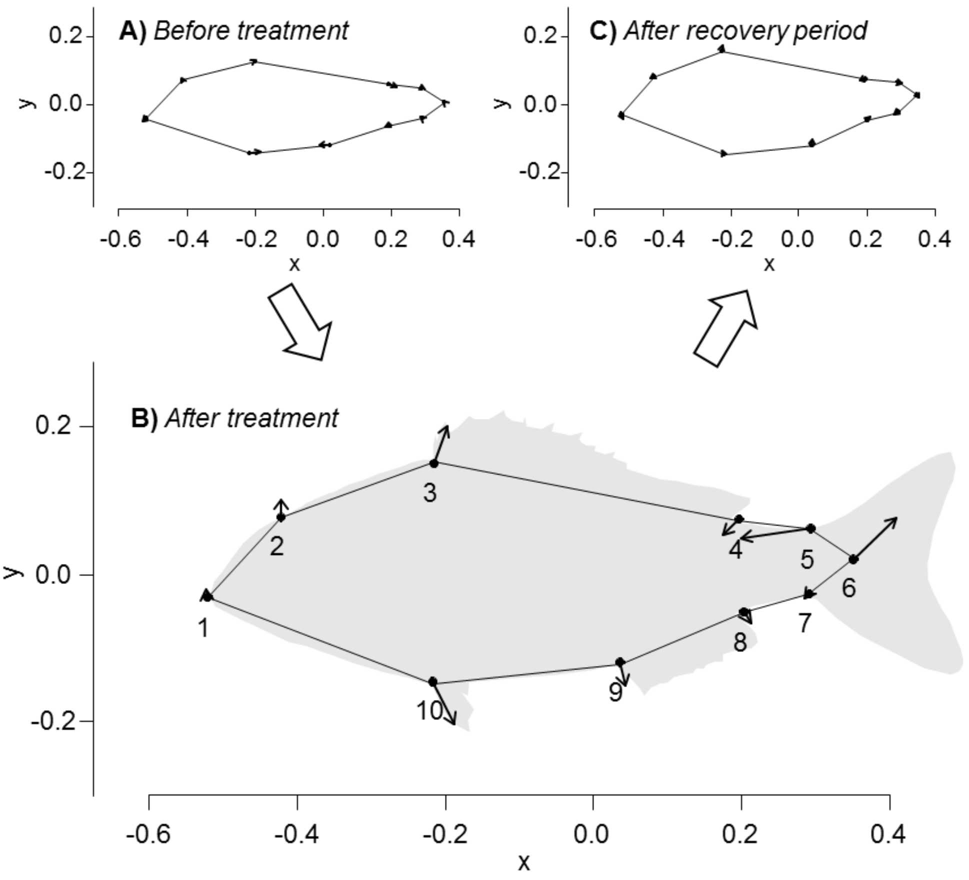

Figure IV.3. Average allometry-corrected body shapes of individuals from the three sampling dates. Vectors show the direction and magnitude (x20 to facilitate the visualisation in all the cases) of the landmarks that present differences. A) Before treatment; B) after one month of treatment; C) after the recovery period. x and y are the coordinates of the projected landmarks and vectors.

There are several examples of PIMDs induced by predatory or conspecific alarm cues in fish, mostly in freshwater species. Examples of these induced adaptations include: incrementing the number and size of anti-predator morphological defences (Januszkiewicz and Robinson 2007; Ab Ghani et al., 2016); differentiations in the body shape that include larger caudal peduncle region (Langerhans et al., 2004); enhanced body depth as we have found in juveniles of *S. aurata* (Brönmark and Miner 1992; Brönmark and Pettersson 1994; Eklöv and Jonsson 2007); or a combination of the latter two strategies (Leinonen et al., 2011). In our case, the body shape differences resulted from a combination of increased body depth (landmarks 3 and 10

Figure IV.3, B) and elongation of tail insertion muscle (landmark 6,

Figure IV.3, B). These PIMDs could be associated with ecological benefits like higher manoeuvrability in front of predators (Domenici et al., 2008), enhanced thrust and faster startle response (Langerhans et al., 2004; Langerhans 2009) or simply shift towards a predatory safe state due to gape limitation of predators (Brönmark and Miner 1992).

Many of the studies on PIMDs are conducted in fast-growing phases as tadpoles in amphibians (Schoeppner and Relyea 2009; Orizaola et al., 2012) or juvenile fish (Svanbäck and Eklöv 2002; Eklöv and Jonsson 2007; Russo et al., 2007), which may rapidly divert energy into shape changes for metabolic reasons with rather direct consequences for survival. However, these morphological differences may not be related solely to predatory presence but also ontogenetic effect directly associated with habitat or diet shift during these stages (Svanbäck and Eklöv 2002; Russo et al., 2007) or confounded by other environmental conditions as hydrodynamic constraints (Johansson and Andersson 2009). Thus, under equally controlled conditions as in the present experiment, the detection of these PIMDs in marine species may help to understand some of the variability in the recruitment dynamics by revealing anti-predation responses. Obviously, our study only represents the first step (evidence) of such predatory responses in marine environment, but we contend that it opens novel opportunities for understanding settlement and recruitment dynamics, and certainly on how selection forces may not only depend on mutation but also on the high phenotypic plasticity (West-Eberhard 2005), which is particularly high in fish (e.g. Pittman et al., 2013).

Whether these PIMDs are reversible or not is much less studied. Macroscopic reversible morphometric changes have been observed as a response to external environmental stressors in characiform fishes in Argentinian floodplains (Scarabotti et al., 2011) and microscopically in crucian carp gills (Sollid et al., 2003), both as an adaptive response to hypoxia events. However, the capacity of revert a strong macroscopic morphological change induced by predatory/conspecific cues was earlier suggested plausible for crucian carp (Brönmark and Pettersson 1994). However, the reversibility of the PIMDs has only been described in tadpoles (Schoeppner and Relyea 2009) and *Carassius auratus*, a freshwater fish (Chivers et al., 2007b). Therefore, we provide novel findings in the plastic nature of PIMDs. Furthermore, there is an energetic cost associated with these morphological changes that may only be possible at certain stages/physiological status (Pettersson and Brönmark 1999; Ferrari et al., 2010). In our work, juvenile *S.*

aurata exposed to predator cues grew at the same rates as control fish revealing an inherent morphological change associated with the energetic demand. The present experiment was performed at the same density and food supply conditions for all the tanks, although in conditions where food is limited due to high densities the energetic cost associated to PIMDs should be compensated with a reduction in growth associated rates (Pettersson and Brönmark 1997). Whether only one trait or both (deeper body and longer tail peduncle) have adaptive value, or whether they are correlated due to metabolic constraints, remain as open questions. On the other hand, morphological changes may not be possible while shifting the allocation of energy from somatic to gonadal growth during maturation, and therefore, the capability of reversing the body shape towards the standard morphotype may be species and stage specific (Relyea 2003; Chivers et al., 2007b; Ferrari et al., 2010).

Species-specificity and stocking conditions have profound effects when observing shape changes of fish. During adulthood, *S. aurata* present different morphotypes induced by aquaculture conditions, since morphological traits are influenced by farming conditions, broodstock origin, or applied for selective breeding programs in hatcheries (Karaiskou et al., 2009; Rogdakis et al., 2011). It was comparatively demonstrated that the majority Mediterranean gilthead sea bream producers target higher body-height individuals (Brown 2003; Hurtado-Rodríguez et al., 2010; Arechavala-Lopez et al., 2012). In fact, due to the plasticity of sea bream individuals, morphological differences have been used to determine the origin (wild or farmed) of individuals (Loy et al., 2000; Grigorakis et al., 2002; Arechavala-Lopez et al., 2012). However, they hypothesised that farmed individuals that spent a time in the wild would be difficult to recognise morphologically since they adopt wild-like shapes, suggesting the use of scales as better escapees' predictors (Izquierdo-Gómez et al., 2017). The present study confirms the reversible nature of phenotypical changes in *S. aurata* and the overall high plasticity in the morphology of this species, since body shape changes diffused towards the end of the experiment, moving backwards to the original wildtype phenotype of their progenitors.

Notwithstanding the large number of unknowns regarding the consequences of the PIMDs and reversibility of the phenomenon along development, we contend that PIMDs in *S. aurata* may be used as a new tool for restocking programs for several practical reasons: a) primarily they are deeper-bodied, which may imply a better survival in front of gape-limited predators (Brönmark and Pettersson 1994); b) deeper bodied individuals may exhibit higher manoeuvrability, increasing escaping opportunities (Domenici et al., 2008); c) the longer tail section has been related with more thrust during startle responses (Langerhans 2009), and potentially with higher escape responses; and d) this is a low-cost technique to be applied in small volumes of water. We proved that the probability of finding body shape differences due to the odour preparation treatment was severely increased after one month of treatment (only 20 doses), although it is likely that a longer treatment period, or an earlier application of the treatment, or a combination of both, would increase such differences. Henceforth, a couple of months before fingerling restocking a treatment of fear similar to this one, for example a predator cohabiting inside a mesh cage, may induce some morphological features that might increment their survival opportunities in the wild, which has been reported to be one of the keys for maximizing success of fish restocking programs (Gil *et al.,* 2015). Moreover, another possibility is to use hatchery selection programs, since deeper-body individuals could be used as broodstock for restocking programs with the aim of obtaining anti-predator enhanced individuals.

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Chapter V: Ocean acidification increases fatty acids levels of larval fish

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Abstract

Rising levels of anthropogenic carbon dioxide in the atmosphere are acidifying the oceans and producing diverse and important effects on marine ecosystems, including the production of fatty acids (FAs) by primary producers and their transfer through food webs. FAs, particularly essential FAs, are necessary for normal structure and function in animals and influence composition and trophic structure of marine food webs. To test the effect of ocean acidification on the FA composition of fish, we conducted a replicated experiment in which larvae of the marine fish red drum (*Sciaenops ocellatus*) were reared under a climate change scenario of elevated CO_2 levels (2100 µatm) and under current control levels (400 µatm). We found significantly higher whole-body levels of FAs, including 9 of the 11 essential FAs, and altered relative proportions of FAs in the larvae reared under higher levels of CO_2. Consequences of this effect of ocean acidification could include alterations in performance and survival of fish larvae and transfer of FAs through food webs.

Introduction

Anthropogenic CO_2 in the atmosphere is dissolving into the oceans and acidifying them (Caldeira and Wickett, 2005; Doney et al., 2012; Rhein et al., 2013). This decline in pH is expected to be greater in coastal areas where the effects will be especially important because of the high biodiversity, presence of areas of special conservation interest (e.g., coral reefs), or importance to seafood production (Doney et al., 2012; Rhein et al., 2013). Ocean acidification (OA) has been demonstrated to affect fundamental processes of early stages of fish, such as growth and survival (Baumann et al., 2012), behaviour (Dixson et al., 2010; Welch et al., 2014), auditory and olfactory function (Munday et al., 2009; Simpson et al., 2011), otolith calcification (Checkley et al., 2009), and even cause tissue damage (Frommel et al., 2011). The effect of OA on the synthesis or metabolic pathways of important biomolecules is less known.

Fatty acids (FAs) are biomolecules that are structural components of cell membranes, metabolized for energy, or stored for future use. FAs are designated as X:YωZ, where X is the number of carbon atoms, Y is the number of double bonds and ωZ indicates the position of the first double bond from the methyl terminus (Brett and Müller-Navarra, 1997; Tocher, 2003). Some FAs can be assembled from precursors, but most animals cannot synthesize *de novo* enough of the long-chain (≥ 18 carbon atoms) FAs that contain multiple double bonds to meet their physiological requirements (Brett and Müller-Navarra, 1997). These highly unsaturated FAs are manufactured by primary producers, and animals obtain them almost exclusively from their diet. For that reason, they are known as essential FAs (EFAs) (Tocher, 2003). Some EFAs (e.g., eicosapentaenoic acid [EPA, 20:5ω3] and arachidonic acid [ARA, 20:4ω6]) are precursors of other important biomolecules, such as eicosanoids and prostaglandins. Moreover, EFAs are indispensable for development of neural and retinal tissues and proper neural functioning in many animals, including humans (Crawford and Broadhurst, 2012).

Changes in the EFA composition of organisms at lower trophic levels due to ocean acidification are currently under scrutiny (Leu et al., 2013; Torstensson et al., 2013). For example, the majority of primary production in the oceans is expected to shift from diatoms to other microalgae (e.g., *Phaeocystis*) (Desvilettes and Bec, 2009), and as a result EFA production in the oceans is expected to decrease (Brett and Müller-Navarra, 1997; Desvilettes and Bec, 2009). Beyond this shift in availability of EFAs, OA may alter the way that animals process and store FAs obtained from their diet, which would have consequences for the animal’s survival and the transfer of FAs to higher trophic levels (Faulk and Holt, 2008; Fuiman and Ojanguren, 2011). We selected the marine fish red drum (*Sciaenops ocellatus*) as a model species to conduct the first experiments on the potential effect of ocean acidification on FA composition of fish larvae. Red drum is a species of high economic importance in aquaculture and recreational fisheries, inhabiting estuarine and coastal areas on the east coast of North America which are endangered by global change and ocean acidification (Rhein et al., 2013), and the species has been the subject of intense research on the dynamics and ecological significance of variations in FA composition of eggs and larvae (Faulk and Holt, 2008; Fuiman and Ojanguren, 2011).

Material and methods

Ocean acidification experiment

Two batches of fertilized red drum eggs were collected from natural spawns from a single tank of broodstock. Each batch was divided into two treatment levels: control CO_2 level (400 µatm) and high CO_2 level (2100 µatm), and reared at a constant temperature (27.4 ± 0.3°C) and salinity (36.6 ± 0.9 ppt). Both high CO_2 and control groups (2 tanks per group) were fed equally with the same highly enriched FA diet. At day 23 posthatching all the fish surviving in each tank were euthanized and measured. For each tank, all the fish were then combined, lyophilized, and homogenized, and then three samples were analysed for FAs composition using gas chromatography (Faulk and Holt, 2008), measuring a total of 27 FAs.

Statistical analyses

The number of fish remaining in each tank was compared between groups using a Mann-Whitney U Test; fish length distribution in each tank was compared across treatments using PERMANOVA, and a t-test was used to compare total FA content. For each fatty acid, ANOVA or a Wilcoxon signed-rank test was used on raw or log-transformed FA concentrations and relative % for comparisons of the control and high CO_2 groups (see Supplementary table IV.1 and Supplementary table IV.2). A redundancy analysis (RDA) was performed on the complete FA composition for control and high CO_2 groups, including egg batch as a factor. Statistical analyses were performed using the R package (www.r-project.org) (R Development Core Team, 2011).

Results

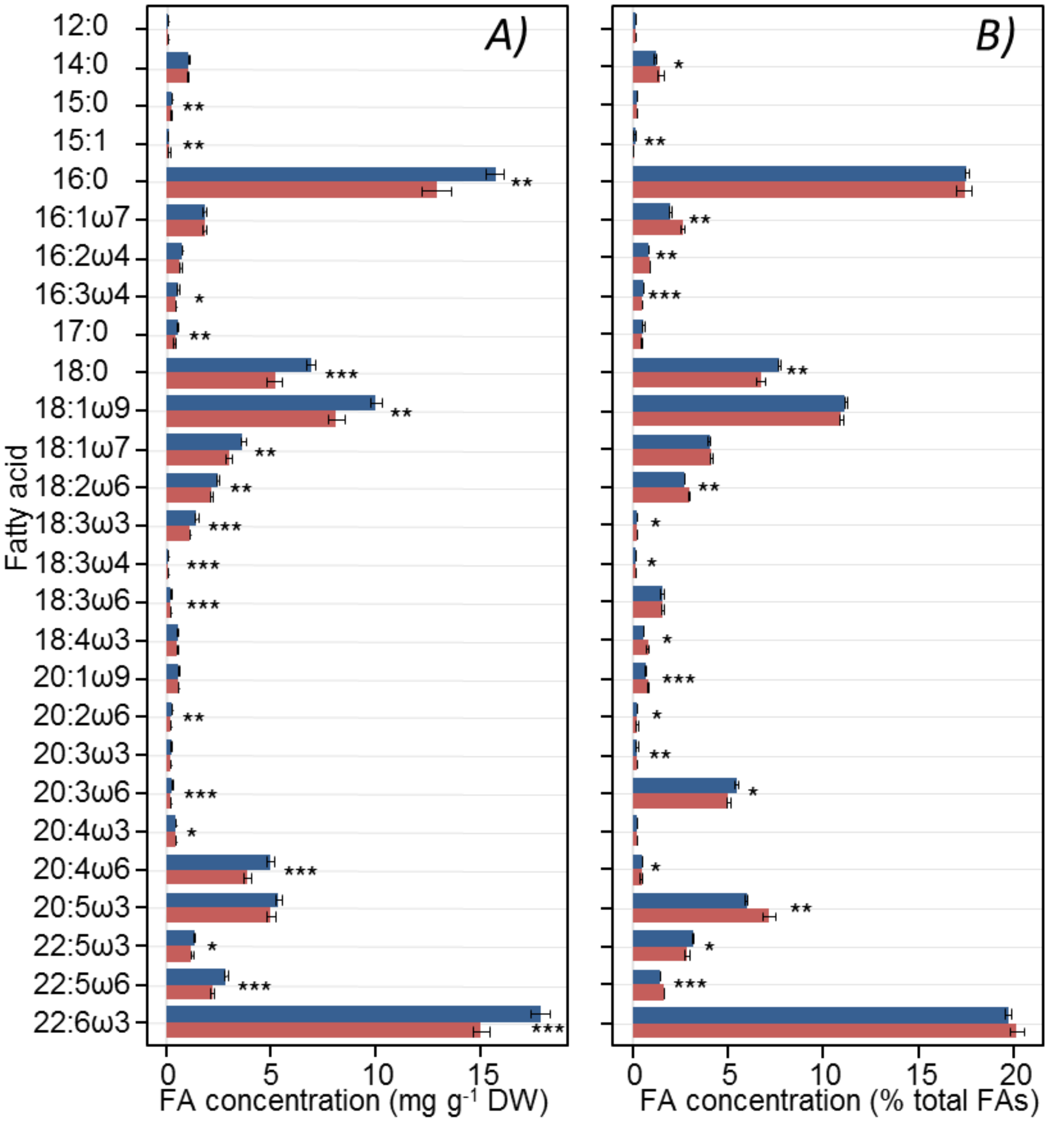

Figure V.1. Mean concentrations of FAs (mg FA g^{-1} dry weight) (*A*) and mean relative % of FAs (*B*); in red drum larvae reared under control (red) and high CO_2 (blue) conditions. Error bars are one standard error (s.e.). Asterisks indicate significant differences (see Supplementary table IV.1 and Supplementary table IV.2) (* P < 0.05; ** P < 0.01; *** P < 0.001).

There was a significant effect of OA treatment on the number of fish remaining (Mann-Whitney U Test, $P \leq 0.01$), with 61.7 % more fish in the high CO_2 group. Mean fish length was significantly smaller (25.3 %) in fish reared under high CO_2 conditions (PERMANOVA, *Pseudo-F*: 26.7; $P \leq 0.001$). Total FA content was significantly higher in the high CO_2 group (T test, $t_{9.77}$: -4.2, $P \leq 0.01$). Analyses of individual FAs (expressed as mg g^{-1} dry weight) showed that 19 of the 27 FAs had significantly higher values under high CO_2 (Figure V.1, *A*; Supplementary table IV.1), including higher levels for 9 of the 11 EFAs. Expressed on a relative basis (% total FAs), 9 FAs were disproportionately higher under elevated CO_2; 9 were disproportionately lower; and 9 remained relatively unchanged (Figure V.1; Supplementary table IV.2).

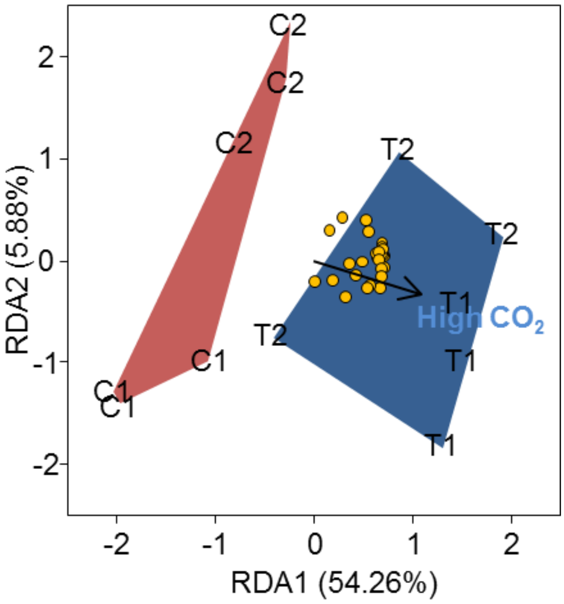

Figure V.2. Ordination plot of RDA results. Letters and numbers identify CO2 level and replicate number (C = control (400 µatm); T = high CO2 (2100 µatm)). Circles represent the FAs measured in red drum larvae. Coloured polygons enclose each treatment group. Arrow shows the direction and intensity of the effect of high CO_2 on FA composition.

The RDA interaction model (model adjusted $R^2 = 50\%$) showed that CO_2 level was a significant factor but egg batch was not (CO_2 level: $F_{1,8}$: 10.1, $P = < 0.001$; Egg batch: $F_{1,8}$: 1.4, $P > 0.05$) (Figure V.2, Supplementary table IV.3). Only the first RDA axis was significant, explaining 54.3% of the model variance (Figure V.2, Supplementary table IV.3).

Discussion

Our results identify a strong effect of elevated CO_2 levels (2100 µatm of CO_2, predicted for the year 2300 (Caldeira and Wickett, 2005)) on the FA content of larval fish. This agrees with recent work that shows an increase in total lipid content of cod (*Gadus morhua*) larvae under high levels of CO_2 (4200 µatm) (Frommel et al., 2011). That prior study reported no differences in the composition of the lipids, while we found 19 of the 27 FAs analysed to be significantly elevated at only 50% of the level of CO_2 used in the prior study. Further, the relative amounts of different fatty acids varied under elevated CO_2, with some FAs increasing significantly and others decreasing significantly. While ocean acidification usually jeopardizes larval survival (Baumann et al., 2012; Dixson et al., 2010), it is worth noting that the increase in some of these FAs may improve ecological performance of the larvae since recent studies of red drum larvae have shown that higher levels of some EFAs are positively correlated with larval escape performance (Fuiman and Ojanguren, 2011; Perez and Fuiman, 2015). Three EFAs – DHA, EPA, and ARA – are especially important to larval fish physiology (Tocher, 2003) and were expected to be closely regulated, regardless of ocean acidification. Surprisingly, DHA and ARA increased on a weight basis and EPA and ARA decreased on a percentage basis under OA conditions. These changes could have significant impacts on physiological functions.

Higher tissue levels of EFAs could potentially result from increased absorption, synthesis, biotransformation, and/or storage. Some marine fish can manufacture EFAs but their capacity is limited (Faulk and Holt, 2008; Norambuena et al., 2013; Tocher, 2003). Increased absorption of ingested EFAs is an unlikely explanation because absorption of other nutrients would have increased as well, leading to more growth, but larval growth decreased while tissue levels of EFAs increased. Rather, we suggest that red drum larvae under this stressor deposit a larger portion of the ingested FAs in tissues. Nevertheless, the mechanism through which this response to OA operates is unknown.

Ocean acidification can affect organisms and ecosystems by altering FA production (e.g., changes in communities of primary producers (Wynn-Edwards et al., 2014)) or through effects on uptake of FAs by higher trophic levels (Litzow et al., 2006). We showed that storage of many FAs by red drum larvae increases and that FA proportions differ under ocean acidification. The consequences of these changes in FAs in tissues on ecological performance of fish larvae and on food web structure and function need to be explored for a more complete understanding of the impacts of OA on marine ecosystems.

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Section III: Otoliths as indirect indicators of life history

"Take the risk of thinking for yourself, much more happiness, truth, beauty and wisdom will come to you that way."

Christopher Hitchens

Chapter VI: Otolith fluctuating asymmetry: a misconception of its biological relevance?

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Abstract

For decades, disruption of the bilateral symmetry of body structures has been related to underperformance and, hence, to fitness. In fish, this concept coupled with the claimed evidence for increased fluctuating asymmetry (FA) of pairs of hard structures, such as otoliths under conditions of stress, has led to the use of otolith fluctuating asymmetry (OFA) as a proxy for individual fitness and population success. Interpreting those significant differences in OFA in relation to stress-inducing environments and using them to identify suboptimal conditions for survival is especially appealing in the context of, for example, assessing climate change using historical otolith collections. Despite several works that give evidence to support the usefulness of OFA approach, we report on a number of unpublished and published datasets from a wide range of both, wild populations and experimental designs that show no correlation between OFA and any of the usual indicators of fitness or between OFA and expected stress gradients. Our results suggest that there may be a strong bias in the published literature toward positive relationships, despite the enormous importance of negative results for understanding the significance of OFA. These results might shed light on the interpretability of OFA in both laboratory and field studies.

Introduction

The development of paired body structures in bilateral symmetrical organisms may deviate from the ideal pattern (i.e. the genetically designed likeness between right and left structures) under different environmental or genetic stressors (Vøllestad and Hindar, 1997; Graham, Freeman, and Emlen, 1993). Developmental instability can be defined as the inability to reach this ideal pattern during development (Zakharov and Graham, 1992). Even though developmental instability is not directly observable, paired structures present in bilaterally symmetrical organisms offer the opportunity to measure departures from perfect symmetry, and the differences between the sides, known as asymmetry, may be used to interpret the divergence from the ideal form (Palmer and Strobeck, 1986).

There are three types of asymmetry or deviations from perfect symmetry that are characterized by different combinations of mean and variance of the distribution of some measure of the difference between the right (R) and left (L) structure (Palmer and Strobeck, 1986; Palmer, 1994). Directional asymmetry occurs when there is a consistent bias towards one of the sides, resulting in a distribution with a non-zero mean, as examples the morphological asymmetry in flatfishes R always < L or vice versa or the differences in otolith microchemistry between sides (Kajajian, Schaffler, and Jones, 2014; Loher, Wischniowski, and Martin, 2008)). Antisymmetry occurs when there is a difference between the sides of an individual but the direction of the asymmetry varies randomly among individuals, which results in a bimodal distribution (e.g., the signaling claw of fiddler crabs R < L or R > L). Fluctuating asymmetry (FA) which has a normal distribution with mean zero and deviations to each side are considered as differences from this ideal symmetrical form (Palmer and Strobeck, 1986, 1992; Palmer, 1994). It is expected that developmental instability would be positively correlated with environmental stressors, thus fluctuating asymmetry, as a measure of the divergence from the ideal pattern, has been used for the past few decades as a negative proxy for developmental instability and fitness, i.e. more fit organisms are expected to show less FA.

Examples of the use of FA as a measure of fitness can be found in a wide range of organisms from insects to humans but not always with the expected results (reviewed Palmer, 1992; Polak and Trivers, 1994; Lens et al., 2002). Focusing on fish, there is a lack of consistent results when comparing several external meristic and morphometric characters (e.g., pectoral fin rays, gill rakers, pelvic fin rays or lateral line scales, among others see Valentine *et al.,* 1973; and Allenbach, 2010). The comparison of internal inert structures, such as otoliths, has provided even more controversial results: otolith FA (OFA) in sagittal otoliths has been usually studied in early stages of fish (but see Kristoffersen and Magoulas, 2009), since their growth rates are faster, their capability to tolerate stress is lower, and the link between growth performance and fitness is stronger (e.g., Anderson, 1988; Houde, 1989). The number of significant inverse relationships between OFA and performance (a proxy for fitness) is relatively high (Alados *et al.,* 1993; Escós *et al.,* 1995; Somarakis *et al.,* 1997a; Franco *et al.,* 2002; Grønkjær and Sand, 2003; Gagliano *et al.,* 2008; Lemberget and McCormick, 2009). Recently, non-significant results have become more common in the literature (Folkvord *et al.,* 2000; Fey and Hare, 2008; Kristoffersen and Magoulas, 2009; Munday *et al.,* 2011a, 2011b; Maneja *et al.,* 2013; Novak *et al.,* 2013), but the danger of a bias toward publication of positive correlations has been pointed out (Palmer, 1999; and see Palmer et al., 2010 and references therein).

The controversial use of OFA is further overshadowed by several varied techniques that have been employed over the years, and comparability of results across techniques is dubious. The more widely used methods are based on univariate analyses of direct measurements, such as maximum otolith radius, perimeter, or otolith area (Somarakis *et al.,* 1997a); maximum length and width (Alados *et al.,* 1993); or weight (Alados *et al.,* 1993; Lychakov and Rebane, 2005). Other popular analyses have been based on the ratio between right (R) and left (L) otolith radius (Folkvord *et al.,* 2000). Multivariate techniques also have been used on otolith shape descriptors, such as Fast Fourier Analysis (e.g.,Gagliano and McCormick, 2004; Gagliano *et al.,* 2008). Geometric morphometry has also been used to compare R and L otoliths (Palmer *et al.,* 2010). In these analyses, otolith shape is decomposed into a number of shape descriptors, which may be derived either from Fourier decomposition of the outline or from the methods developed by landmark-based geometric morphometry (Rohlf and Marcus, 1993). In both cases, shape of an otolith is described by a vector, and left-right differences are evaluated using multivariate analysis (see Palmer et al., 2010 for details).

Independently of these diverse methodologies the use of OFA is thought to be of interest in fisheries management. For example, it could provide a measurement of past individual and population fitness because it can be measured from historic collections of otoliths. In this context*, a posteriori* quality control of assessment failures linked to stock collapses, could inform and improve future management decisions. Similarly, assessing current fitness status at the individual or population level is sometimes complicated, and OFA could be an alternative, more affordable approach. Another potential application of OFA is stock differentiation (Palmer *et al.,* 2010). Thus, in the case that OFA–fitness or OFA–stress relationships were robust, OFA could be routinely included in the tool-kit for stock assessment.

In this study, we present 11 case studies, only one of which has been published (Maneja *et al.,* 2013), from both natural populations and experimental studies (laboratory and mesocosm) in which FA in sagittal otoliths was measured. In these studies we tested if there was a relationship between OFA and different proxies for fitness, under a wide range of stressors including starvation, natural predatory field and ocean acidification, among others. We include examples from different temperate fish species and life history stages (from larvae to adults) covering a wide range of experimental designs, to test the hypothesis that OFA can be used as an indicator of stress.

Material and methods

For the sake of simplicity, a short description of the methodology used in each experiment is provided to better understand the underlying hypothesis being tested. Case studies are divided in two blocks, natural populations and laboratory experiments (Table VI.1).

Ethical guidelines

All experimental laboratory or mesocosm studies based on live animals complied with the prevailing national laws for ethical treatment of experimental animals at that time. For these experiments, individuals were euthanized by an overdose of anesthesia (tricaine methanesulfonate or MS-222) and all efforts were made to minimize suffering.

Experiments #1, #4 and #5 were carried out at the University of Texas Marine Science Institute (MSI) (Port Aransas, Texas, USA) under the Institutional Animal Care and Use Committee (IACUC) approved Animal Use Protocols AUP-2013-00083, AUP-2011-00039, AUP-2013-00041, AUP-2013-00155 and AUP-2012-00133. Experiments #2 and #3 were conducted at Espegrend Marine Station from the University of Bergen (Norway) using the animal experimentation permit ID2346, which was approved by the national regulatory Committee on the Ethics of Animal Experiments under the Norwegian Animal Welfare Act (LOV 2009-06-19 nr 97: Lov om dyrevelferd). Experiments #6, #7 and #8 were carried out at the Laboratory of Marine Research and Aquaculture (LIMIA) from the Balearic Government (Port d'Andratx, Balearic Islands, Spain) in strict accordance with the recommendations from the Directive 2010/63/UE, and in strict compliance with the Spanish law (RD53/ 2013, BOE n. 34 February 8th 2013)

Laboratory and mesocosm experiments

1. Atlantic croaker (*Micropogonias undulatus*) juveniles under three different diets

Eggs were collected from natural spawns of captive Atlantic croaker brood stock at the MSI. The eggs were hatched in 150 L conical tanks and, afterwards larvae were fed with enriched rotifers (*Brachionus plicatilis*) and *Artemia* at increasing densities (0.25-2 mL^{-1}) as fish grew. Upon reaching juvenile size, fish were weaned onto a marine dry pellet feed (Otohime from Reed Mariculture, Campbell, CA, USA) until reaching experimental size (TL = 110 ± 10 mm TL, TW = 17 ± 5 g) and then randomly assigned to one of twelve 350 L experimental circular tanks. Experimental treatments included 'low' (32% protein; 5% fat) and 'medium' (45% protein; 12% fat) quality diets, which contained lower protein and lipid content than the control 'high' (48% protein; 15% fat) quality diet. Each treatment included four replicate tanks, with 8 fish sampled daily at days 8, 10, 14, 20, and 52 (n=40 per treatment group). Fish were humanely euthanized, sagittal otoliths were extracted, cleaned, air dried, and weighed to the nearest 0.001 g. Unsigned FA in otolith mass was calculated as the absolute value of the difference between right and left otoliths divided by their arithmetic mean (|R-L|/mean of R and L).

The hypothesis in this experiment was that the high quality diet would produce better growth and fitness and therefore more symmetrical otoliths than the low quality diet.

Table VI.1: Resume of CS and results. Laboratory (lab), mesocosm (mes) or field; life-history stage; otolith fluctuating asymmetry (OFA) and statistical model used (ANOVA: Analysis of Variance; GLMMs: Generalized Linear Mixed Models; LRT: Likelihood Ratio Test). In bold the stressor being tested against OFA; nested design by variables; data transformation prior to the statistical analysis; and results.

CS	Lab/ mes/ field	Species	Stage	OFA measurement /Model	Variables	Nested	Transform	Statistic	p-value
1	Lab	Atlantic croaker	Juveniles	Unsigned mass ANOVA/Permutation Test (i=1000)	**Diet** + age (dph)		None	F obs: 0.668	p-value: 0.514
2	Mes	Atlantic cod	Larvae	Unsigned area GLMMs/LRT	**CO_2 levels** + age (dph)	Tank	BoxCox	$\chi^2_{(d.f=2)}$: 3.361	p-value: 0.186
3	Mes	Atlantic herring	Larvae	Unsigned area GLMMs/LRT	**CO_2 levels** + age (dph)	Tank	BoxCox	$\chi^2_{(d.f=2)}$: 0.806	p-value: 0.668
4	Lab	Red drum	Larvae	Geometric morphology GLMMs/LRT	**CO_2 levels**	Tank	BoxCox	$\chi^2_{(d.f=1)}$: 0.021	p-value: 0.885
5	Lab	Red drum	Larvae-juveniles	Geometric morphology GLMMs/LRT	**Age (dph)**	Tank	BoxCox	$\chi^2_{(d.f=1)}$: 0.123	p-value: 0.725
6	Lab	Meagre	Larvae	Geometric morphology Linear regressions	**GIcore** **GIedge** **CS**		None	F $_{(d.f=1,24)}$: 0.026 F $_{(d.f=1,24)}$: 0.182 F $_{(d.f=1,24)}$: 0.239	p-value: 0.873 p-value: 0.673 p value: 0.629
7	Lab	Meagre	Juveniles	Geometric morphology ANOVA	**Food levels**		Log	F $_{(d.f=2,46)}$: 0.199	p-value: 0.820
8	Lab	Gilt-head seabream	Juveniles	Geometric morphology GLMMs/LRT	**Treatment** + age (dph)	Tank	BoxCox	$\chi^2_{(d.f=1)}$: <0.001	p-value: 0.999
9	Field	Painted comber	Adults	Geometric morphology ANOVA	**Fishing pressure** + age (year class)		Log	F $_{(d.f=1,87)}$: 0.399	p- value: 0.529
10	Field	Atlantic menhaden	Juveniles	Geometric morphology GLMMs/LRT	**River area**	Site	BoxCox	$\chi^2_{(d.f=1)}$: 4.945	p-value: 0.084
11	Field	Atlantic menhaden	Juveniles	Geometric morphology GLMMs/LRT	**Harmful Algal Bloom**		Log	F $_{(d.f=2,26)}$: 0.355	p-value: 0.703

2 and 3. Atlantic cod (*Gadus morhua*) and Atlantic herring (*Clupea harengus*) larvae under ocean acidification conditions

A land-based mesocosm experiment was conducted in March to May 2010 at the University of Bergen's Espegrend Marine Station (Norway) to investigate the influence of elevated pCO_2 on the calcification process of otoliths of Atlantic cod and Atlantic herring larvae (Maneja *et al.,* 2013; Frommel *et al.,* 2014). Both species were co-reared in 2300 L, flow-through experimental tanks at a stocking density of 4 larvae L^{-1}. Three levels of pCO_2 treatments (control-370, medium-1800, high-4200 µatm) with three replicates were implemented. The carbonate chemistry of the experimental tanks was controlled by bubbling CO_2 into the bottom of the tanks through a computer-controlled system. The larvae were fed daily with fresh and appropriately-sized zooplankton filtered over 24 h from the adjacent fjord. Sampling was carried-out eight times during the experiment (15-29 individuals per group and day), which corresponded to the following ages of Atlantic cod: 7, 11, 14, 18, 25, 32, 39 and 46 days post hatch (dph) (n=169 Control group; n=195 Medium CO_2 group; n=202 High CO_2 group). Otoliths from all the cod larval samples were extracted. In the case of Atlantic herring, only otoliths from 25, 32 and 39-dph were processed (7-28 individuals per group and day; n=54 Control group; n=58 Medium CO_2 group; n=54 High CO_2 group). Atlantic herring larvae were 7 d younger than cod larvae because of differences in the main hatching date of the two species during the experiment. Immediately after sampling, calibrated pictures of larvae were taken and used to calculate standard length. The larvae were stored at -80°C and later freeze-dried to obtain the dry weights. The sagittal otoliths were then extracted taking into account the left and right sides and mounted on microscope slides using crystalbond^{TM} 509. Each otolith was photographed using an Olympus BX61 compound microscope. The surface area and dimensions of each otolith were then measured from the pictures using ImageJ software.

It was hypothesized that changes in the carbonate chemistry of seawater, particularly the pH and the availability of carbonate and bicarbonate ions, will influence the precipitation of calcium carbonate into the otolith matrix. Under decreased seawater pH condition, fish regulates its internal carbonate chemistry (e.g., blood, plasma, intercellular spaces) to counteract the side effects of acidosis (Maneja *et al.,* 2013). The process might have direct or indirect influence on the calcification process, which in turn could increase the magnitude of OFA.

4. Red drum *(Sciaenops ocellatus)* larvae under ocean acidification conditions

Three batches of red drum eggs were collected from natural spawns from a single tank of brood stock at MSI. Initially 1000 eggs were hatched and maintained in six cylinder-conical tanks of 100 L each, three of them at control CO_2 levels (400 µatm) and three at 2100 µatm of CO_2. Carbon dioxide concentration in the tanks was stabilized by periodically injecting CO_2 from a cylinder connected via a solenoid valve controlled by an injection timer to the tanks. The pH of the tanks was measured several times per day (Accumet AR15, Fisher Scientific). Titratable alkalinity was measured twice per week in each tank (AS-ALK2, Apollo SciTech) and hence CO_2 levels were calculated using alkalinity and pH values (CO_2calc software, Robbins et al., 2010). Temperature and salinity were kept stable and equal in all the tanks by means of daily measurement and slight corrections in the heaters and the addition of distilled water. Larvae were fed following the institution's standard protocol for this species: first enriched rotifers (3-12 dph), *Artemia* sp. nauplii, metanauplii (11-22 dph), live prey were enriched using Algamac 3050 according to manufacturer's recommendations (Aquafauna Bio-Marine, Hawthrone, CA, USA), and finally commercial dry pellet diet (20-23 dph) (Otohime Reed Mariculture, Campbell, CA, USA). At 23 dph, larvae were euthanized (n=91 Control group; n=97 High CO_2 group), photographs were taken for length measurement and otolith extraction was carried out under a binocular microscope (Olympus SZH). Otoliths were analyzed using geometric morphometry, following the protocol published by Palmer et al., (2010).

The working hypothesis under this design was to test if high predicted levels of CO_2 in the future ocean will alter the otolith size as predicted by some studies (Checkley *et al.,* 2009; Bignami *et al.,* 2013) as well as the OFA levels.

5. Red drum *(Sciaenops ocellatus)* larvae and juveniles under intraspecific competition

Red drum eggs were collected from natural spawns of captive broodstock at MSI. They were hatched in conical tanks and larvae were transferred at 2 dph to two, 265 L cylindrical tanks partially submerged in a 4500 L reservoir for controlling temperature, salinity and nitrates/nitrites in a bigger closed recirculation system. Initial larval density was 50 larvae L^{-1}. The number of larvae was estimated every week to adjust the amount of feed, and the fish were fed 20% below the recommended protocol established for this species to induce competition. The feeding protocol was similar to the previous experiment (#4) but live prey were not enriched and fish were weaned onto commercial dry pellet feed of increasing particle size (Otohime Reed Mariculture, Campbell, CA, USA). Larvae were sampled at 21 (n=46) and 51 dph (n=67), euthanized and photographed for length measurements. Pairs of sagittal otoliths were extracted and analyzed using geometric morphometry, as described above.

The purpose of this experiment was to test if OFA was reduced under increased intraspecific competition. If those individuals that have a higher fitness are those that survive, the degree of OFA in the late sampling (51 dph) should be lower than in the first sampling (21 dph) reflecting selection towards the well faring individuals. In addition, bigger individuals should have less OFA and smaller and weak individuals should display higher OFA in their otoliths.

6. Meagre *(Argyrosomus regius)* larvae under normal culture conditions

Meagre eggs were obtained at the LIMIA by using Ovaprim hormone (Syndel Inc., Vancouver, Canada) to induce a male and female to spawn, since this species does not spawn naturally in captivity. Eggs were incubated at 18.5 ^{o}C in conical hatching tanks and after 3 d gently transferred into 1200 L tanks at a density of 50 larvae L^{-1}. Temperature, feeding and maintenance of the tanks followed established protocol for this species (Gil *et al.,* 2014). Larvae were sampled at 22 dph (n=27) and frozen with liquid nitrogen. Dry weight (24 h drying at 60 ^{o}C) was used as a measure of larval size. Sagittal otoliths were extracted after rehydrating the dry larvae under a stereoscopic microscope (Leica MZ16). Otoliths were mounted on microscope slides and photographed three times each (Camera Leica DC300) to specifically account for observational error. Otolith shape was measured with geometric morphometry landmarks and daily growth increments were calculated from these otoliths. Two growth estimates were calculated from daily growth marks on the otoliths, otolith core growth (GIcore) from the first five daily growth marks, and otolith edge growth (GIedge) (five last daily growth marks). In addition mean centroid size (CS) of R plus L otoliths was calculated.

This experimental design tested whether OFA was higher in individuals with slower growth rates, meaning that individuals with faster growth would have more symmetrical otoliths and potentially relating OFA with survival of the bigger individuals.

7. Meagre (*Argyrosomus regius*) juveniles under three different quantities of feed

A total of 60 juveniles, 5 months old (wet TW = 83.7 ± 24.5 g and TL = 19.5 ± 1.9 cm), were distributed in a 10000 L tank divided in 6 sections (10 fish per group) at the LIMIA. The tank circulation was open, and the water temperature was maintained at 18.0 ± 1.3 ^{o}C with a heating system. The fish were subjected to three different treatments over 4 months (112 days): control, starvation and low diet; with two replicates for each treatment. Control fish were fed daily with fresh fish (until satiation) and the starved fish were not fed throughout the experiment. Low diet fish were fed once a week. At the end of the experiment, fish were sacrificed and sagittal otoliths were extracted (n= 12 Control; n=19 Low diet; n=18 Starvation). Otoliths were photographed, always oriented in the same direction, using a digital camera (Leica DC300) attached to a stereoscopic microscope (Leica MZ16). The outline of the otoliths was extracted and geometric morphometry analysis was performed to calculate the OFA.

The hypothesis of the experiment was that well-fed fish should have more symmetrical otoliths than fish fed once a week and/or unfed fish.

8. Gilt-head sea bream (*Sparus aurata*) juveniles under predatory stimulus

Gilt-head sea bream juveniles were obtained from a collaborative aquaculture fish farm (Aquicultura Balear, CULMAREX, Balearic Islands, Spain) and transported to the LIMIA. The juveniles (50 dph, initial wet weight TW = 0.33 ± 0.15 g and LT = 32 ± 4.5 mm) were stocked in six cylinder-conical tanks of 120 L each (with open flow system) at a density of 1.7 juveniles liter^{-1}. This density is below the commercial densities used and allows for better water quality and welfare of the individuals. Three of the tanks were subjected daily to the presence of an olfactory stimulus of predators. This was achieved by freezing water with predator and prey smell following (Stabell and Lwin, 1997) and introducing one of these ice cubes each day into the tank while closing the open flow system. Control seawater ice-cubes were introduced in the remaining tanks as well. Fish were sampled three times: at the start of the experiment and after one month of treatment. The experiment continued for another month without treatment and fish were sampled again after this last month (n=90 Control; n=90 Treatment). Individuals were measured and weighed and sagittal otoliths were extracted and photographed (Leica MZ16 stereo microscope equipped with Leica DC 300 camera). OFA was estimated using geometric morphometry.

This experiment tested two hypotheses: the predatory stimulus may induce developmental instability and this should be reflected in the amount of OFA; fast growers/bigger individuals should have less OFA than smaller ones.

Field sampling

9. Painted comber (*Serranus scriba*) along a fishing pressure gradient

The painted comber is a small-bodied serranid species highly vulnerable to recreational fishing. The research project ROQUER (http://www.roquer.org/) focused on disentangling the effects of recreational fishing on the life-history of *Serranus scriba* in the Mediterranean Sea, and found an empirical evidence for downsizing of adults and the high vulnerability of overexploitation of *S. scriba* due to the recreational angling activity (Alós *et al.,* 2014). Two populations were analyzed. The first sample was composed by 62 individuals of 137.7 ± 15.6 mm and age 4 ± 0.8 years, sampled at the National Park of Cabrera (declared a no-take marine protected area in 1991) which renders a pretty unique unexploited and control population of *S. scriba* (Francour *et al.,* 2001). The second sample was composed by 29 individuals of 141.2 ± 17 mm of fish size and 3.52 ± 0.74 years, sampled at Palma Bay, the most important area for recreational fishery in the Balearic Islands (Morales-Nin *et al.,* 2005). All individuals were sampled using conventional recreational angling gear in similar environmental conditions in terms of depth and *Posidonia oceanica* seagrass habitat.

We hypothesized that the degree of OFA should differ among populations from Palma Bay and National Park of Cabrera responding to the stress imposed by high fishing pressure present in Palma Bay that have already caused downsizing of the adults in this species (Alós *et al.,* 2014)

10. Atlantic menhaden (*Brevoortia tyrannus*) along a migratory corridor

Adult menhaden spawn in the continental shelf waters off the US east coast and larvae are transported to estuarine nursery locations (Nicholson 1978). The estuarine waters of Chesapeake Bay are the primary nursery for the largest fraction of juvenile recruits in the adult population (Ahrenholz et al., 1987; Ahrenholz 1991). Similarly, striped bass (*Morone saxatillis*), makes extensive use of Chesapeake Bay and is one of the dominant predators of menhaden (Griffin and Margraf 2003; Overton et al., 2009). Further, low abundance of menhaden have been implicated as a potential cause of poor condition in striped bass and increased disease prevalence in Chesapeake Bay (Hartman and Margraf 2003). Striped bass in Chesapeake Bay generally increase in abundance from the mouth toward the headwaters of Chesapeake Bay (Richards and Rago 1999). Juvenile menhaden were collected from three sites nested within each of three locations in Chesapeake Bay. These three locations were varying distances from the natal source and are respectively designated as upper (n=36), middle (n=37), and lower (n=30) Chesapeake Bay. Upon collection, menhaden were immediately placed on ice and later frozen in the laboratory. Sagittal otoliths were extracted and cleaned of tissue under a compound microscope. Both the left and right otolith was photographed and the outline was extracted following Palmer et al., 2010.

The hypothesis under consideration was that OFA would decrease with increasing distance from source locations and with increasing predation pressure. Because these two stressors are confounding, we could not separate their effects. Regardless of the mechanism, individuals with poor performance will be selected against and will have a smaller chance to arrive at the upper locations of Chesapeake Bay. Therefore we expected to see the greatest OFA in individuals from the lower Chesapeake Bay because of its closer proximity to the natal source and least exposure to predation and the least OFA in individuals from the upper Chesapeake Bay because of its greater distance from the natal source and larger exposure to predation.

11. Atlantic menhaden (*Brevoortia tyrannus*) during an algal bloom.

The lower Chesapeake Bay has been plagued by re-occurring blooms of a dinoflagellate (*Cochlodinium polykrikoides*) which is caused by excessive urban runoff and the subsequent higher nutrient loading (Mulholland *et al.,* 2009). These harmful algal blooms (HABs) occur in productive menhaden nurseries and have the potential to displace juvenile menhaden into less productive habitats or interfer with their foraging by clogging their gill rakers. Juvenile menhaden were collected before the onset of a HAB (n=9), during the period of maximal HAB extent (n=10), and after the HAB had subsided (n=10). The same collection and analysis protocols were followed as in the previous menhaden case study (#10).

The hypothesis under consideration was that fluctuating asymmetry would decrease after exposure to the HAB. Two mechanisms, direct exposure to the HAB and subsequent reduced feeding ability or indirectly being forced into suboptimal habitat were believed to negatively impact juvenile menhaden in the lower Chesapeake Bay. Consequently, only menhaden above a threshold fitness level were expected to be seen after the HAB and thus FA was expected to be less in this group than in menhaden seen before or during the HAB.

Statistical analysis

As a direct consequence of the differences among the experiments and observations presented in these case studies, the methods for analyzing the different datasets are diverse, but still comparable. In all cases, we used absolute values of OFA instead of signed values (R or L otolith) to ensure non-directional asymmetry or clear antisymmetry, assuring unimodal, centered OFA distributions. Statistical analysis was maintained as consistent as possible for all data sets, as follows. All statistical analyses were performed using the R software (www.r-project.org). ANOVA was performed for studies that employed factorial designs, after successfully satisfying the assumptions of normality (Shapiro-Wilk test) and homogeneity of variance (Levene Test). For experiments that did not satisfy these assumptions, logarithmic transformation was checked first, and if deviation persisted, Box-Cox transformation (as implemented in the *boxcox* function of the *MASS* library (Venables and Ripley, 2002)) was applied. Residuals remained non-normal even after applying this sequence of transformations for only one case study (#1). For that study, a Monte-Carlo randomization test (Manly, 1991) was applied using the *shuffle* function of the *permute* library (Simpson, 2014). For nested factorial designs, data were first fitted to two generalized linear mixed models (GLMMs) (including or not the variable of interest) using the function *lmer* from the *lme4* library (Douglas *et al.,* 2014). These two models were then compared using a likelihood ratio test. Finally, in case study #6, the variables of interest were not categorical, but continuous (growth rate), thus linear regression models were fitted.

Geometric morphometry was used for describing otolith shape in 8 of the 11 experiments. In those cases, otolith outlines were analyzed using several functions implemented in the *geomorph* library (Adams and Otárola-Castillo, 2013) to produce a vector of shape descriptors. Two replicates of the same otolith were produced (i.e.*,* four replicates per fish), which allowed application of the interaction model described by Palmer and Strobeck (1986). According to this MANOVA model, *side* was considered as a fixed factor, *individual* was a random factor, and the *size*individual* interaction represented OFA. In those 8 case studies, the procedures detailed in the paragraph above were only applied after OFA was compared statistically (and found to be significantly different) against measurement error. MANOVAs were computed using the *rda* function of the *vegan* library (Oksanen *et al.,* 2013). An example of the customized R code used in the analyses can be consulted as Supplementary material.

Results

Independently of the method used in the evaluation of the OFA (otolith mass asymmetry, otolith area asymmetry and geometric morphometric analysis of the otolith asymmetry), the analyses did not provide evidence for supporting the general hypothesis that OFA can be used as a tool for detecting an *a priori* assumed level of stress. In this section, we detail the case study-specific results.

Experiment #1 (Atlantic croaker under three different diets), the only case study that measured asymmetry of otolith mass, no significant differences were found in the degree of otolith asymmetry (unsigned mass FA) between the diets (Table VI.1; Figure VI.1).

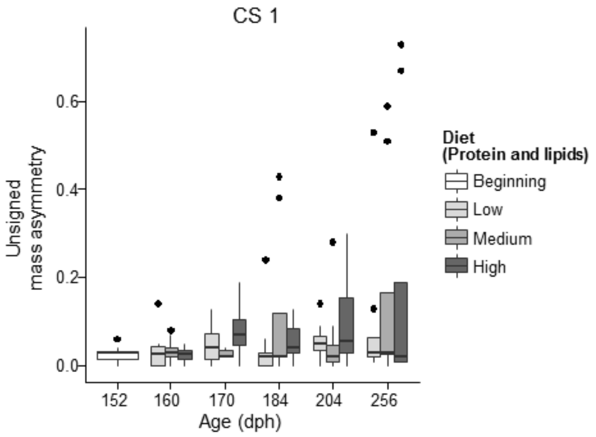

Figure VI.1. Unsigned mass asymmetry (|R–L|/meanRL) along the duration of the CS 1. Atlantic croaker was fed three different diets with different levels of protein and lipids.

For those studies that measured asymmetry in otolith area (Experiments #2 and #3, cod and herring larvae), there was no significant effect of either medium or high levels of carbon dioxide on the OFA (Table VI.1; Figure VI.2, Experiments #2 and #3). However, there was a significant effect of the same stressor (CO_2) on growth and otolith size in cod (see Maneja et al., 2013 for further details).

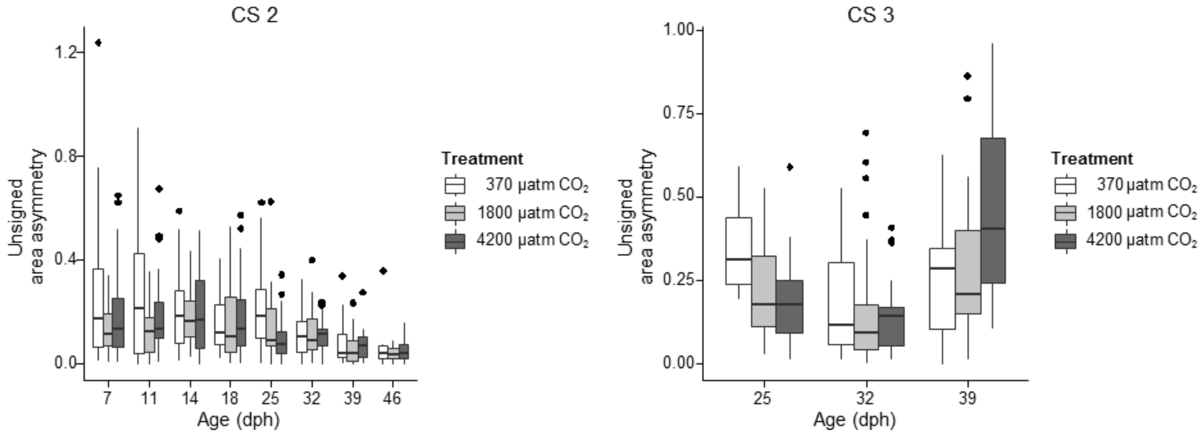

Figure VI.2. Unsigned areaasymmetry (|R–L|/meanRL) along the duration of the mesocosm CS (2and3). Both Atlantic cod (CS 2) and herring (CS 3) larvae were subjected to different ocean acidification levels.

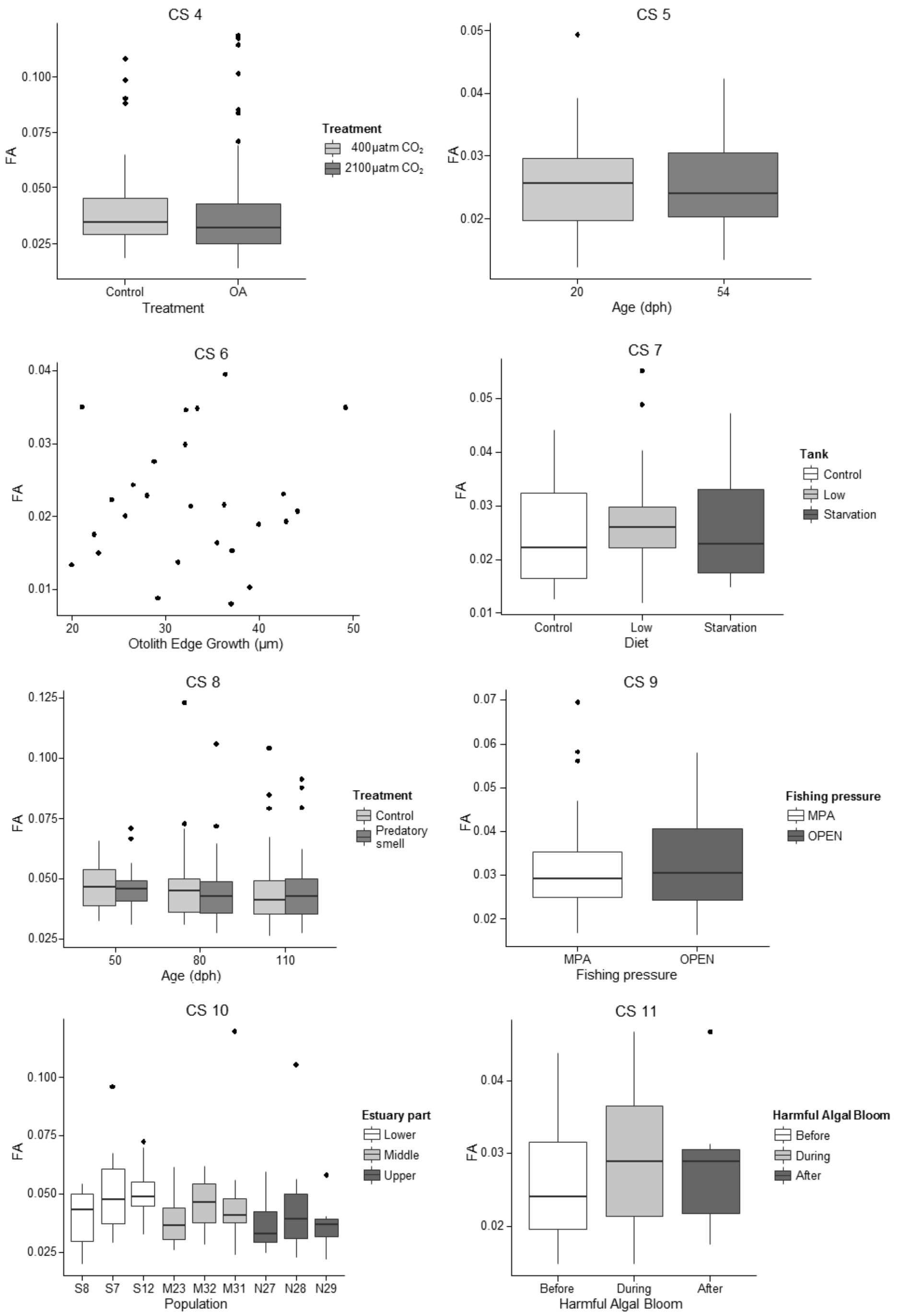

Figure VI.3. Absolute otolith FA levels (unsigned asymmetry) for the CS 4–11 measured through geometric morphometrics. Hypotheses tested include CO_2 effects in red drum larvae (CS 4), differential survival vs. OFA in larvae and juveniles of red drum (CS 5) or larval meagre (CS 6), effects of food levels on juvenile meagre (CS 7), effect of predator odour on OFA of juvenile sea bream (CS 8), effect of fishing gradient on adult painted comber OFA (CS 9), and effect of river area (CS 10) or HABs (CS 11) on juvenile Atlantic menhaden. Results summarized in Table VI.1.

Landmark-based analysis of otolith shape using geometric morphometry was used in the rest of the experiments. In Experiment #4 (red drum under ocean acidification) even though the higher level of CO_2 in the treatment did affect growth of the larvae (reduced standard length; Kruskal-Wallis test, $\chi^2_{(d.f=1)} = 43.2475$, p-value < 0.001) the OFA was not significantly influenced by the ocean acidification treatment (Table VI.1; Figure VI.3, Experiment #4).

In case study #6, none of the two growth estimates that were calculated from daily growth marks on the otoliths, otolith core growth (GIcore) and otolith edge growth (GIedge) or the mean centroid size (CS) was significantly correlated with OFA (Table VI.1; Figure VI.3, Experiment #6 presents OFA vs GIedge; similar scattered patterns were observed for GIcore and CS).

The remaining experiments had no extra features to be addressed and are summarized in Table VI.1 and Figure VI.3, Experiments #5, #7, #8, #9, #10 and #11

Discussion

In this study, we provide evidence for a general lack of support for the hypothesized relationship between OFA and the commonly used indicators of individual fitness for a suite of experiments. The idea of OFA has been widely used as a proxy for fitness since Somarakis et al., (1997b) suggested its potential use as a measurement of physiological condition. OFA is extremely appealing and easy to implement, and it has been demonstrated to vary among populations (Palmer *et al.,* 2010) and therefore has potential usefulness for fisheries assessment. OFA has been used as an indicator of life history changes (e.g., settlement; Arneri and Morales-Nin, 2000), for evaluating stress related with the effect of different environmental factors (Franco *et al.,* 2002), cohort variability and productivity (Somarakis *et al.,* 1997a), decreasing recruitment success in larval coral fish (Gagliano *et al.,* 2008; Lemberget and McCormick, 2009), or population responses to extreme stressors (e.g., starvation; Grønkjær and Sand, 2003). However, some studies have published non-significant relationships between OFA and a stressor, usually as an extra result accompanying some other, more striking results (Folkvord *et al.,* 2000; Munday *et al.,* 2011a; Maneja *et al.,* 2013). Fewer works publish the lack of differences of OFA among populations (Kristoffersen and Magoulas, 2009) or the lack of correlation between OFA and stressors like high temperatures (Novak *et al.,* 2013).

Therefore, scarce previous literature coupled with the unpublished data reported here suggests a potential bias in the published literature in favour of significant relationship between OFA and fitness.

We suggest four explanations for the widespread lack of relationships between OFA and measures of fitness that stem from our results and from previous literature on OFA. First, the large amount of non-significant results we found may be due to a lack of precision in the measurements of OFA. Moreover, we provide here three different methods for estimating OFA: mass asymmetry (Experiment #1), area (Experiments #2 and #3) and geometric morphometrics (Experiments #4-#11); which can be susceptible to be biased due to the technological limitations of the microscopes or other instrumentation used. In fact, since OFA is observed at small scales (in many cases the measurements are made close to the resolution of the optical microscope), we might not be detecting FA when it exists (i.e., the deviations from perfect symmetry are smaller than the measurement error). Assuming that FA existed, that problem may further vary in importance depending on the size of the otolith (few microns for larvae, as in Experiments #2 and #3, to several mm for adults, Experiment #7 or #9), species (pelagic as in Experiments #3, #10 and #11 or demersal as in Experiment #8), technique used and *a posteriori* image processing. Among our case studies we specifically tested for the measurement error (two photographs per otolith or even three in Experiment #6) but it is not the general case. The technological development of the image acquisition devices, image processing and shape analysis algorithms is challenging and continuously improving. However, for future studies we strongly recommend understanding the sources of measurement error, in terms of first, increase the minimum sample size, both in total number of individuals and samples per individual (true replicates, i.e., number of OFA estimates per individual); second selecting the highest number of analysed traits (multivariate, e.g., shape analyses should be preferred against univariate analyses); and third, fix the scale and processing protocols for a given range of sizes so the magnification of the pictures to be processed does not vary with age, or if it does, include an explicit analysis of this measurement error. Concerning statistical analysis, the interaction model (Palmer and Strobeck, 1986; Palmer *et al.,* 2010) or other models that disentangle FA from measurement error should be preferred. Nevertheless, negative results may reflect true lack of differences in OFA or type 2 error (failure to detect an effect that is present) which would emerge from both small effect size and small sample size. Therefore, to maximize sample size is always advisable. Certainly, sample size of two of the experiments (#7 and #11) were modest (10 to 20 fish per group) but in other cases sample size seems not to be a concern (e.g., more than 100 fish per group in #4). Obviously, to detect tiny effects of a stressor on OFA will demand huge sample size, but to measure OFA in, for example, thousands of fish per treatment may limit the practical applicability of OFA.

The second issue that could also obscure the relationship between OFA and stress may be an inadequate knowledge of the biological processes and correlations with environmental variables governing the otolith formation. The otolith is an inert structure that accrues protein and calcium carbonate (normally aragonite see Wright *et al.,* 2002) at a rate that varies with species, metabolic rate (correlated with temperature and life-stage), food supply and ambient ions concentrations (Campana, 1999). Some authors have used the OFA concept based on the assumption that it would be greater in individuals growing suboptimally as they are experiencing stressful conditions (e.g., Grønkjær and Sand, 2003). However, in our case study #1 (Atlantic croaker), the high and medium protein and lipid diet groups exhibited faster growth but, unexpectedly, highest levels of OFA. Therefore, there is not a solid understanding on how reduced metabolism (or up-regulated metabolic pathways) might affect OFA. This is partly linked to our lack of knowledge of the importance of maintaining high otolith symmetry at different life-stages. Attaining the fastest growth during early stages has been theorized to support higher survival probabilities (bigger-is-better hypothesis Anderson, 1988; Bailey and Houde, 1989; Houde, 1989), and there is no evidence that it should be detrimental to have impaired otolith growth while maintaining fast somatic growth (e.g., by investing all the energy in optimal regulation of tissue growth at the expense of ion balancing in the endolymph surrounding the otolith (Payan *et al.,* 1998)). For example, we did not find a relation between OFA and somatic growth (as total length and three different otolith growth indices) in Experiment #6. Moreover, in Experiment #5, the levels of OFA of the remaining individuals after intraspecific competition period (54 dph) were equal to the levels of OFA at the beginning of the experiment (21 dph), despite of expecting lower OFA levels in the survivors. Additionally, the survival of adult fishes with abnormal and asymmetrical otoliths produced by calcite (Morales-Nin, 1985) or vaterite precipitation (Tomas and Geffen, 2003) provide direct evidence of an unclear relationship between OFA and mortality. Therefore, fundamental knowledge on the meaning and consequences of OFA is needed before generalizing the use of OFA as a proxy for fitness.

Thirdly, even in the case that OFA was detectable and that it responded in the predicted direction, there is much debate on what can be considered an exogenous stressor (Allenbach, 2010). Linking a stressor to a change in OFA requires a mechanistic understanding between the driver and the response. For example, minimizing the asymmetry in a pair of otoliths enhances their acoustic functionality (Edds-Walton and Fay, 2002; Lychakov *et al.,* 2006) and this can be seen in the recruitment success of larval reef fish that was strongly diminished in larvae with higher OFA since otoliths play a key role in their ability to detect sounds from coral reefs that induce directional swimming on this species (Gagliano *et al.,* 2008). But this relationship is not always that clear. Assuming that only correlational evidence was available (e.g., significant variation in OFA in a population in and out of a marine protected area described in case study #9), it is not clear what the stressor might be. In this case study #9, fishing pressure through angling is likely to generate stress and differences in the remaining individuals since there is selective removal (Alós *et al.,* 2014). However, ever since recreational fisheries were abandoned in the National Park of Cabrera (Balearic Archipelago, Mediterranean Sea, Spain), top-predator species and potential predators of small individuals of *Serranus scriba* have increased notably in abundance (e.g., *Epinephelus marginatus* Reñones *et al.,* 1999). Both stressors, recreational angling and predation risk may have confounding effects because they act in the same direction of selection producing similar levels of OFA and, therefore failing to attribute them to the initially hypothesized stressor.

The fourth cause of the lack of significant relationships between OFA and fitness could be related to experimental design. There is some evidence for a lack of relationship in laboratory conditions (e.g., Munday *et al.,* 2011a, or experiments #4 and #5). Laboratory conditions can be beneficial for the individuals (controlled temperature, salinity, *ad libitum* diet) even in the presence of some kind of stressor (for instance ocean acidification as in Experiment #4). Under these conditions, expression of the relationship between OFA and the stressor could be difficult to detect. However, among our case studies we also include experiments performed in mesocosm (with the same stressor, ocean acidification Experiments #2 and #3) subject to daily variations which are more representative of the natural processes. However, no differences were found in this design either. Going further we also included field data from natural populations, Experiments #9, #10 and #11, none of which exhibited the hypothesized relationship between OFA and fitness. One of the causes that may contribute to the abundant negative results is the high natural mortality observed in the early life stages of fish (Bailey and Houde, 1989). Sampling usually occurs after this period of high natural mortality which may have already removed the more asymmetrical individuals (Downhower *et al.,* 1990; Gagliano *et al.,* 2008) thus the observer may be skipping the correct temporal window to link OFA to fitness. However, as shown in some experiments (e.g., #2 and #3), even observing at those scales may produce non significant results. Individuals sampled from the field may have been already biased by this fact, since individuals with larger OFA are no longer available, thus potentially reducing the amount of variability present in OFA levels within different sampling locations and/or time (as may have happened in case study #10). We strongly recommend researchers that special care is taken in the experimental design to reduce these issues through including a careful selection of the stressor and control all other potentially confounding variables and improving the sampling scheme to accurately include this early life temporal window, and if possible include the corpses of naturally dead individuals, in order to have a representative sample of the entire population.

Our results suggest that the application of OFA to assess fitness is a technique that has less supporting evidence accumulated than required to be generally used in the analysis of many natural and laboratory based scenarios, in concordance with the questionable evidence that OFA is generally correlated with fitness (Allenbach, 2010). This suggests a potential publication bias that should be mitigated by journals being more receptive to publishing negative results, especially when new scientific ideas or techniques arise (Jennions *et al.,* 2002). By failing to report non-significant results, a new technique cannot be fairly evaluated and the danger of technique over-rating is dramatically increased.

Finally, we do believe that otolith FA can be used if certain assumptions are met, but it should not be applied generally without careful consideration. New laboratory-based studies are needed to improve knowledge of the physiological mechanisms that produce changes in OFA. This might yield insight into the potential types and levels of stressors that can produce OFA, which is the best way to generate reasonable and testable hypotheses.

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Chapter VII: Fishing-related effects on fish life-history revealed by otolith microchemistry

This chapter is under review in:

Ignacio A. Catalán, Josep Alós, Carlos Díaz-Gil, Silvia Pérez-Mayol, Gotzon Basterretxea, Beatriz Morales-Nin and Miquel Palmer. Submmitted to *Fisheries Research*

Abstract

Inferring habitat selection and movement in wild fish by using otolith microchemistry has been the subject of much debate. Experimental results suggest that complex environmental vs. life-history interactions affect the incorporation of certain chemical elements in otoliths. We hypothesized that under relatively homogeneous hydrographic conditions, individual life-history trait strategies should dominate the signals of geochemical signatures. We tested our hypothesis by analysing the individual chronologies in the otolith signals of strontium to calcium (Sr:Ca) and barium to calcium (Ba:Ca) ratios from a sedentary coastal fish (*Diplodus annularis*). Two areas of the same NW Mediterranean region with different fishing pressure were sampled: a no-take marine protected area where the bigger-is-better strategy should prevail (energy allocation biased towards growth to compensate the large natural mortality at small sizes) and a highly exploited population where the smaller-is-better strategy should dominate (energy allocation biased towards reproduction to compensate the large fishing mortality of large sizes). Significant differences in reproduction vs. growth trade-offs were observed between sub-populations. The chronologies of Sr:Ca and Ba:Ca were compared between back-calculated ontogenetic stages (larvae, juveniles and adults) and sub-populations/life-histories using a General Linear Mixed Model. The Sr:Ca chronology reproduced well the allocation of energy to growth or reproduction according to the life-history strategy of the individual. Matching between ontogeny and otolith microchemistry was less obvious in the case of Ba:Ca. We contend that the effects of fishing pressure on microchemical signals should receive more attention in

field studies, as trade-offs between mortality and growth/reproduction might alter microchemical patterns, under a given a set of conditions.

Introduction

The coupled analysis of otolith microchemistry and age from individual fish has become a widely used technique in exploring relevant aspects of fish ecology, such as the association of individuals with a particular habitat through their life-cycle and in disentangling migrations and population structures (Campana 1999, Elsdon et al., 2008, Phillis et al., 2011, Sturrock et al., 2012, and references therein). Seven elements (Li, Mg, Mn, Cu, Zn, Sr and Ba) are routinely used to infer past location in fishes, and strontium (Sr) and barium (Ba) are the most-studied elements due to the positive relationship between their ambient concentrations and their concentrations in otoliths (Elsdon and Gillanders 2005, Elsdon et al., 2008, Tabouret et al., 2010). Typically, marine fishes derive between 83% and 98% of their otolith Sr and Ba from the surrounding water, and the changes produced by marked variations in water properties (i.e. salinity, chemical composition) or productivity are indicative of a particular habitat (Chen and Jones 2006, Walther and Thorrold 2006).

Although it is clear that minor or trace elements accumulated in the otoliths might be associated with ambient concentrations (Limburg 1995, Campana 1999, Milton and Chenery 2001, Zimmerman, 2005, Ranaldi and Gagnon 2010), the relative bioavailability of different elements taken up by fishes has received less attention (reviewed in Elsdon et al., 2008). It is likely that these elements will be fractioned during transport across the gut and gills from water and food to the blood, hen to the endolymph and finally to the otolith (Borelli et al., 2001, Payan et al., 2010), and their proportion with respect to calcium also depends on the calcium deposition rate and organic matrix content (McFadden et al., 2016). Kalish (1991) was among the first authors to argue that physiological factors, in addition to environmental, influence the chemical composition of otoliths. He suggested that changes in blood plasma composition during gonad development, in particular the composition and concentration of ion-binding proteins, appeared to be important and that the apparent relationship between temperature and otoliths elemental composition was due to the effect of temperature on gonad development. Since then, It is known that changes in otolith microchemistry cannot solely reflect the ambient water concentrations; mounting evidence has revealed an important role of ontogeny and physiology that might lead to the misinterpretation of the field patterns inferred from microchemical analyses (Alós et al., 2017, Hoff and Fuiman 1995, Brown and Severin 1999, Clarke and Friedland 2004, Miller and Kent 2009, Morales-Nin et al., 2014, Miller 2011, Gronkjaer 2016). For example, several studies have demonstrated that Sr:Ca and Ba:Ca ratios in the otolith respond to seasonal variations in water temperature, and several studies have reported negative relationships between the somatic growth rate and Sr:Ca (Sadovy and Severin 1994) and Ba:Ca (Miller 2011). Brown and Severin (2009) concluded that the water Sr:Ca ratio is the primary factor affecting otolith Sr:Ca for freshwater and estuarine fishes, but not marine species. Even though lifetime Sr:Ca profiles of the marine fish analysed varied as much as those of estuarine fish, there was no apparent relationship with water chemistry, suggesting that other factors are able to introduce significant variability in Sr:Ca. Recently, Sturrock et al., (2015) showed experimentally that physiological bio-kinetic factors (e.g. associated to reproductive stage) likely exert a large effect on otolith microchemistry.

Fish vary widely in their life-history strategies within the same species. Indeed, the direction imposed by local sources of mortality should inevitably generate populations that respond to the local selective pressures by allocating energy to growth or reproduction according to an optimal life-history strategy (Reznick et al., 1990, Hutchings 1993, Puebla et al., 2007). The natural mortality imposed by predators to small-sized individuals should move the trade-off between growth and reproduction to life histories, where individuals allocate more energy to somatic growth according to the "bigger-is-better" strategy (Reznick 1982, Hutchings 1993, Edeline et al., 2007, Olsen and Moland 2011). In contrast the gear-selective properties of fishing shift mortality towards large-sized individuals, and should indirectly induce a higher investment to reproduction and a lower allocation of energy to somatic growth. The outcome at the population level is to maximize the life-span fitness in exploited environments according the "smaller-is-better" strategy (Olsen and Moland 2011, Alós et al., 2014). Thus, we hypothesized that in cases in which the hydrographic conditions and seawater composition were not too dissimilar (i.e., within the same water mass), effects such as life-history optimization to contrasting mortality pressures might strongly imprint a geochemical tag through fish life. This idea is supported by the findings of Sturrock et al., (2014, 2015), who suggested that physiological influences were especially strong for Sr and Ba, and were stronger than variations in water chemistry. Significant relationships were observed between element:calcium ratios and physiological-related factors such as length, sex, growth rate, condition and spawning in fishes kept in captivity. In this respect, our hypothesis is also founded on the theory that individual growth curves can actually be modelled as functions of energy investment, driven by combinations of temperature, life stage, sex and reproductive stage (Lester et al., 2004, Pecquerie et al., 2011).

Existing data suggest that, in areas located away from major river plumes, Sr and Ca in seawater of the NW Mediterranean are relatively stable, even with depth (Bernat et al., 1972). The absence of permanent rivers and scarce industrial pollution in the Balearic Archipelago (NW Mediterranean) offers a good environment for examining the effects of differential pressures on life-history traits in wild fish in their natural environment. Moreover, highly contrasting areas in terms of natural and human-induced mortality pressure were reported to be compatible with life-history divergence of a sedentary fish (Alós et al., 2014). In addition, because of weak circulation during the reproductive season, the spatial scales of hydrodynamic connectivity are low, favouring phenotypic differentiation (Basterretxea et al., 2012, Alós et al., 2014, Álvarez et al., 2015). Here, we compared the otolith microchemistry in two relatively unconnected sub-populations of the annular seabream (*Diplodus annularis* Linnaeus 1758), a coastal sedentary fish with reduced adult dispersion (March et al., 2011), as a case-study with contrasted life-history divergence. One zone is located in the Marine Protected Area of Cabrera National Park (CNP), where recreational fishing is forbidden; the other is Palma Bay (PB), which is characterized by high recreational fishing pressure (Morales-Nin et al., 2005, Alós et al., 2014) (Figure VII.1).

The primary aim of the present study was to examine the patterns of otolith microchemistry variations along the life history of annular seabream in two wild subpopulations in their natural environment. We hypothesized that the microchemical signal through different life periods might be plausibly explained by diverging life history strategies shaped by external pressures other than water chemistry.

Materials and methods

Fish and otolith collection

Adult annular seabream (*D. annularis*) were obtained in the spring-summer of 2007 and 2008 from experimental fishing sessions conducted in two different areas in the southern Majorca Island (NW Mediterranean) as in Alós et al., (2010) (Figure VII.1), and complying with the ethical guidelines in the Declaration of Helsinki and even with the current more restrictive Spanish Laws (RD 53/2013). One sampling site corresponds to Cabrera National Park (CNP), a set of small islands to the Southeast of Majorca that are characterized by the highest level of protection from recreational fishing since 1991. Since the establishment of the CNP, top-predator species and potential predators of small individuals of *D. annularis*, such as the dusky grouper (*Epinephelus marginatus*), have notably increased (Reñones et al., 1999). The other sampling site is a wide bay to the south-west of the main island, Palma Bay (PB), which is located approximately 30 km from CNP. PB was identified as the most important recreational fishery for *D. annularis* in Majorca Island (Morales-Nin et al., 2005, Alós et al., 2014). The recreational fishery of *D. annularis* is highly body-size dependent as gear (hook) selectivity reduces the vulnerability (fishing mortality) to individuals larger than 120 mm (Cerdà et al., 2010). Both areas are subjected to the same ocean dynamics, which is characterized by weak wind-driven shelf circulation (e.g. Jordi et al., 2011, Basterretxea et al., 2013); however, the salinity can vary slightly (less than 0.5 psu) during few days at PB following heavy rains, which reflect the discharge of several creeks with seasonal discharge of freshwater along the coastline (e.g. Font-Muñoz et al., 2015). Because of the high degree of protection, human activities at CNP are minor, and coastal waters are markedly oligotrophic. Conversely, PB is more productive (Jordi et al., 2009). All sampling sites had depths between 10 and 25 m over *Posidonia oceanica* seagrass beds. We collected 81 individuals from PB and 38 individuals from CNP, and both samples had a sex ratio of approximately one.

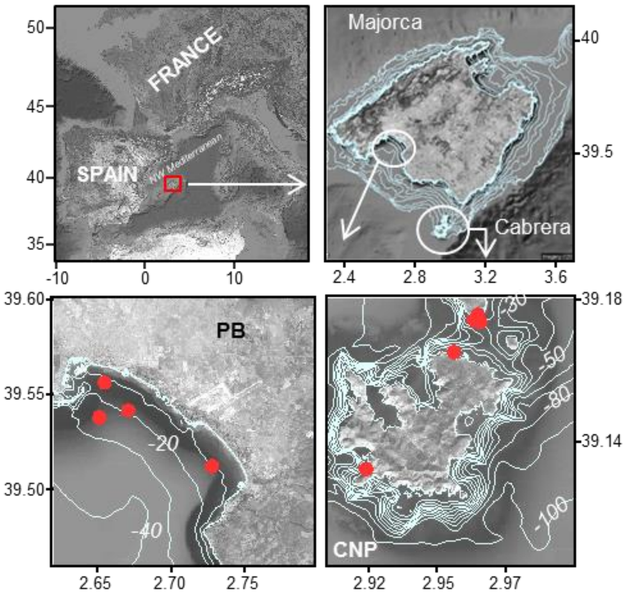

Figure VII.1. Location of the two sampling areas, Palma Bay (PB, Majorca Island) and the national maritime-terrestrial Park of Cabrera (CNP). In red, specific collection sites for geochemical analyses of otoliths.

Sagittal otoliths of *D. annularis* were removed using clean methods as in Morales-Nin et al., (2014) and stored in acid-cleaned, plastic vials until further handling. The right otoliths were stored dry for annual increments age interpretation as in Alós et al., (2010). A subsample of 34 fish of similar ages (21 from PB, 13 from CNP), ranging 120 to 150 mm TL, was processed for microchemistry analysis and finer-scale age determination. These otoliths were individually mounted on glass microscope slides using a thermoplastic resin (Crystalbond TM) with the distal side up and processed to obtain sagittal sections at the core level for Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICPMS) analysis.

Estimation of individual length-at-age life-history traits

We extracted and analysed the length-at-age data from each fish using two methods. In one method (procedure A), the individual length at a given age was used to estimate the life-history strategy and to compare the sub-population life-history characteristics between areas. In the other method (procedure B), the age in months from birth was assigned to each laser spot by using cubic spline interpolation on the ablated otoliths.

For procedure A, we relied on the existence of a clear relationship between otolith and somatic size (Alós et al., 2010), thus enabling the back-calculation of length-at-age using annual growth increments contained in the otoliths (Pilling et al., 2002, Mollet et al., 2010). Indeed, the extraction of individual life history from otoliths or other calcareous structures has been a prominent tool for the study and detection of the “bigger-is-better” and “smaller-is-better” life history strategies within PB and CNP (Alós et al., 2014). This method is applicable to *D. annularis* and the otolith length-at-age data from the otolith was back calculated for each individual following the method developed in Alós et al., (2010). Length-at-age data were transformed from annual width increments (in mm of otolith) to fish total length (in mm) using the model of Alós et al., (2010). We then fitted the individual back-calculated length-at-age data to the growth-based life-history model of Lester et al., (2004) to estimate of the life-history parameters of annual reproduction investment (g), asymptotic size at infinite age (L∞) and immature growth rate (h) (Arlinghaus et al., 2009, Matsumura et al., 2011). Briefly, the basis of this life-history model lies in the existence of a trade-off between the energy allocation to somatic growth and reproduction (Alós et al., 2014), determining the optimal life-history strategy that maximizes the fitness in different mortality scenarios (Lester et al., 2004, Shuter et al., 2005). According to this model, potential somatic growth for ages prior to sexual maturation (T, years) is considered as linear and is given by:

$$L_t = h(t - t_1) + \varepsilon \qquad \text{for } t \leq T \qquad \text{eq. 1}$$

where Lt is the length at age t (mm), h is the immature growth (mm year-1), t1 is the age at size 0 and ε is a Gaussian distributed error term (Lester et al., 2004).

After sexual maturation, the somatic growth of an individual is represented using the von Bertalanffy growth model (VBGM) with parameters that provide explicit information on the energy allocated to reproduction (g, annual reproduction investment, year-1) and is given by

$$L_t = L_\infty\left(1 - e^{-k(t - t_0)}\right) + \varepsilon \qquad \text{for } t \geq T \qquad \text{eq. 2}$$

where L_∞ = 3h/g, k = ln(1 + g/3) and to = T + (ln(1 - g(T - t1)/3)/ln(1+g/3) and ε is a Gaussian distributed error term (Lester et al., 2004).

These two equations provide a biological interpretation of the VBGM, which had been traditionally considered purely as a mathematical descriptor (Lester et al., 2004). Thus, k is proportional to reproduction investment (k ≈ g/3) and reflects the relative investment averaged over the entire reproductive cycle (g) (see also Charnov (2008)), L∞ (asymptotic size at infinite age) is set as the ratio of net production to reproduction investment (L∞ = 3h/g), and t0 is a simple function of T and g (Lester et al., 2004). *D. annularis* mature (T, age in years) during their first year (first annulus of the otolith) in the Balearic Islands, and thus, all length-at-age data correspond to individuals that reached sexual maturation (the first age class are from the mature stage as samples were obtained in spring-summer, see Alós et al., 2010 for otolith increments formation). Therefore, we fitted the individual length-at-age data obtained from each individual sampled in this study to the VBGM (eq. 2) and subsequently derived the life-history parameters g, L∞, to and h using the VBGM parameters according to Lester et al., (2004).

The VBGM (eq. 2) was fitted for each individual using a Bayesian approach. The detailed model design, the R script and a user-friendly interface to derive the parameters for any set of data are supplied in the free Shiny application website at https://fishecology.shinyapps.io/B-VBGM/ (last accessed on November 25, 2016). Briefly, the model was implemented and run using the R2jags library of the R package (at http://www.r-project.org/, last accessed November 25, 2016), which opens JAGS (at http://mcmc-jags.sourceforge.net/, last accessed July 10, 2016). A hierarchical (observations [length-at-age] within individuals within population) structure and minimally informative priors and hyper-priors were used. Three Monte Carlo Markov chains (MCMC) were run using randomly selected initial values. The first 100000 iterations were discarded, and adequate thinning (one out of ten consecutive values were kept) was adopted to ensure the independence of successive iterations within the chain. After assessing the convergence of the three chains (using the Gelman-Rubin diagnostic, (Plummer et al., 2006)), the number of valid iterations after burning and thinning oscillated between 3000 and 9000.

For procedure B, after the LA-ICPMS analyses (see below), calibrated digital images from the slides were used to measure the otolith radius (posterior axis) and the laser ablation spot distances to the otolith primordium using the ImageJ software (Schneider et al., 2012). Pictures were also used to determine whether laser spots were conducted on translucent or opaque areas of the otolith. The otolith primordium was assigned age 0 and always appeared in an opaque area (warm months). The transitions from translucent (cold months) to opaque months (warm months) were used to assign a year transition. The average distance between the spots closer to the opaque and translucent areas was used to derive the transitional point estimate for a given year. It was assumed that these individuals were born in June (Ayyildiz et al., 2014), and that the first transition from translucent to opaque corresponded to 10 months (0.83 years). Subsequently, each translucent to opaque transition was assigned a full year, following Alós et al., (2010). Spots falling on the last incomplete years were assigned an extrapolated to fractional years based on the average otolith distance-age relationships from individuals with complete annual rings.

Determination of the microchemical composition along the otolith radius

The detailed protocol for otolith preparation is described in Morales-Nin et al., (2014). Otolith chemical quantifications for the most commonly analysed elements, Ca43, Ca44, Sr88 and Ba138, were determined using a Nd:YAG UP-213 laser ablation system (NewWave Research) coupled to an ElementXR plasma mass spectrometer (Thermo-Finnigan). Primordium to posterior axis transects on the sagittal sections were performed to embrace the entire signal from the larval period to the age of collection. Laser spots were 55 µm in diameter separated by 80 µm centre-to-centre. Laser conditions were set to a frequency of 10 Hz, 60% energy and acquisition time of 66 s (40 s of sampling time). Certified Reference Materials (CRMs) as otolith powder pressed into pellets (FEBS-1, Sturgeon et al., (2005); NIES-22, Yoshinaga et al., (2000); and glasses (NIST614 and NIST616)) were sampled using the same instrument conditions at the beginning and end of each working session and after every 20 LA-spots. The LA-ICPMS output data were processed using the Glitter software (GEMOC, Macquarie University), which corrects for any instrumental drift and calculates the elemental concentrations (⍰gMe/gOtolith) with Ca43 used as an internal standard. CRMs were used to calibrate element concentration, and the election of one specific CRM for one element in a given session (day) was based on Geffen et al., (2013). In brief, element values were accepted using a specific CRM when mean estimates of analytical precision (% RSD, relative standard deviation) were below 10% and analytical accuracy (observed value/certified reference value in %) was between 90 and 110%. To reduce bias, the detection of outliers on the CRMs and the election of the concrete CRM were achieved using an ad-hoc script from R software. Elements that were not under the detection limit (DL) for at least 80% of the spots were retained for further analysis, together with the standards showing no significant differences between sessions (ANOVA). The elemental ratios (molar ratios) used for comparisons were Sr:Ca and Ba:Ca.

Environmental variables

Environmental information from the two areas was extracted and only used to refute/support our hypothesis that it was life history and not ambient the major driver of otolith microchemical patterns. Monthly averages of sea surface temperature (SST) and chlorophyll concentration (Chl-a) for PB and CNP were extracted from NASA GES DISC (MODIS-Aqua) at a spatial scale of 4 km. The register covered the precise period embracing the life span of fish for which microchemistry was conducted (2003-2008). The influence of runoff on sea-surface salinity at PB was assessed from hourly measurements at an oceanographic buoy located at this site (SBE-37 conductivity and temperature recorder) from February to December 2015 (http://www.socib.es, Tintoré et al., 2013). Seasonality is marked and rainfall regime is relatively stable in the area, so it is assumed that the only complete available year (2015) with a high resolution time-series of salinity is representative of a typical year. Further, it was assumed that effects of coastal runoff on salinity at CNP are low since the watershed at this site is small (< 5 km2).

Statistical analysis

Initial comparisons between the two subpopulations with respect to size and age, and their relationship with microchemical signals in the otolith border (selecting the average value of the last two spots which were at < 500 microns from the border) were performed using either ANOVA via General Linear Model (GLM) using normal error structure or Mann-Whitney-Wilcoxon Test (W) when the normality of the residuals was not met, even after transformation (Zuur et al., 2010). Between-population comparisons of life histories were conducted by comparing the Bayesian population means of each parameter using the degree of overlap of the Bayesian credibility intervals (BCI, 2.5% - 97.5%). To generate robust conclusions, we considered differences in the life-history traits only when the BCI did not overlap at all. Further, in order to ensure that the subsample used for the microchemistry analysis was representative of each subpopulation growth dynamics, comparison of the Lester growth parameters (see above) between the subsample and the subpopulation was conducted through ANOVA on properly transformed variables.

To analyse how geochemistry varied through life stages, standardisation according to age at a fine scale through time was needed. Therefore, we elaborated a model which accounted for individual data, and both life stage (see below) and area-related differences in growth. The data for that model were characterized by their longitudinal nature and high individual variability. For each individual we assumed three differentiated life stages with respect to growth and mortality trade-offs and energy investment (Lester et al., 2004, 2014): pelagic stage (larvae/postlarvae), juvenile stage and adulthood. Pelagic stage in *D. annularis* lasts less than one month (Macpherson and Raventós 2006), and is characterized by prioritizing energy investment in fast growth (Peck et al., 2012). Individuals progressively spend higher energy in developing more complex structures after metamorphosis, including those required for reproduction, which demands a large amount of energy during the reproductive season. In *D. annularis*, most individuals mature within the first year (Alonso-Fernández et al., 2011). After maturation, a relevant fraction of the energy is transferred to both the maintenance of reproductive structures and to the generation of gametes. Using the estimated age of each spot (months), we divided each otolith into three life Stages common for both areas: larvae (< 1 month), juvenile (between 1 and 10 months, just prior to the first reproductive period), and adults (11-40 months). The distribution of Sr:Ca and Ba:Ca within each phase was plotted for each individual, and the median adequately described each distribution. To examine whether the elemental composition varied between the marine protected area and the exploited area depending on the

Stage, we applied a General Linear Mixed Effect Model (GLMM) using lme4 package (Bates et al., 2015) from R (R Development Core Team, 2011).

$[Yijk]\ \lambda \sim Areak+Stagej+ (Area\cdot Stage)kj+ ai + \varepsilon ijk,$ eq. 3

where Y is the dependent variable (median value of elemental ratio) for each individual otolith i, in a given otolith portion corresponding to a Stagej=larva, juvenile, adult and a given Areak= PB, CNP. These factors and their interaction Area·Stage were considered as fixed effects. To account for the lack of independence between stages of a given individual, we declared the individuals as a random factor ai, which was considered a normally distributed error term with zero mean

$$ai \sim N\ (0,\sigma.between\text{-}fish2)$$

where σ.between-fish2 refers to between-fish variance. Additionally, εijk was considered the normally distributed error term with zero mean and variance σ2

$$\varepsilon ijk = N(0,\sigma 2)$$

Moreover, the term λ refers to the box-cox transformation value (R package MASS, Venables and Ripley 2002) which was necessary to fulfil the model assumptions inherent to the selected Gaussian error structures (Zuur et al., 2010, Jones et al., 2016). The λ values were very close to integers, and thus the latter were selected, being $\lambda = -1$ (median Sr:Ca) and a logarithmic transformation for median Ba:Ca due to $\lambda = 0$. Approximate P values for significance of terms were obtained using the package lmerTest (Kuznetsova et al., 2016). The sample size for this last model was slightly lower than that for general comparisons (N = 19 for PB, N = 12 for CNP), because we had to discard three fish for which too few laser spots were detected in the larval area.

Results

The analysis of the environmental data revealed similar seasonal SST dynamics but, conversely, chlorophyll was higher at PB (Figure VII.2, A and B). Temperature typically ranged from 13ºC in late winter to 26 ºC in August, but exceptionally reached 28ºC during summer 2003. Slightly higher winter temperatures (<0.5ºC higher on average) were recorded at CNP. We took 2015 as an assumed "typical year" for studying seasonal changes in salinity and interpreting potential influences on the water microchemistry. Salinity at PB displayed maximum variations of 0.4 psu, that are associated with the influence of different oceanic water masses in the area, and only during few days in September 2015 values dropped below 37. This short episode was coincident with the highest precipitation event observed during that year (Figure VII.2, C). Although generally following a similar seasonal trend, clear differences were observed between the phytoplankton biomass at both PB and CNP. Fuelled by coastal nutrient sources, PB is a more productive area often exceeding 0.6 mg m-3 (monthly mean) in the seasonal peak. The winter maximum also occurs earlier in this bay, and enhancement of phytoplankton biomass during fall is not rare, as reported by Jordi et al., (2009). Conversely, variations at CNP are coupled with oceanic conditions and productivity enhancement is restricted to late winter months.

As shown in Figure VII.3, two key life-history parameters of the subpopulations were significantly different between the areas. Individuals from CNP (the no-take marine protected area) showed a lower reproduction investment (g) and a larger maximum size ($L\infty$) suggesting a "bigger-is-better" life-history strategy. In contrast, individuals sampled in PB (the highly exploited population) showed a larger g and a smaller $L\infty$ suggesting a "smaller-is-better" life-history strategy. The Bayesian Credibility Intervals (BCI 2.5% - 97.5%) did not overlap in those life-history traits and the existence of between-population differences was therefore accepted. The BCI of the immature growth rate (h) and the theoretical age at size zero (to) clearly overlapped discarding any between-population differences in these traits (Figure VII.3).

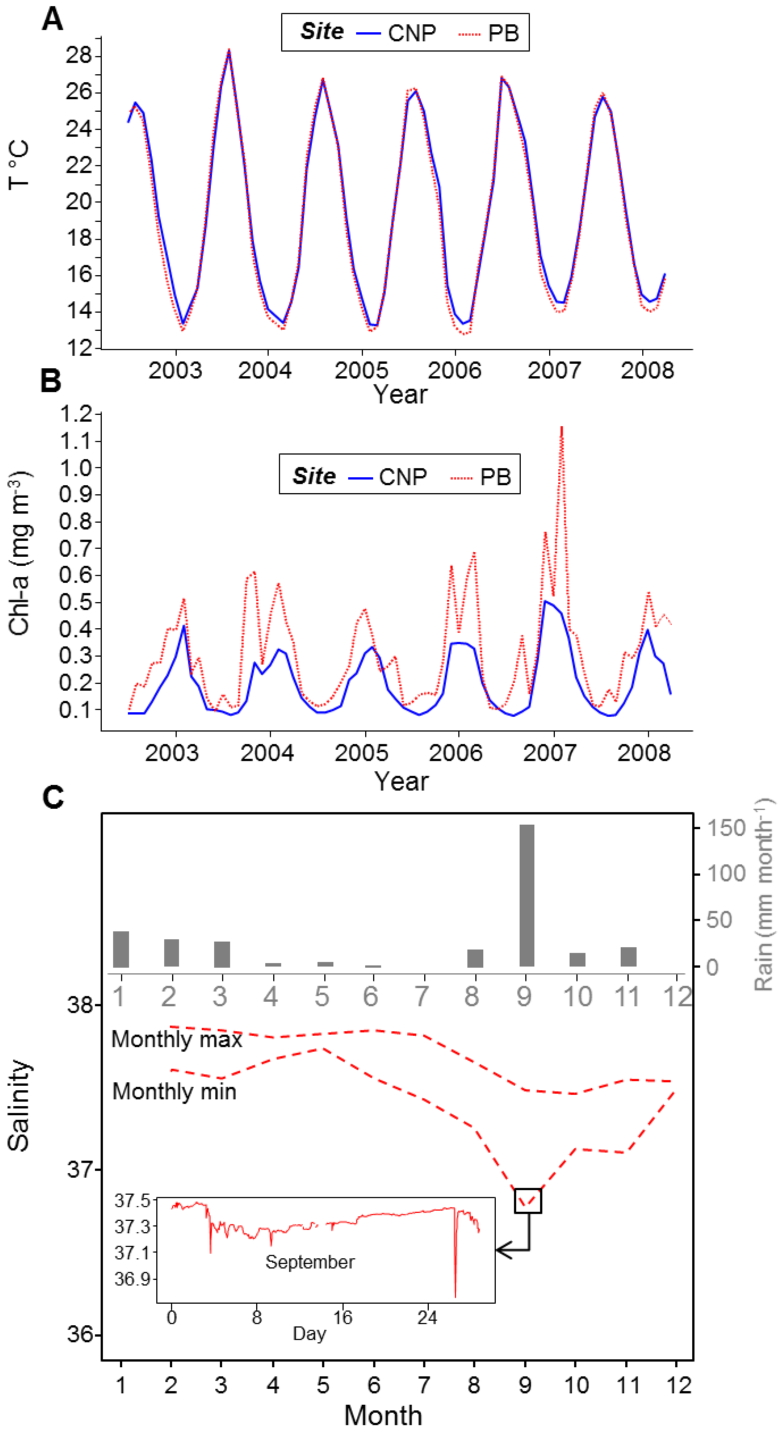

Figure VII.2. Evolution of monthly means of satellite-derived Sea Surface Temperature (SST) (A) and Sea Surface Chlorophyll (SSC) (B) at the two collection sites, embracing the life span of the analysed fish. Panel (C) shows an example (for year 2015) of the impact of rainy months (upper part of the panel) on the monthly maximum and minimum salinity values (lower part) in Palma Bay at 1 m depth (SOCIB, http://www.socib.es). A zoom of the rainiest month shows that relatively low salinity episodes in Palma Bay caused by rain are of very short duration. CNP, Cabrera National Park; PB, Palma Bay.

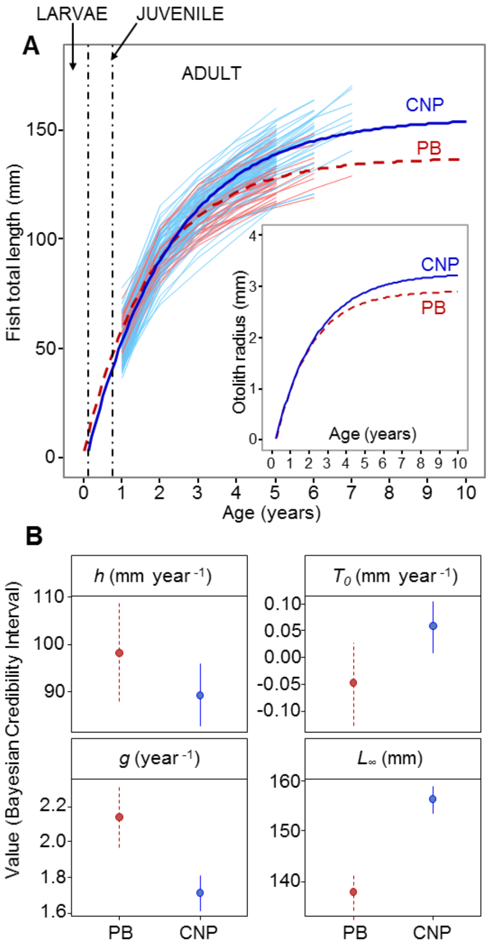

Figure VII.3. A), Individual-based von Bertalanffy growth model (VBGM) growth estimates showing the differences between the sub-populations; the protected area (CNP) and Palma Bay (PB). Raw data on annual length-at-age readings (thin lines) and life-history periods used in later analyses are also shown. The inner graph shows the corresponding variation in otolith radius. B) Parameter estimates of life-history traits based on VBGM, after applying the model of Lester et al., (2004). h, juvenile growth; g, investment in reproduction; T0, function of T and g. L∞, length at infinite age.

The subset selected for microchemical analyses was comparable in length and age for both areas (length: PB = 129 ± 11.5 mm, CNP = 133 ± 9.5 mm; age: PB = 38 ± 6.8 months, CNP = 37 ± 5.0 months) (GLM, all p>0.25). A comparison of the Lester Parameters between the subsamples used for otolith analyses and the Lester parameters of the rest of the population showed no significant differences, with ANOVA significance values oscillating between P=0.12 and P=0.96 The analyses of the Sr:Ca and Ba:Ca ratios in the otolith edge showed significantly higher values in PB vs. CNP for Sr:Ca ($F_{1,22}$ = 23.36, p<0.001) but not for Ba:Ca ($F_{1,22}$ = 3.391, p = 0.079; both transformed through box-cox method, λ = -1/2).

The averaged data from each individual depicted marked differences in the geochemistry of the otolith through life (Figure VII.4, A and C). The GLMM showed a significant interaction term in both Sr:Ca and Ba:Ca (Figure VII.4, B and D; Table 1). According to Figure VII.4, this finding implied that Sr:Ca was significantly different during the larval stage (individuals < 1 month old, average number of laser spots = 2.7 ± 0.96, higher values in CNP), and that the signals then converged during the juvenile stage (average number of laser spots = 13.0 ± 1.38) and the effect changed its sign (higher values in Palma) during the adult stage (average number of spots = 13.4 ± 2.47). The effect was the same for Ba:Ca except in the adult stage. Furthermore, a clear inverse effect between fish age and Sr:Ca was observed (Figure VII.4, A and B), regardless of the area analysed.

Table VII.1. Results of fixed and random effects for the GLMM on Sr:Ca and Ba:Ca (Box-Cox transformed). Approximate P values for fixed effects are derived from ANOVA type III and Satterthwaite's approximation for degrees of freedom, whereas significance tests for random effects are performed using a likelihood ratio test.

	Sr:Ca					
	Fixed effects					
	Sum Sq	Mean Sq	df	Dendf	F	Pr(>F)
Area	$5.10\cdot10^{-10}$	$5.11\cdot10^{-10}$	1	5.97	0.274	0.619
Stage	$3.73\cdot10^{-7}$	$1.86\cdot10^{-7}$	2	6.69	100.387	<0.0001
*Area*Stage*	$4.04\cdot10^{-8}$	$2.02\cdot10^{-8}$	2	6.69	10.856	<0.01
	Random effect					
Individual	Variance	S.D.	χ^2	df		
	$2.82\cdot10^{-10}$	$1.68\cdot10^{-5}$	1.36	1		
	Ba:Ca					
	Fixed effects					
	Sum Sq	Mean Sq	df	Dendf	F	Pr(>F)
Area	1.140	1.140	1	30	13.890	<0.001
Stage	0.769	0.384	2	60	4.681	<0.05
*Area*Stage*	2.383	1.192	2	60	14.516	<0.001
	Random effect					
Individual	Variance	S. D	χ^2	df		
	0.0360	0.1897	7.03	1		

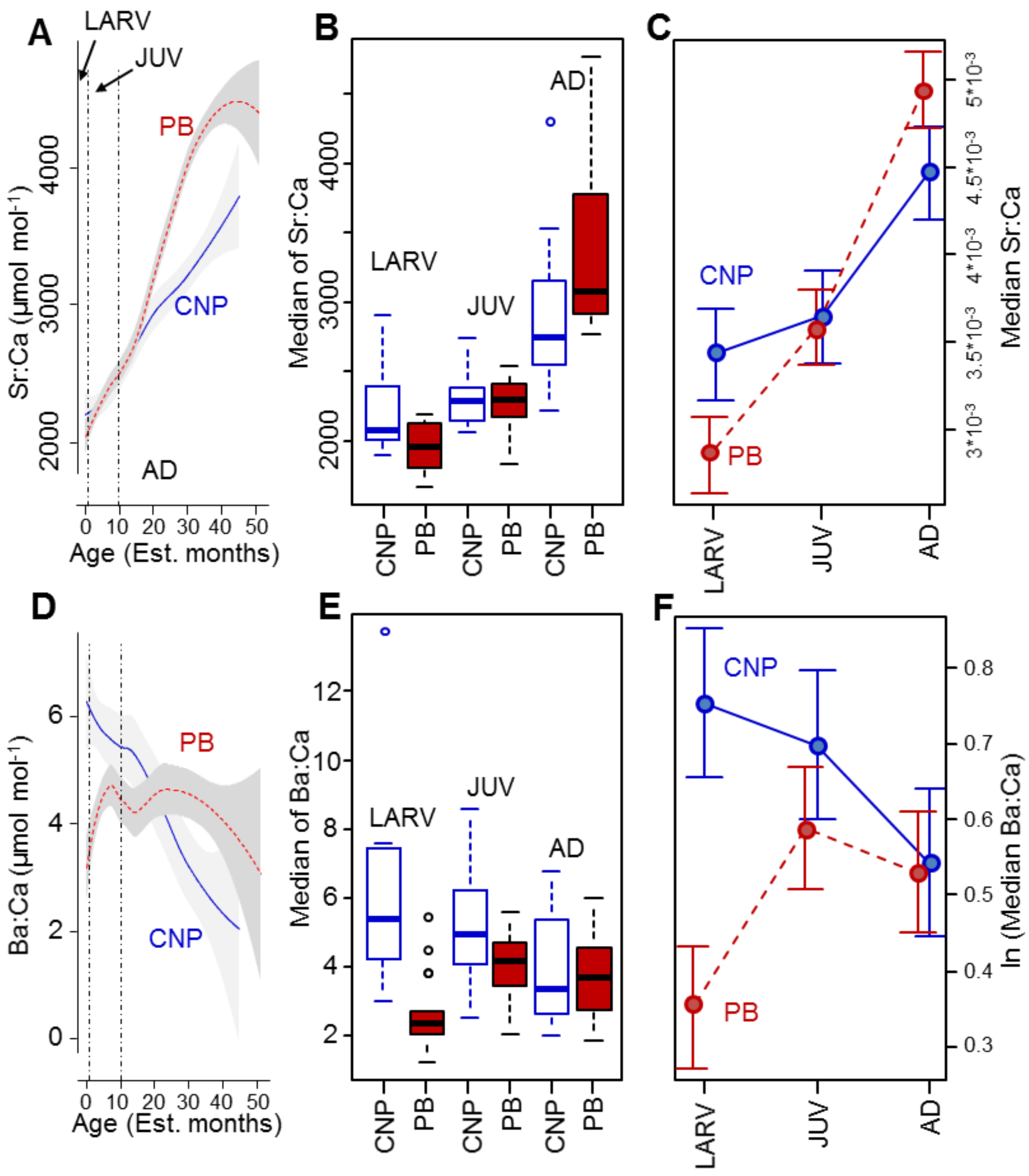

Figure VII.4. Upper panel, results for Sr/Ca. Lower Panel, results for Ba/Ca. A and D, smoothed (local polynomial regression) individual trajectories for Palma (PB) and Cabrera National Park (CNP). B and E, raw data (box-plots). C and F, interaction plots between Stage and Area (error bars are 95% CI, data are Box-Cox transformed) of the GLMM. LARV, larvae; JUV, juveniles; AD, adults.

Discussion

The usefulness of Sr:Ca and Ba:Ca in otoliths for detecting changes in salinity and/or productivity has been highlighted in general terms and has become the basis to study fish habitat selection and movement using otolith microchemistry (Campana 1999, Elsdon et al., 2008). When these links are claimed, the relationship between salinity and Sr:Ca is in an overwhelming number of cases positive, whereas the contrary is commonly observed for Ba:Ca, the latter also correlated with high productivity (Thorrold et al., 1997, Hamer et al., 2006, Mercier et al., 2011, Webb et al., 2012). However, recurrent cautionary notes remark that interpretation must account for multiple sources of variation including species-specificity, temperature and/or growth effects (reviewed in Campana 1999; Elsdon et al., 2008; Chang and Geffen 2013). Here we give evidence supporting that effects of life-history dynamics, not accounted for in these kind of field studies, are the most likely explanation for the main observed microchemical patterns.

In the present study, we present field evidence supporting 1) significantly different life-history traits between PB and CNP according to our hypothesis, and 2) strong interaction effects between the area of collection and the life stage in otolith Sr:Ca and Ba:Ca ratios, compatible with a strong effect of life-history strategy adopted in each subpopulation on Sr:Ca and Ba:Ca deposition. Before interpreting the implications of our results in a frame of life-history strategies (see below), we shall remark the reasons why we do not think that alternative hypotheses such as temperature of water chemistry can explain the bulk of the observed patterns. First, if water chemistry played a major role, we would not expect a significant interaction between area and life stage (which we observe): the chemical signals of the subpopulations would differ through their entire life, and curves would be similar in shape and rates. The likelihood that water chemistry suddenly differed between sites coinciding exactly with the shift in life stage, is low, and should persist for years. On the contrary, by overlaying the differences in growth through life we can more parsimoniously explain the observed results. Furthermore, and taking Sr:Ca as an example (whose dynamics are far more clear and consistent in the literature than those of Ba:Ca), even if salinity was much lower in PB (which is not, as shown in Figure VII.2, C), we would expect lower Sr:Ca levels in PB (the relationship between salinity and Sr:Ca is direct and in some cases non-significant see review in Elsdon and Gillanders (2003), Elsdon et al., 2008), but the observed results are in the opposite direction. In the case of Ba:Ca, however, we acknowledge that, as the literature reports, the microchemical dynamics may be linked to primary production, which was higher in PB. Second, the potential effects of temperature are negligible. The effect of temperature on otolith microchemistry is one order of magnitude less than that of salinity (Campana 1999), and our temperature differences were minimal and only detected in few weeks during the slow-growing period (Figure VII.2, A). The individuals, on the contrary, presented sustained (through life) differences in their microchemical patterns. Because of all these evidences, we believe that the following explanation may more plausibly explain our results.

Sturrock et al., (2015) showed an inverse relationship between growth and Sr:Ca, and a weaker relationship with Ba:Ca. Such a negative relationship can be understood within a simple mechanism of a surface-dependent ion incorporation (depending on the surface of the otolith capsule, which it is in direct contact with the endolymph) in comparison to a volume-dependent otolith growth. This would explain why, when an otolith rapidly grows, the unregulated incorporation of the ions, will occur at a relatively lower rate with respect to the calcium matrix. If we assume such an effect (observed in many papers, see reviews before) and account for the fact that we standardized for age and that seasonality was comparable between areas, we would expect that faster growing individuals, at comparable stages, would show reduced elemental ratios. If we account for the fact that energy investment changes from larval phases (growth translates into a somatic increase) to adult phases (energy investment will partly translate into reproductive costs), we can reconcile the results from changes in life-history traits and geochemical changes both through time and between areas.

Within this interpretative context, our data show that fast-growing larvae consistently showed lower Sr:Ca ratios than did older individuals, regardless of the place of origin (Figure VII.4, A and B). In the case of Ba:Ca, a weak growth vs. Ba:Ca relationship was observed (Figure VII.4, C and D). As reported elsewhere, the biokinetics of Ba incorporation is less clear compared with that of Sr and is influenced by a more complex array of factors (Campana 1999, Zhao et al., 2017). To this respect, weaker relationships between growth and Ba:Ca are generally reported in the laboratory (Sturrock et al., 2015). However, the significant interaction term showed that in CNP both Sr:Ca and Ba:Ca were higher (at the larval stage) than in PB, suggesting a lower larval growth in CNP.

The shift in geochemical relationships between PB and CNP was evident after maturation (Figure VII.3) and clearly matches with the life histories observed in both populations. We hypothesized that the life-history of the individuals would generate a different microchemical signal in the otolith. According to the recent relationship between the Sr:Ca and Ba:Ca ratios and growth and reproduction mentioned above, one would expect that life strategies favouring large body sizes (bigger-is-better) should show smaller values of Sr:Ca-Ba:Ca ratios at the adult stage than would life-history strategies favouring small body sizes and higher reproduction investment (smaller-is-better). The results of the present study clearly support this hypothesis. First, we observed that the recent signal (otolith edge) from individuals sampled in PB (smaller-is-better) had larger Sr:Ca-Ba:Ca values than did CNP (bigger-is-better), explained by high reproduction investment and low growth rates in PB. Second, the Sr:Ca ratio along the otolith shifted to a higher Sr:Ca after maturation in the individuals sampled in PB, supporting the hypothesis that higher reproductive investment and small adult body size may be favoured by size-biased mortality. This shift was not as evident for the individuals sampled in the CNP suggesting a higher investment into somatic growth and lower investment in reproduction (somewhat expected to maximize their older life-spans). Therefore, the individuals sampled in PB, having invested a larger fraction of the energy in reproduction, were likely crystallizing a smaller amount of aragonite than the individuals of CNP, resulting in an increased Sr:Ca. This pattern was not as evident for Ba:Ca although some divergence patterns were also observed among the individuals of both populations. According to these results we can certainly conclude that the life-history of the individual affects the deposition in the otolith of Sr and Ca, and to a lower extent of Ba, generating different microchemical patterns.

These results in terms of diverging life-history traits between PB and CNP are in the same direction of those previously published for the painted comber (*Serranus scriba*), a small-bodied serranid captured in the same areas and subjected to the same angling pressure and lack of hydrodynamic connectivity. *D. annularis*, as the painted comber, shows high site-fidelity and a small home range of ~ 1 km2 (Alós et al., 2012), suggesting that in addition to slight ontogenetic depth displacements (Harmelin-Vivien et al., 1995), the potential change in habitat characteristics occurs at a scale orders of magnitude smaller that the distance between the sampled areas. We therefore contend that we might be analysing a species that responds similarly to the painted comber in terms of downsizing in front to recreational fishing pressure. Our geochemical data support the concept that the effects of fishing on sedentary fish inhabiting relatively homogeneous waters can indirectly (i.e., by selecting particular growth rates and reproduction investment histories) induce large variations in otolith chemistry. Therefore, this source of variations adds to the covariables considered in future studies using otolith microchemistry to track habitat changes. We acknowledge the need for further studies in the laboratory under controlled conditions, but rearing these small bodied coastal species has been unsuccessful to date. We also acknowledge the need for a deeper understanding in the interplay between reproduction investment and the physiology of the otolith. Our results not only offer support for the claimed importance of individual physiology on the elemental ratios but also highlight the potential effects of unaccounted external factors (trade-offs between growth/reproduction and mortality at different life-stages). Notably, we do not suggest a lack of validity of the approach of otoliths microchemistry as biotracers in the case of strong gradients of, for example, salinity or temperature. However, we highlight that any other unaccounted factors affecting growth rates (e.g., fishing and/or natural predation), might exert strong regulation in the microchemical signal via highly differentiated individual energy allocation, and should be not only accounted for in field analyses but this fact could be even used to identify long-term alteration of population traits due to fishing.

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Chapter VIII: General Discussion, remarks and future opportunities

Understanding the complexity of the processes that regulate both settlement and recruitment which occur at different time and spatial scales, and how they are affected by anthropogenic uses of the coastal habitats is crucial for the management of coastal fisheries, from both conservationist and socio-economical points of view. In this thesis, we have tried to broad the knowledge about these processes in the Mediterranean Sea using a wide multidisciplinary lens. Throughout the different chapters, we have tried to focus in different specific questions along several key moments of the life cycle of the coastal fish species (Figure VIII.1). Along each of the chapters, we have provided a detailed discussion on each specific issue, thus in this section a relatively short yet integrative view is provided, together with my perspective on future work, in the light of the work provided herein.

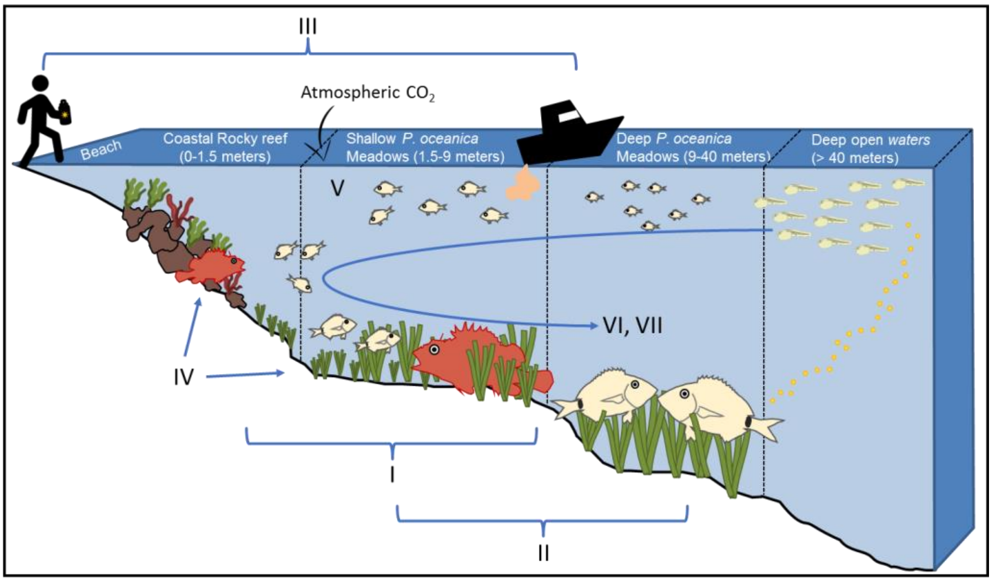

Figure VIII.1. Typical Mediterranean coastline seascape, from shallow sand or pebble beaches, to rocky and macrophytes areas, shallow and deeper seagrasses and gross sand/maerl deeper than 40 meters. The life cycle of *D. annularis* is used to illustrate the workflow of this thesis; in Chapter I we focused on shallow *P. oceanica* meadows as juvenile habitats; in Chapter II we explored a method for analysing juvenile dynamics at wider depth-ranges using SBRUVs; Chapter III is devoted to assess the anthropogenic impact on the juveniles' olfactory capacities; in Chapter IV analysed the effect of predatory olfactory cues on the development of morphological defences; along Chapter V we assessed the impact of acidification on the fatty acid composition of recently settled juveniles; finally,

in Chapters VI and VII we used otoliths to analyse life history traits throughout the different developmental stages.

The ecological and fishery values of the Balearic Archipelago underwater seascapes have been recognised for long time and protection measures have increasingly been promoted for the last 20 years, with the creation of several Marine Protected Areas and the continuous interest and collaboration of the local government and research institutes. In section I we gave a glimpse to the value of the iconic *Posidonia oceanica* meadows for the juvenile fish communities. Thanks to the early studies from Massuti and colleagues (Massuti, 1965; Oliver, 1966), we could perform a comparison of the community change from a unique historical perspective. Specifically, we found a community shift that have happened along the last 50 years' period using an old fishing traditional gear to capture coastal shrimps: the ganguil (Figure VIII.2).

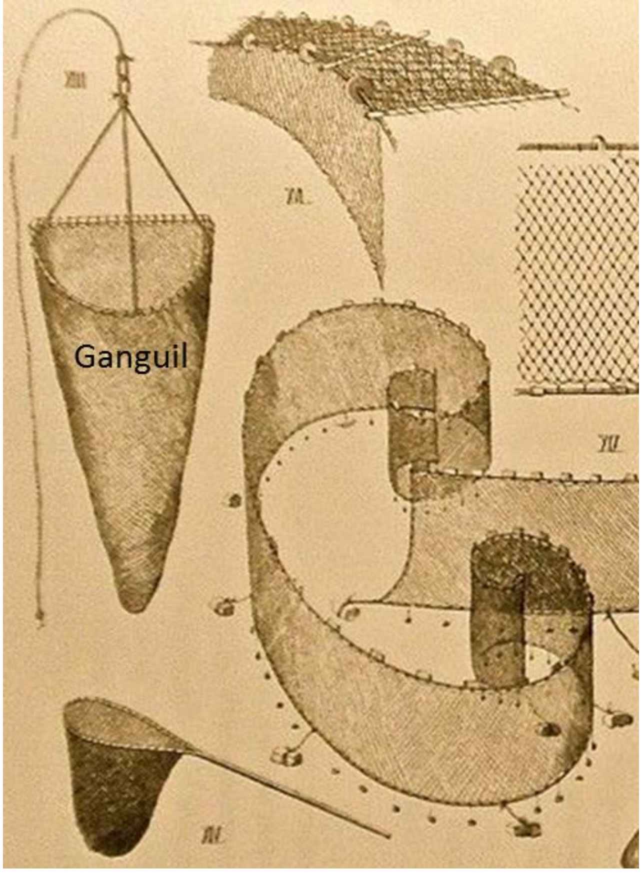

Figure VIII.2. A piece of an art sheet from Die Balearen (Salvator, E.L., 1897) showing some of the traditional Balearic fishing gears. Ganguil for shrimp fishing is depicted at the top left.

On the other hand, we found that shallow (2-6 m) *P. oceanica* meadows are used sequentially by settlers of different species, most notably by their abundance by *Diplodus annularis*, *D. vulgaris*, *Sarpa salpa* and *Symphodus ocellatus.* These species progressively disappeared from these shallow areas as they grew, probably in a combination of ontogenetic movement/habitat shift and mortality processes. The differences between the two periods, 1960 and 2012-2013 might not directly be related with the decrease in the intense fishing over these areas (actually using "ganguil"), but also due to habitat loss, increment of pollution or physical anthropization of the nearshore habitat. However, the absence of nowadays' very common species such as *D. annularis* and *D. vulgaris* in 1960's samples suggests a potential effect of the intensive use of "ganguils" in the Balearic archipelago, especially in Palma Bay where peaks of 200 boats per night were observed trawling during Saturdays and Sundays (Massuti, 1965). Furthermore, in Massuti's samples the fraction of early settled juveniles of sparids seemed to be absent, and we hypothesised that they may be settling at deeper areas that those swiped by ganguil users in order to maintain the adult populations. In addition, not only other species of local commercial interest, such as the black scorpion fish (*Scorpaena porcus*) had increased their abundances into the *P. oceanica* meadows in recent years, but also the protection of this habitat may have helped species of high ecological interest like members of Syngnathidae family (e.g. seahorses, pipefish etc).

Based on the results of from Chapter I, it was clear that many questions on the changes in settlement patterns could not be answered if the settlement habitat is not properly sampled. For example, the size-related occupancy of seagrass meadows along depth and time is unclear for many species. Based on this, and on the compelling need to develop robust and low-cost sampling methods, we developed a tool to advance this knowledge getting intensive sampling at deeper areas than using the ganguil, and even more without altering the communities nor the *P. oceanica* meadows: the stereoscopic camera system. We presented the design and performance of this system in detail along the Introduction and Chapter II. We contend that this is a small yet potentially useful contribution to coastal ecology and fisheries science, since it allows for the estimation of community composition and relative abundance of several species, and it provides accurate estimates of early stages size structures, using the accompanying software for measuring fish. As an example, we found similar abundances of *D. annularis* early juveniles both at the shallower and deepest strata analysed (2,10 and 20 m, deeper than previously reported 5-8 m (Harmelin-Vivien et al., 1995)), supporting our prior hypothesis that this species may settle throughout most of the depth range of *P. oceanica* meadows. Other potential uses of video-based technology include measurements of behavioural variability among different species (e.g. Alós et al., 2014), communities' assemblages or among different behavioural types from the same species (e.g. Alós et al., 2015), which can be of great interest for evaluating the effects of rapid changes in fish populations induced by for example recreational fisheries. The use of stereo-cameras, like this one or similar ones adapted to deeper habitats, incorporating illumination, baited or not, deployable or fixed, open a wide field of research for future work. In this context, and in collaboration with the Government of the Balearic Islands, we have installed an underwater fixed observatory in Port d'Andratx (visit https://imedea.uib-csic.es/sites/sub-eye/) which continuously stores permanent data on fish communities, as well as serving as an excellent informative platform for the general society (Figure VIII.3 and Figure VIII.4). Moreover, it has been included as a part of the international network of underwater observatories (OBSEA, (Aguzzi et al., 2015))

Figure VIII.3. Photograph while cleaning the SUB-EYE: Port d'Andratx underwater cabled coastal observatory situated in front of a wreck. Picture cortesy of P. Arechavala.

Figure VIII.4. Photograp: a group of gilthead seabreams (*Sparus aurata*) captured by the SUB-EYE wreck camera.

Along these two chapters we discussed specifically how the anthropogenic use of the coast (e.g. the growth of Palma harbour, see annex, Supplementary figure I.1), may have altered the structure of juvenile fish assemblages. In Section II we went a step further and investigated whether the anthropogenic impact along the coast was affecting key behavioural issues in juvenile fish. We used gilthead seabream (*Sparus aurata* (Linnaeus, 1758): Sparidae) as model species, since it is widespread in Europe and it has an important economic role in both commercial and recreational fisheries, as well as in aquaculture (Brown et al., 2005). Firstly, in Chapter III, we explored the juvenile ability to detect chemical cues. In particular, we focused on man-related cues associated to port-related products and sunscreen at ambient concentrations. Secondly, in Chapter IV, we explored the effect of chemical cues from a more ecological/adaptive point of view: do predator-prey interactions alter or modify the body shape of these juveniles?

Choice chamber experiments with *S. aurata* suggested that juveniles presented altered behaviours when entering sunscreen polluted water, as well as fewer scape movements (sprints) when confronted with *P. oceanica* and Harbour waters. Therefore, we provided evidence of the capacity of these juveniles to detect different chemical cues, although the interpretation of the results in terms of consequences for the fish is elusive. A step forward in our work was to introduce new metrics for analysing the video data from these type of experiments: instead of using only the time spent in each water type (analysis that many times yields non-significant results (Jutfelt et al., 2016), and may remain unpublished), we showed that a strong behavioural response could be observed through the difference in mean and variance of speed of individual movements triggered by the detection of a chemical cue. This methodology was used in a similar study with the most abundant species in shallow *P. oceanica* meadows (as observed in Chapter I), the Ocellated wrasse (*Symphodus ocellatus* (Linnaeus, 1758)) (Gouraguine et al., 2017). Although that work is not included as part of this thesis, the methodology was developed within this thesis, and has raised quite of an interest among the scientific community, which opens possibilities for further collaborations and potential work within this line of research.

Behaviour and morphology are often hand by hand, and clear examples exist on how a behavioural type can be induced by predators, concurrently including a change in the body shape of the prey (Brönmark and Miner, 1992; Domenici et al., 2008). Regretfully, we could not provide such a direct link within this thesis, but we did find, for the first time in temperate marine fish, odour-related predatory-induced morphological changes in individuals exposed to an odour treatment. In the literature, it is suggested that altering ones' shape can have several advantages in terms of survival probability (Domenici et al., 2008), since a higher number of predatory induced morphological defences (PIMDs) directly correlates with better escaping performance and increased predatory handling time (e.g. Eklöv and Jonsson, 2007). For example, some increases in body depth in freshwater fish are known to provoke difficulties for attacking predators due to a reduction in the mouth gape/prey height ratio (Nilsson and Brönmark, 2000). In our case juveniles of *S. aurata* did not only experience a change in body shape within only one month of treatment with the odour cues of predators and conspecifics, but this response was found to be reversible. Reversibility of PIMDs had only been found in a freshwater species (*Carassius auratus*) (Chivers et al., 2008) and in amphibians' tadpoles (Schoeppner and Relyea, 2009). In Chapter IV we offer the first evidence of reversible PIMDs in marine taxa. Moreover, this reversibility suggests that there must be an energetic associated trade off for developing PIMDs that deserves further attention. For instance, it is unknown whether the PIMDs are inducible at different life stages or only during early life stages. While we did use aquaculture individuals for this experiment, our results may have ecological relevance: in Chapter I we found an increment in the top predator *Scorpaena porcus* (the odour of which was used as a chemical cue in Chapter IV) among the shallow seagrasses meadows a threat that juvenile fish from many species have to face in their early stages.

In both Chapters III and IV we focused on ecological questions involving the recruitment in a highly anthropized coastal environment. However, in Chapter V we aimed for a global threat with potential effects on fish: ocean acidification. The increment of anthropogenic CO_2 dissolved in the ocean is leading to a worldwide acidification that has some undesired (yet largely understudied) effects on marine life. Despite having used typical Mediterranean species along all the chapters of this thesis, in Chapter V we used a model species form the North American's coast line, the sciaenid red drum (*Sciaenops ocellatus*) for an ocean acidification (OA) experiment. We explored the effect of OA on the otolith shape and growth of early developmental stages (discussed in depth in Chapter VI). Additionally, we focused on the OA effects on larvae fatty acid (FAs), because certain FAs correlate with better antipredator performance (e.g. time of startle response or survival) thus there may be a link between OA, FAs and survival. We measured the FA of the larvae under OA and performed behavioural trials on them. Unfortunately, the videos on behavioural responses were damaged and could not be analysed for this thesis. Many questions remain open as to the importance of OA on fish species. Future work must address the large variability in OA in coastal areas, which can be naturally under daily fluctuations in pH due to primary production (e.g. estuaries) or coastal lagoons, and how adaptations to this fact may operate under scenarios of global CO_2 increase. Experiments on transgenerational effects of OA are also required in all species to analyse adaptation, but it is likely that the investment of research funds is firstly diverted towards more clear effects, such as temperature or hypoxia. In any case, the increment of FAs found in fish under ocean acidification remains as an interesting result (first in its kind) and broadens the knowledge of the effects of OA on marine organisms, moreover if results obtained for red drum could be similar for the Mediterranean species remains unknown.

Otoliths have been used extensively in fish and fisheries studies, and along this thesis otoliths have been used as indirect indicators of life history, bot in terms of their capacity to evaluate developmental stress (Chapter VI) and to analyse, via microchemistry, the effect of fishing in shaping sub-population structure at small spatial scales (Chapter VII). Chapter VI is actually an example of the so called "negative results" or results that produce non-significant effects. Under a given hypothesis, the accumulation of studies showing no effects, may be an indication of a lack of generality of that hypothesis. This is what it is shown in Chapter VI. The initial (usually accepted) hypothesis in that case was that individuals with high otolith fluctuating asymmetry (OFA, here the difference between left and right otoliths) have experienced troubles during their development and therefore survival (more stressed individuals would be more prone to death). Therefore, it was expected to see a decreasing trend in OFA during developmental stages due to selection (see Figure VIII.5 below).

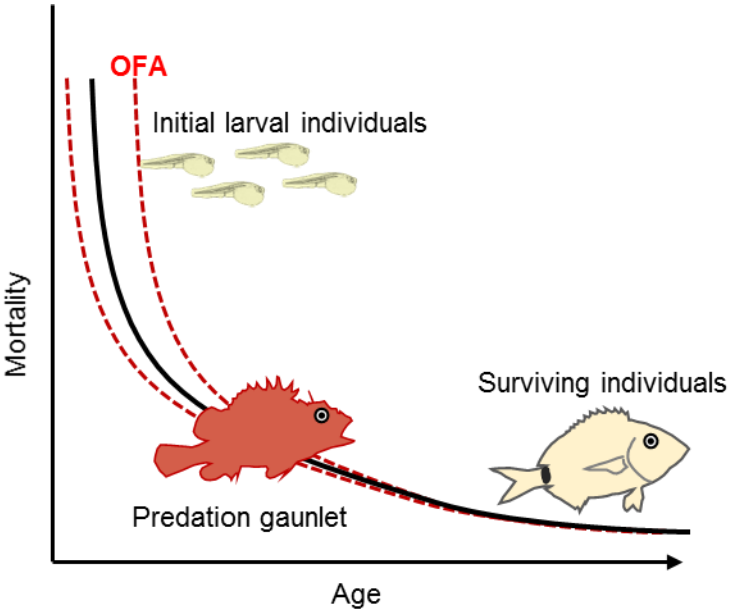

Figure VIII.5. Expected trend on the mortality (i.e. decreasing number of individuals) and "amount" of OFA (reduced from the starting point towards survivals due to mainly predatory pressure on less fit individuals

Although some species did exhibit the expected trends (yet not significantly different), other species were found to present the opposite and unexpected trend (Figure VIII.6).

In this case, the utility of OFA as indicator of stress and or surviving individuals was discarded as a general trend.

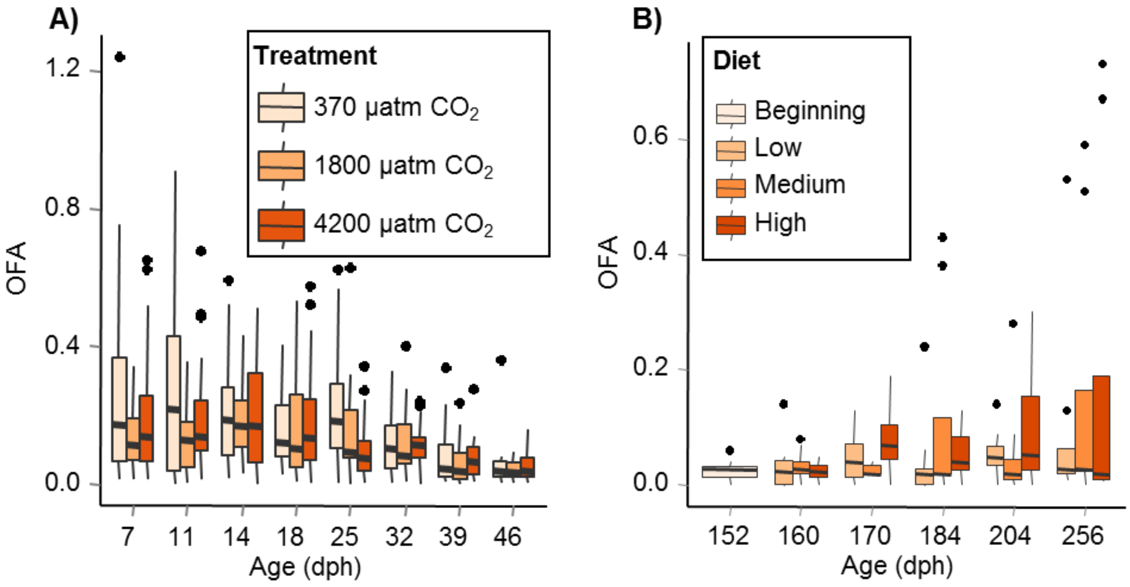

Figure VIII.6. Otolith fluctuating asymmetry (OFA) in two fish species during larvae/juvenile phases. A) Cod (*Gadus morhua*) larvae during 46 days under different levels of Ocean acidificiation; B) Atlantic croaker (*Micropogonias undulatus*) juveniles. Modified from Chapter I.

Whereas in Chapter VI we focused on a certain trait (OFA) in different species at a precise life stage or during a determined short period under a wide variety of stressors, in Chapter VII we used otoliths to compare life history traits (LHT) among different populations of a single specie (*D. annularis*) under three (likely) combination of factors: fishing pressure, oceanographic isolation and predator pressure. Prior laboratory experiments had demonstrated that the incorporation of metals to the otolith matrix was dependent not only of the surrounding water/diet composition but on growth dynamics (Sturrock et al., 2015, 2014). We hypothesized that under strong selection drivers for growth such as fishing pressure (in this case mainly recreational, due to the scarce economical value of *D. annularis*) and predation in contrasting relatively isolated (in terms of connectivity) areas, the resulted populations would differ in their otolith microchemical signatures despite of growing in very similar water masses. Our assumptions are supported by prior studies on oceanographic isolation (Basterretxea et al., 2012) and fishery effects on growth traits of other species between these two areas

(Alós et al., 2014a). We found significant differences in the LHT between both sites, and a concurrent difference in the ratios of Sr:Ba and Ba:Ca further confirming the initial hypothesis, i.e. a well differentiated life-history strategy in each subpopulation of *D. annularis.* Our results, although not absolutely conclusive, strongly suggest that microchemical signatures may be largely affected by impacts such as fishing pressure, if the characteristics of the surrounding water are not largely different.

The Balearic Islands offer an incredible scenario for testing hypotheses on recruitment processes of recreational fish species; they include MPAs with over 20 years of effective protection and the largest array of MPAs in Spain, together with highly impacted areas such as Palma Bay, Port d'Andratx or Pollensa Bay. The fishing recreational activity is one of the highest in the Mediterranean, and there are even opportunities to conduct before-after control-impact experiments, due to the creation of recent MPAs such as Dragonera. This thesis opens more questions than it closes, as expected. Nonetheless, the analysis of settlement/recruitment processes has been here tackled in a wide sense, and no attempt to incorporate this knowledge into the dynamics of a single population has been made. The reason for that is that the knowledge on population dynamics of recreational species is still at its infancy in the Mediterranean. Most research investment on fisheries ecology is directed to commercial species, and therefore many knowledge gaps remain open for less-studied species. However, we believe that the required step forward on population dynamics of coastal fish species (quantification of recruitment, determination of the effects of environmental vs human factors on its variability etc) can benefit from our results and developed methodologies. We expect to see the use of low-cost video-based techniques to advance this new knowledge, and to combine laboratory experiments and field data into modelling exercises to solve practical and scientifically relevant questions on recreational fish species at several spatio-temporal scales.

Conclusions

1. Within a year, shallow *Posidonia oceanica* meadows are sequentially used as nursery habitats in the Mediterranean for species of recreational interest, and can be adequately studied using non-damaging small beam trawls “ganguil”.
2. The prohibition of the intensive use of ganguil in the 60’s may be related to the current higher abundance in the number of juveniles of fish species of interest for both, artisanal and recreational fisheries.
3. Stereoscopic low-cost video cameras can be effectively used to provide accurate measurement of juvenile fish, and open opportunities for a better analysis of recruitment processes.
4. *Diplodus annularis* juveniles were found at all analysed depths in similar abundance, suggesting that the nursery value of *Posidonia oceanica* meadows can be higher than expected for this species.
5. Sunscreen pollutants steamed from bathers affect the behaviour of *Sparus aurata* juveniles making them less reactive.
6. *Sparus aurata* juveniles do not negatively react to harbour collected water. The value of these anthropized areas for some species needs to be further evaluated.
7. Predatory smell induces morphological defences on *Sparus aurata* juveniles. These changes are reversible at the short term, indicating a potential energy trade-off with untested ecological consequences.
8. Ocean acidification alters the internal composition of *Sciaenops ocellatus* early stages in terms of their fatty acid composition, which in turn may have consequences for survival.
9. Otolith fluctuating asymmetry was not related with any stressor in eleven different studies. We thus show the value of negative results, and the lack of generality of the usual hypothesis on OFA.
10. Differences among subpopulations in otolith microchemistry can be explained by differences in life history strategies, under scenarios of comparable water masses and differing pressure selections for growth and reproductive investment.

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Settlement and recruitment processes.

Lists of figures, tables and abbreviatures

List of figures

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List of abbreviations

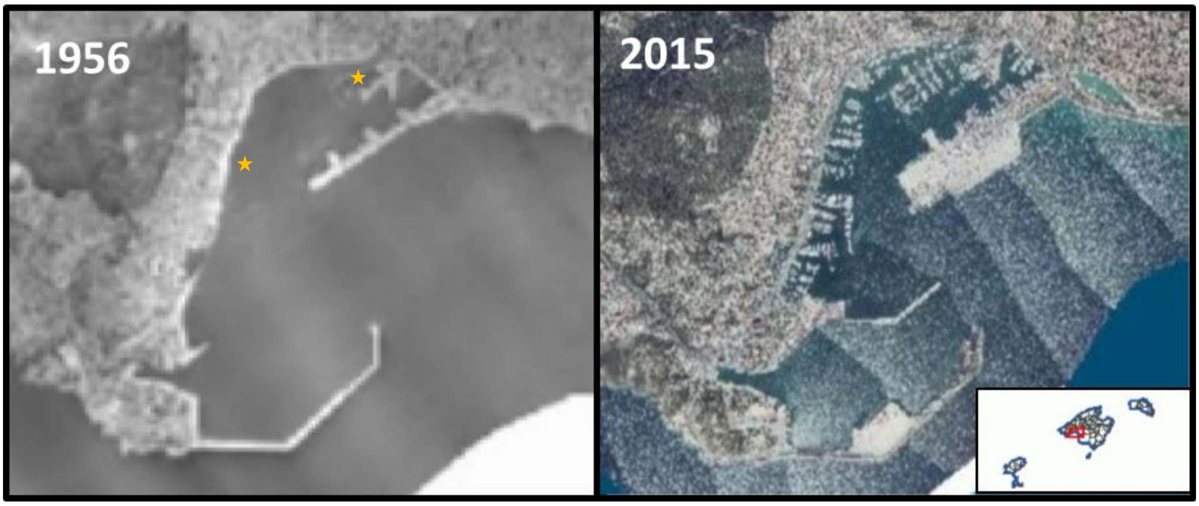
Abbreviature	Complete name
CNP	Cabrera National Park
CO_2	Carbon dioxide
Dph	Days post hatching
FAs	Fatty acids
GLM	General Linear Model
LA-ICPMS	Laser Ablation Inductively Coupled Plasma Mass Spectrometry
LHT	Life history traits
MPA	Marine protected area
OA	Ocean acidification
OFA	Otolith fluctuating asymmetry
PB	Palma Bay
PIMDs	Predatory induced morphological defences
RDA	Redundancy Analysis
SBRUV	Stereoscopic Baited Remote Underwater Video
VBGM	Von Bertalanffy Growth Model

Settlement and recruitment processes.

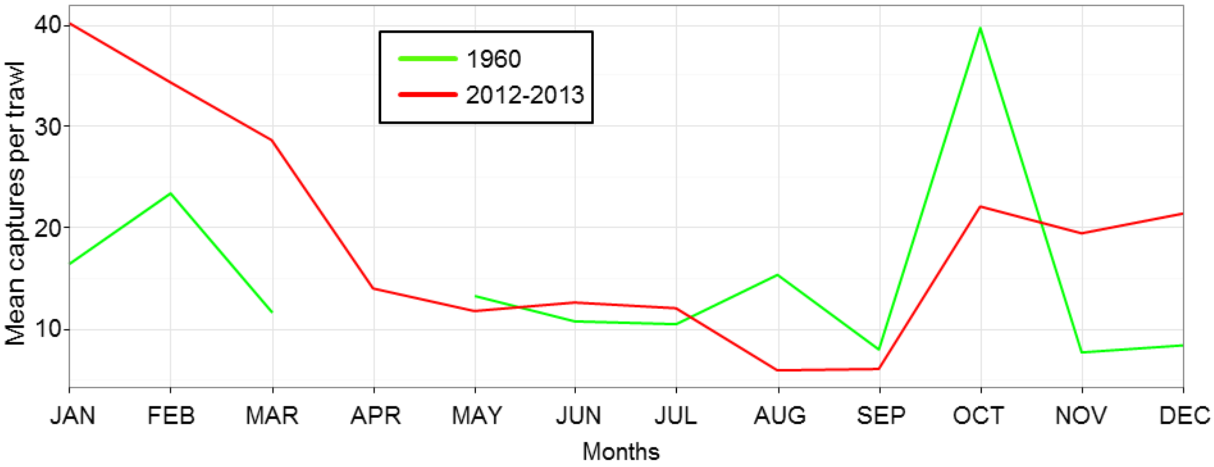
Annexes

I. Supplementary material Chapter I:

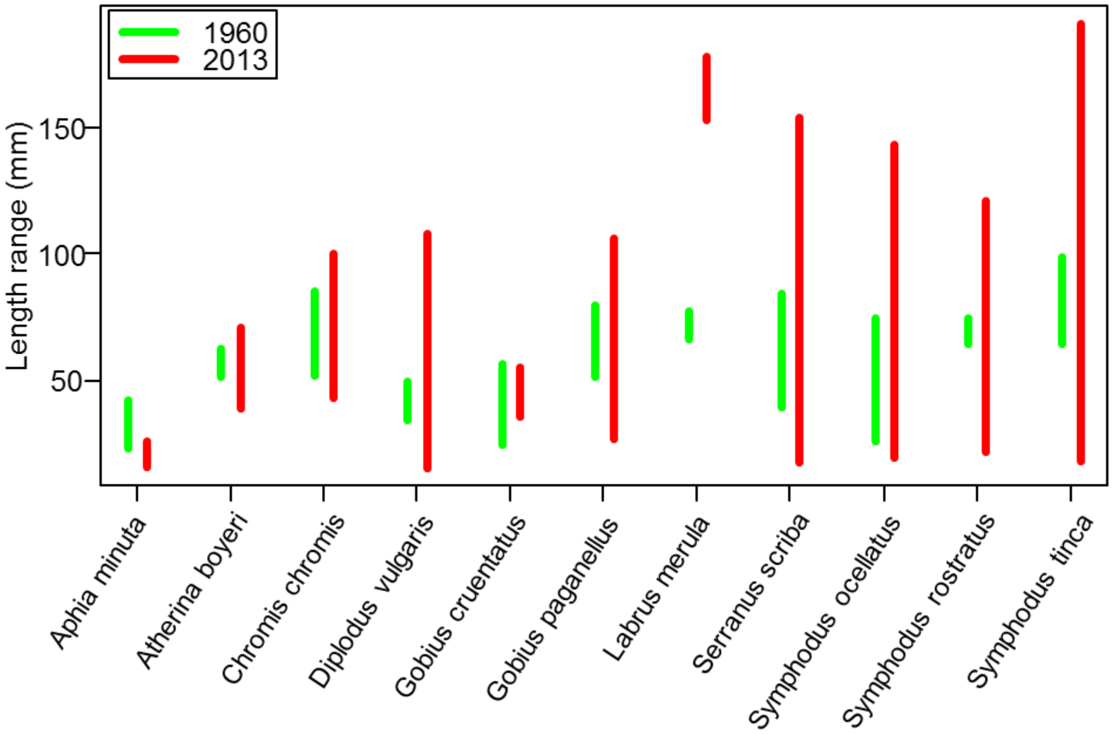
Changes in the juvenile fish assemblage of a Mediterranean shallow seagrass nursery area after half century of nearshore trawl prohibition.



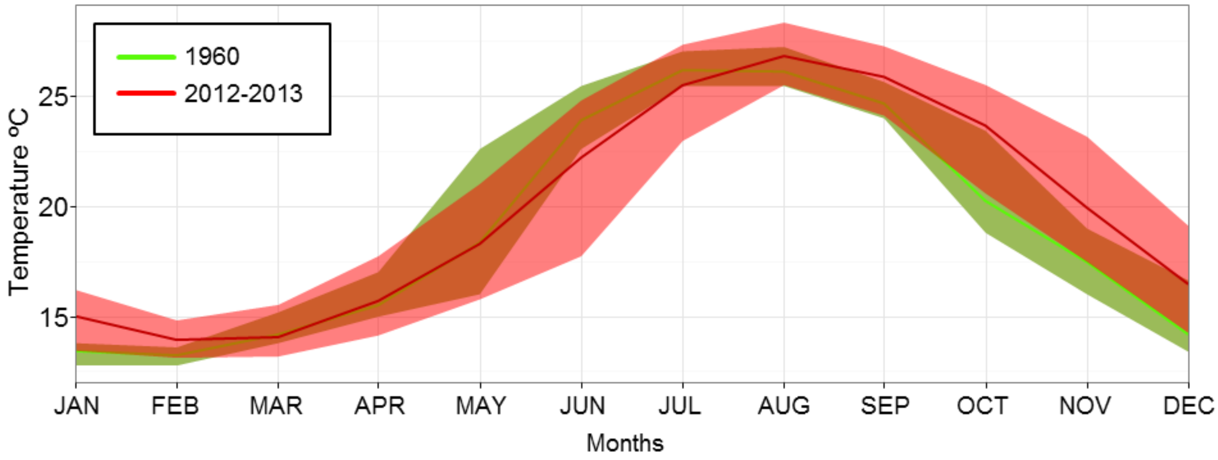
Supplementary figure I.1: Palma Bay in 1956, aerial photography. From the Service of territorial information of the Balearic Islands (Sitibsa). The sampling areas from 1960 Station I and Station II (yellow stars) are nowadays completely anthropized and covered with pontoons (see detail of an aerial photography from 2015, Google).



Supplementary figure I.2: Mean captures per trawl during years 1960 and 2012-2013 (number of individuals).



Supplementary figure I.3: Length range (TL) of individuals captured in 1960 and 2012-2013. Only species captured in both years (abundance >1 individuals) are shown. The rest of species and length ranges can be found in Table 2.



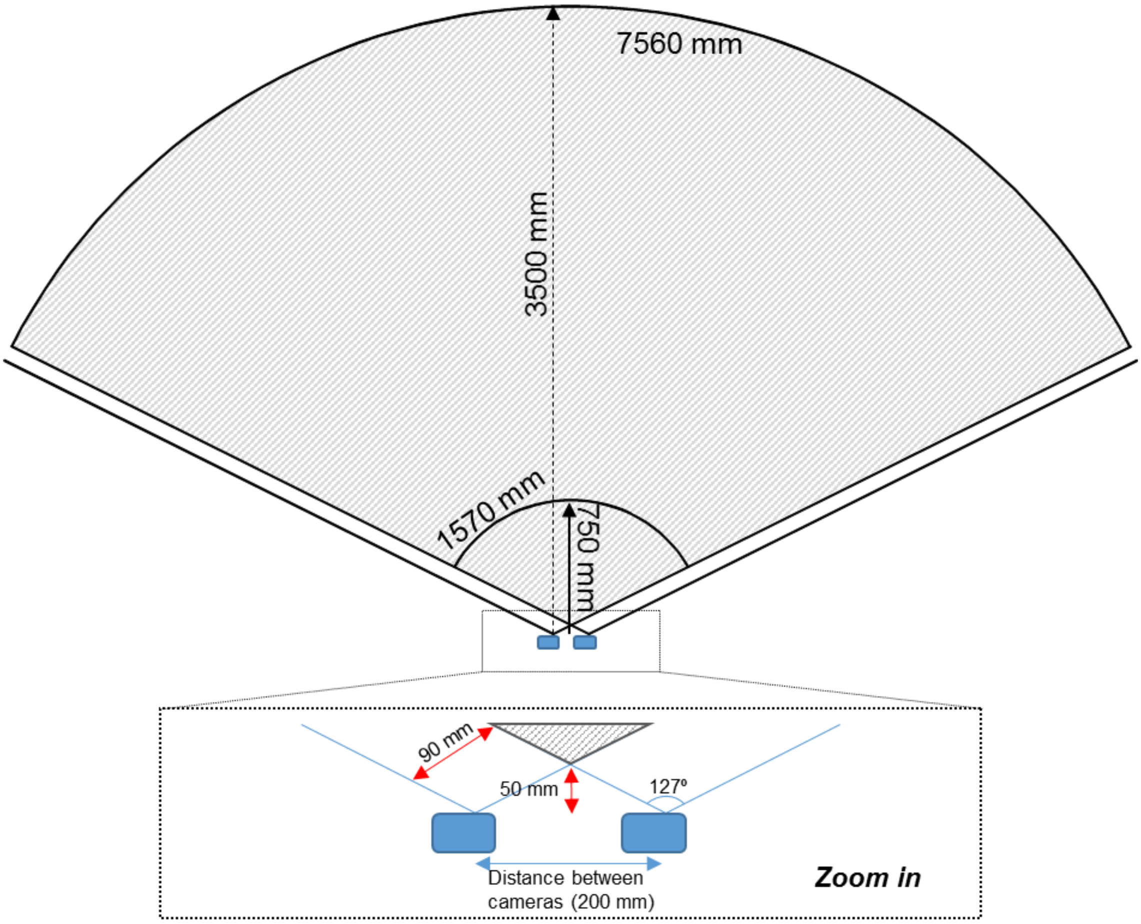
Supplementary figure I.4: Temperatures (ºC) along 1960 and 2012-2013 periods.

Supplementary table I.1. environmental variables used for the 2012/2013 analysis period. Variables marked with a No were highly correlated with others (>60%) and therefore not used in the RDA model.

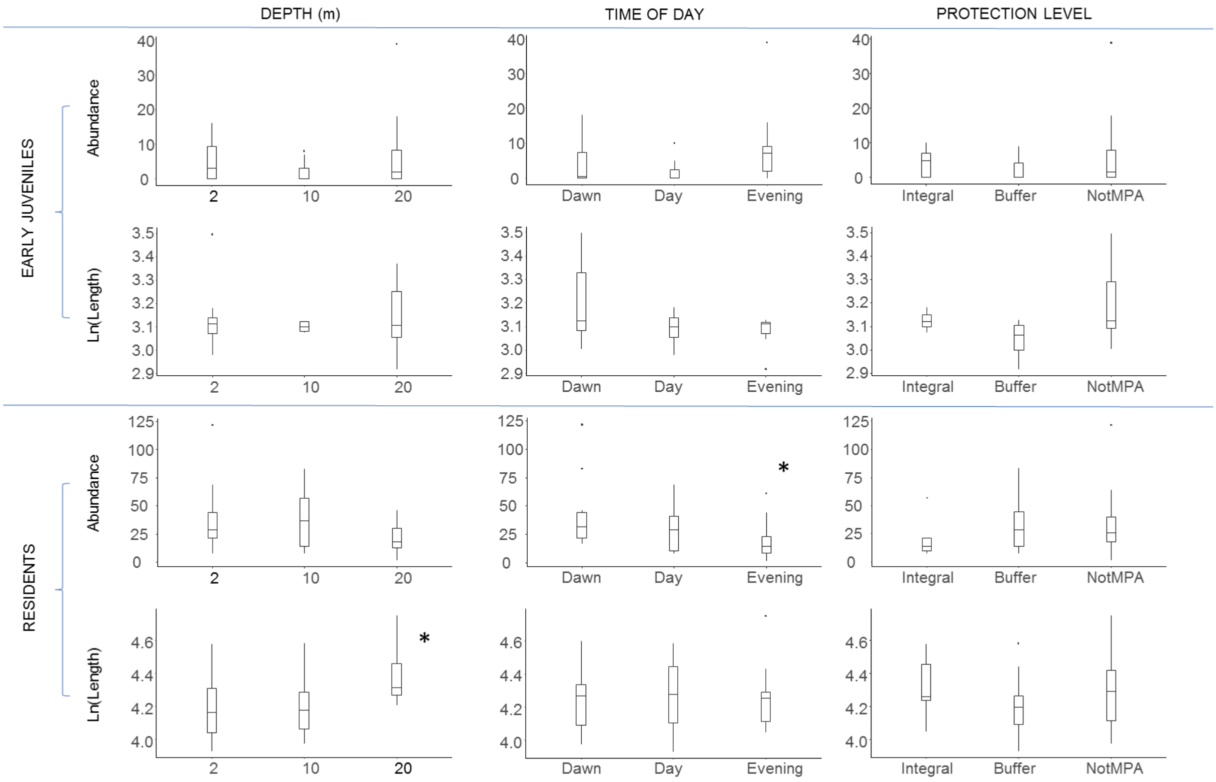
Variable	Origin	Units	RDA model
Sea Surface Temperature	Satellite	ºC	Yes
Salinity	Buoy	PSU	Yes
Significant wave Height	Buoy	m	Yes
WEWave	Buoy	m	Yes
SNWave	Buoy	m	No
SurfaceCurrent	Buoy	m/s	Yes
WECurrent	Buoy	m/s	Yes
SNCurrent	Buoy	m/s	Yes
WindIntensity	Buoy	m/s	Yes
WEWind	Buoy	m/s	Yes
SNWind	Buoy	m/s	Yes
SSTanomaly	Modelled	ºC	Yes
ModelTemperature	Modelled	ºC	No
ModelSurfaceSalinity	Modelled	PSU	No
ModelSurfaceCurrent	Modelled	m/s	No
ModelWECurrents	Modelled	m/s	Yes
ModelSNCurrent	Modelled	m/s	No
Moon Phase	Calculated	% of moon	Yes
Photoperiod	Calculated	h	Yes
Depth	Measured	m	Yes

II. Supplementary material Chapter II:

Using stereoscopic video cameras to evaluate seagrass meadows nursery function in the Mediterranean



Supplementary figure II.1. Complete aerial view of the stereoscopic field of view of the SBRUV system with a close up view of the cameras set-up. At 3.5 m from the cameras a total field of view with 7.76 m amplitude.

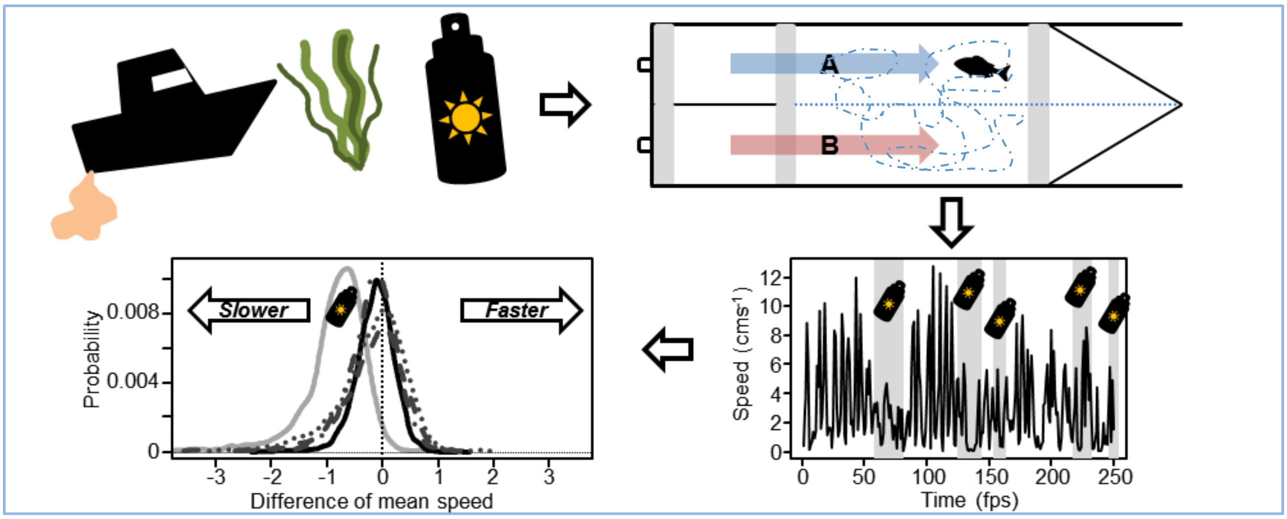


Supplementary figure II.2. Exploratory boxplots of the distributions of abundance (number of individuals per video) and natural logarithm of the fish length for both early juveniles (above) and residents (below) along the factorial design: Depth category (2, 10 and 20 m); Time of day (Dawn, Day, Evening); and Protection Level (Integral, buffer or NotMPA). * denote significant effects.

Supplementary figure II.3. An image taken from the Left Camera's footage displaying two early juvenile *Diplodus annularis* (marked with a red ellipse). The presence of two leaves of *Posidonia oceanica* (Bottom Right) showcase the issue of obscured areas on the footage while measuring juvenile fish.

III. Supplementary material Chapter III:

Anthropogenic chemical cues can alter the swimming behaviour of juvenile stages of a temperate fish.



Supplementary figure III.1: Graphical abstract from Chapter III as published in *Marine Environmental Research*.

IV. Supplementary material Chapter V:

Ocean acidification increases fatty acids levels of larval fish

Supplementary table IV.1. Univariate differences in fatty acid composition on a weight basis (mg FA g-1 dry weight). Effect of treatment was assessed using ANOVA (log-transformed data when needed) or Wilcoxon tests. Significant results are denoted in bold and marked with * P <0.05; ** P < 0.01; and *** P < 0.001.

Fatty acid	Control		High CO_2		Transformation/ Analysis	P	
	Mean	s.e.	Mean	s.e.			
12:0	0.048	0.003	0.059	0.006	ANOVA	0.114	
14:0	1.007	0.050	1.064	0.037	ANOVA	0.384	
15:0	0.206	0.010	0.262	0.009	ANOVA	**0.002**	**
15:1	0.041	0.009	0.100	0.020	Log/ANOVA	**0.010**	**
16:0	12.910	0.728	15.719	0.420	ANOVA	**0.007**	**
16:1ω7	1.804	0.096	1.818	0.051	ANOVA	0.904	
16:2ω4	0.648	0.037	0.717	0.026	ANOVA	0.164	
16:3ω4	0.390	0.019	0.548	0.059	Log/ANOVA	**0.016**	*
17:0	0.382	0.027	0.521	0.014	Log/ANOVA	**0.003**	**
18:0	5.172	0.340	6.920	0.199	Wilcoxon test	**0.004**	***
18:1ω9	8.116	0.407	10.016	0.265	ANOVA	**0.003**	**
18:1ω7	3.000	0.137	3.655	0.104	ANOVA	**0.003**	**
18:2ω6	2.114	0.068	2.464	0.073	ANOVA	**0.005**	**
18:3ω3	1.104	0.017	1.421	0.061	ANOVA	**0.001**	***
18:3ω4	0.071	0.004	0.102	0.003	ANOVA	**0.001**	***
18:3ω6	0.150	0.007	0.199	0.006	ANOVA	**0.001**	***
18:4ω3	0.508	0.030	0.521	0.017	ANOVA	0.710	
20:1ω9	0.555	0.028	0.573	0.024	ANOVA	0.649	
20:2ω6	0.164	0.019	0.245	0.011	ANOVA	**0.005**	**
20:3ω3	0.185	0.011	0.221	0.023	ANOVA	0.196	
20:3ω6	0.185	0.013	0.270	0.013	ANOVA	**0.001**	***
20:4ω3	0.365	0.006	0.407	0.012	ANOVA	**0.013**	*
20:4ω6	3.879	0.178	4.959	0.145	ANOVA	**0.001**	***
20:5ω3	5.001	0.172	5.376	0.161	ANOVA	0.142	
22:5ω3	1.194	0.038	1.318	0.035	ANOVA	**0.038**	*
22:5ω6	2.181	0.101	2.828	0.084	ANOVA	**0.001**	***
22:6ω3	15.047	0.422	17.850	0.482	ANOVA	**0.001**	***

Supplementary table IV.2. Univariate differences in relative fatty acid composition (% total fatty acids). Effect of treatment was assessed using ANOVA (log-transformed data when needed) or Wilcoxon tests. Significant results are denoted in bold and marked with * P <0.05; ** P < 0.01; and *** P < 0.001 with higher proportions identified by ↑ and lower proportions by ↓.

Fatty acid	Control		High CO$_2$		Transformation/ Analysis	P		
	Mean	s.e.	Mean	s.e.				
12:0	0.067	0.006	0.063	0.006	ANOVA	0.696		
14:0	1.492	0.121	1.182	0.023	ANOVA	**0.031**	*	↓
15:0	0.277	0.004	0.292	0.007	ANOVA	0.097		
15:1	0.045	0.008	0.110	0.023	Log/ANOVA	**0.005**	**	↑
16:0	17.398	0.381	17.537	0.138	ANOVA	0.740		
16:1ω7	2.600	0.118	2.018	0.017	Wilcoxon test	**0.005**	**	↓
16:2ω4	0.907	0.019	0.803	0.017	ANOVA	**0.002**	**	↓
16:3ω4	0.498	0.018	0.582	0.005	ANOVA	**0.001**	***	↑
17:0	0.515	0.030	0.607	0.065	Wilcoxon test	0.199		
18:0	6.767	0.223	7.747	0.063	ANOVA	**0.002**	**	↑
18:1ω9	10.953	0.155	11.168	0.054	ANOVA	0.220		
18:1ω7	4.093	0.044	4.080	0.019	ANOVA	0.789		
18:2ω6	2.928	0.041	2.733	0.027	ANOVA	**0.003**	**	↓
18:3ω3	0.202	0.002	0.223	0.002	Wilcoxon test	**0.003**	*	↑
18:3ω4	0.095	0.002	0.113	0.002	Wilcoxon test	**0.004**	*	↑
18:3ω6	1.520	0.053	1.557	0.062	ANOVA	0.663		
18:4ω3	0.753	0.063	0.578	0.010	ANOVA	**0.021**	*	↓
20:1ω9	0.817	0.033	0.643	0.017	Log/ANOVA	**0.001**	***	↓
20:2ω6	0.207	0.023	0.277	0.009	Log/ANOVA	**0.014**	*	↑
20:3ω3	0.242	0.014	0.303	0.010	ANOVA	**0.006**	**	↑
20:3ω6	5.027	0.117	5.468	0.089	ANOVA	**0.013**	*	↑
20:4ω3	0.272	0.014	0.247	0.021	ANOVA	0.344		
20:4ω6	0.512	0.021	0.450	0.007	ANOVA	**0.017**	*	↓
20:5ω3	7.132	0.337	5.940	0.071	ANOVA	**0.006**	**	↓
22:5ω3	2.817	0.086	3.125	0.051	ANOVA	**0.012**	*	↑
22:5ω6	1.637	0.026	1.463	0.013	ANOVA	**0.001**	***	↓
22:6ω3	20.168	0.368	19.717	0.179	ANOVA	0.295		

Supplementary table IV.3. Results of RDA interaction model by factors and constrained axis computed for fatty acid composition of red drum larvae exposed to control and high levels of CO2 (Treatment) for n = 2 replicate experiments (Batch). Only Treatment and axis RDA1 explained a significant amount of the variance of multivariate fatty acid composition.

		Eigenvalue	Proportion explained	Variance	F	P	
Factors	Treatment			12.36	10.06	< 0.001	***
	Batch			1.68	1.37	0.23	
	Treatment × Batch			3.15	2.56	0.08	
	Residual			9.82			
Axis	RDA1	14.65	0.54	14.65	11.93	0.005	**
	RDA2	1.59	0.06	1.59	1.29	0.280	
	RDA3	0.94	0.03	0.94	0.77	0.550	
	Residual			9.82			

V. Supplementary material Chapter VI:

Otolith fluctuating asymmetry: a misconception of its biological relevance?

R code provided as example in Chapter VI.

```
#-----------------------------------------------
# Otolith fluctuating asymmetry: A misconception of its
biological relevance?
# Supplementary material
# ICES Journal of Marine Science
# Carlos Díaz-Gil, Miquel Palmer, Ignacio A. Catalán, Josep
Alós, Lee A. Fuiman,
# Elena García, María del Mar Gil, Amalia Grau, Andrew Kang,
Rommel H. Maneja,
# John A. Mohan, Bernat Morro, Jason J. Schaffler, Lucie
Buttay, Inmaculada Riera-Batle,
# Borja Tolosa, Beatriz Morales-Nin.
#-----------------------------------------------
#
# Objective: To demonstrate the method used for estimating
Fluctuating Asymmetry from otolith shape
# Last update: Jan 26, 2015
# Statistical details in Palmer et al., (2010, Marine Ecology
Progress Series)
# palmer@imedea.uib-csic.es
#
# Section 1 generates simulated otoliths and shows the format
needed for analyzing real data
# Section 2 aligns configurations
# Section 3 measures fluctuating asymmetry and test for
between-group differences
#
#
#-----------------------------------------------
# 1: Loading libraries and simulating otoliths
#-----------------------------------------------
remove(list=ls())
# Required libraries
library (geomorph) # shape analysis
library (vegan)    # multivariate statistics
par(ask=F)

# Setting one real otolith from which other otoliths will be
simulated
k=31        # Number of landmarks
```

```
m=2         # Number of dimensions
#Configuration of an otolith (x1,y1,x2,y2...)
p=c(734.1000, 307.45111, 990.9310, 725.33776, 772.4534,
172.50383, 857.7490, 82.71775, 992.6118, 66.27043, 1120.4296,
108.13021, 1239.3777, 170.99375, 1363.9466, 196.15934,
1459.3865, 287.70574, 1540.4280, 393.63562, 1594.5291,
517.55693, 1572.4778, 649.68038, 1576.0848, 785.29470,
1512.5876, 903.41064, 1446.1706, 1022.17428, 1386.9853,
1144.62132, 1300.8843, 1248.46049, 1189.6094, 1325.35902,
1090.8697, 1416.23693, 957.0162, 1427.17581, 822.5715,
1414.92931, 710.7513, 1337.81896, 612.2683, 1244.82369,
540.3685, 1129.68996, 473.6102, 1012.35064, 416.2209,
890.39064, 401.1600, 755.20759, 446.0428, 627.77256, 490.8340,
499.46208, 516.1674, 366.19907, 598.3370, 268.40118)
oto=array(0, c(k, m))
for (j in seq(2,2*k,2)){
  oto[j/2,1]=p[(j-1)]
  oto[j/2,2]=p[j]
}
plot(oto)   #Shows this otolith.

# Simulating fish
n=c(30,30)   # Number of fish per group (two groups)
sd.fish=10    # Between fish variability
sd.fa1=0.8     # Asymmetry-related standard deviation for the
first group of fish
sd.fa2=0.75*sd.fa1    # Asymmetry-related standard deviation
for the second group of fish
sd.fa=c(rep(sd.fa1,n[1]),rep(sd.fa2,n[2]))       # Asymmetry
standard deviation for all fish
config=array(0, c(k, m, 2*2*sum(n)))    #template for all
configurations (two otoliths per fish and two replicates per
otolith)
sd=0.1          # Measurement error
for (i in 1:sum(n)){
  # Shape (defined by 31 landmarks)of the fish (averaged
left/rigth otolith)
  fish=oto + cbind(rnorm(k,0,sd.fish),rnorm(k,0,sd.fish))
  # Shape of the left and rigth otoliths
  temp=cbind(rnorm(k,0,sd.fa[i]),rnorm(k,0,sd.fa[i]))
  left=fish+temp
  rigth=fish-temp
  #two replicates per otolith
  temp=left+cbind(rnorm(k,0,sd),rnorm(k,0,sd))#measurement
error
  config[,,(4*(i-1)+1)]=temp
  temp=left+   cbind(rnorm(k,0,sd),rnorm(k,0,sd))#measurement
error
  config[,,(4*(i-1)+2)]=temp
  temp<-rigth+cbind(rnorm(k,0,sd),rnorm(k,0,sd))#measurement
error
```

```
  config[,,(4*(i-1)+3)]=temp
  temp=rigth+cbind(rnorm(k,0,sd),rnorm(k,0,sd))#measurement
error
  config[,,(4*(i-1)+4)]=temp
}

dim(config)
# config compiles the 31 landmark positions (x,y coordinates)
of the 240 otoliths
# (in the example there are 60 fish, 2 otoliths per fish, and
2 replicates per otolith)
# This config object is the base for the following analyses,
and in this example is simulated data,
# but in real data this kind of files (x, y coordinates of the
landmarks) can be obtained using for example
# tpsDig2 software (Rohlf 2004) that can be freely donwloaded.


#-------------------------------------------------
# 2: Registering the configurations (generalized procrustes
alignment)
#-------------------------------------------------
#Semilandmarks: defining sliding landmarks (landmarks #1 and
#2 are fixed)
curves=array(NA,dim=c((k-3),3))
colnames(curves)=c(" before"," slide","after")
curves[,1]=c(3:(k-1))
curves[,2]=c(4:k)
curves[,3]=c(5:k,1)
curves=rbind(c(1,3,4),curves)

#Alignment
gpa=gpagen(config, Proj = TRUE, ProcD = TRUE, curves=curves,
ShowPlot=TRUE)

plot(gpa$coords[,1,1],gpa$coords[,2,1],asp=1,type='n')
for (i in 1:dim(gpa$coords)[3]){

lines(gpa$coords[c(1,3:30,1),1,i],gpa$coords[c(1,3:30,1),2,i]
)
}

# Checking plot (pca of the replicates)
plotTangentSpace(gpa$coords)

#-------------------------------------------------
# 3: Estimating FA
#-------------------------------------------------
# Model for FA estimation
# The ind*side interaction represents the FA-related effects
```

```
# Note that the partial effects of fish and side are left out
("Condition")

# Restructuring gpa$coords as a matrix
shape=two.d.array(gpa$coords)
#plot(gpa$coords[,1,1],gpa$coords[,2,1],asp=1,lwd=3)

# ANALISYS
ID=as.factor(rep(1:sum(n),each=4))   # fish ID, check each=4
because there are 2 otoliths and 2 replicates
SIDE=as.factor(rep(c(1,1,2,2),sum(n))) # side of the otolith,
RIGHT OR LEFT coded as 1 or 2
int=rda(shape ~ ID*SIDE+Condition(ID+SIDE)) # this is the
MANOVA model
#anova(int, by="terms", permu=200) #this step need to be
checked in order to confirm the FA (ind*side) is bigger than
the measurement error. WARNING (time comsuming)

# Checking plot (looking for outliers)
#plot(int,display=c("wa"))

# The fitted values for each row (replicate) should be used
latter for OFA estimates
# Note that the two replicates of the same otolith must have
the same score
fa<-fitted(int)
fa=fa[seq(1,dim(fa)[1],by=2),] #selecting only one of the two
replicates

# Estimating Otolith Fluctuating Asymmetry OFA (=Euclidean
distance between the scores corresponding to left and right)
OFA=rep(NA,sum(n))
for (i in 1:sum(n)){
  OFA[i]=sqrt(sum((fa[2*(i-1)+1,]-fa[2*(i-1)+2,])^2))
}
hist(OFA)

# Differences between treatments
TREATMENT=as.factor(c(rep(1,n[1]),rep(2,n[2]))) #assigning 1
or 2 to the two groups defined in the example
boxplot(log(OFA)~TREATMENT,ylab="Otolith        Fluctuating
Asymmetry")
glm<-glm(log(OFA)~TREATMENT) #Checking if the OFA is different
between groups.
summary(glm)
shapiro.test(resid(glm)) #Important to note the checkout of
the normality

# In this example with simulated fish the two groups had
different otolith fluctuating asymmetry.
# the parameters to simulate fish can be altered in section 1.
```

```
# It is worthy to check what happens when you alter the sample
size, in case of real differences the sample size
# can be drastically reduced and still the differences will
clearly arise and be detected.
# Nevertheless if the OFA differences between groups are tiny
or almost undetectable it does not matter how much the
# sample size is increased.
# With real data this parameters are not manually entered but
calculated from the real otoliths.
# Calculating Otolith Fluctuating using R.
```

Supplementary tables and figures lists.

Supplementary figures

Supplementary tables

Notes & errata