Universitat de Girona

EFFECTS OF PARASITISM ON THE CONDITION AND REPRODUCTIVE CAPACITY OF THREE COMMERCIALLY EXPLOITED FISH SPECIES IN THE MEDITERRANEAN SEA

Dolors Ferrer Maza

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DOCTORAL THESIS

Effects of parasitism on the condition and reproductive capacity of three commercially exploited fish species in the Mediterranean Sea

Dolors Ferrer Maza

2015

Universitat de Girona

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2015

Doctoral Programme in Experimental Sciences and Sustainability

Supervised by

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Thesis submitted in fulfilment of the requirements for the Degree of Doctor (PhD) at the Universitat de Girona

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DECLARE:

That the thesis entitled *Effects of parasitism on the condition and reproductive capacity of three commercially exploited fish species in the Mediterranean Sea*, presented by Dolors Ferrer Maza in the pursuance of a doctoral degree, has been completed under our supervision and meets the conditions required to opt for an International Doctorate.

In witness thereof, we hereby sign this document.

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Girona, December 2015

"Imagine a world where parasites control the minds of their hosts,
sending them to their destruction.
Imagine a world where parasites are masters of chemical warfare and camouflage,
able to cloak themselves with their hosts' own molecules.
Imagine a world where parasites steer the course of evolution,
where the majority of species are parasites.
Welcome to Earth."

Parasite Rex: Inside the Bizarre World of Nature's Most Dangerous Creatures

Carl Zimmer (American science writer, 1966 -)

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List of abbreviations

The abbreviations and acronyms used in this thesis are listed below. As it can be seen, in several cases, two different abbreviations stand for the same thing. It should also be noted that the terms *weight* and *mass* are both used to denote the same measurement. These issues are due to the specific styles of each journal in which the articles have been published. Since the thesis is presented as a compendium of publications, the section of the results has been transcribed as close as possible to the original text of the scientific papers.

ABSG **(also** ABS_{G}**)**: absolute lipid content in gonads (g)

ABSL: absolute lipid content in liver (g)

ABS_{M}: absolute lipid content in muscle (g)

AS: actively spawning (ovarian developmental phase)

BF **(also** F_{B}**)**: batch fecundity (eggs per batch)

BFrel **(also** F_{Brel}**)**: relative batch fecundity (eggs per gram of eviscerated body weight)

CA: cortical alveolar (oocyte developmental stage)

CI: confidence interval

cox 2: mitochondrial cytochrome oxidase II gene

DEV: developing (ovarian developmental phase)

EW **(or** M_{E}**)**: eviscerated body weight (g)

F_{B} **(also *BF*)**: batch fecundity (eggs per batch)

F_{Brel} **(also *BFrel*)**: relative batch fecundity (eggs per gram of eviscerated body mass)

FDR: false discovery rate

GLM: general linear model

GSI **(also** I_{G}**)**: gonadosomatic index

GW **(also** M_{G}**)**: gonads weight (g)

H: hydration (oocyte developmental stage)

IA **(also** I_{A}**)**: relative intensity of atresia (%)

I_{A} **(also *IA*)**: relative intensity of atresia (%)

I_{G} (also *GSI*): gonadosomatic index

I_{LG} (also *LGSI*): lipid gonadosomatic index

I_{LM}: lipid musculosomatic index

***LGSI* (also I_{LG})**: lipid gonadosomatic index

LHSI: lipid hepatosomatic index

L_{T}: total body length (cm)

LW: liver weight (g)

M_{DO} (also *ODW*): dry mass of hydrated oocytes (mg)

M_{E} (also *EW*): eviscerated body mass (g)

MEDITS: International bottom trawl survey in the Mediterranean

M_{G} (also *GW*): gonad mass (g)

MLS: minimum landing size

M_{M}: muscle mass (g)

M_{T} (also *TW*): total body mass (g)

NaCl: sodium chloride

***ODW* (also M_{DO})**: dry weight of hydrated oocytes (mg)

OM: maturation (oocyte developmental stage)

***P* (also P_{P})**: prevalence of parasites (%)

***PA* (also P_{A})**: prevalence of atresia (%)

PG: primary growth (oocyte developmental stage)

POFs: post-ovulatory follicles

P_{P} (also *P*): prevalence of parasites (%)

REG: regenerating (ovarian developmental phase)

REGR: regressing (ovarian developmental phase)

SC: spawning capable (ovarian developmental phase)

SD: standard deviation

***TW* (also M_{T})**: total body weight (g)

VIT1: early vitellogenesis (oocyte developmental stage)

VIT2: late vitellogenesis (oocyte developmental stage)

List of figures

GENERAL INTRODUCTION

RESULTS

Chapter I: European hake (*Merluccius merluccius*)

***Chapter II*: Red mullet (*Mullus barbatus*)**

Chapter III: **European anchovy (*Engraulis encrasicolus*)**

GENERAL DISCUSSION

List of tables

GENERAL INTRODUCTION

RESULTS

Chapter I: European hake (*Merluccius merluccius*)

Chapter II: Red mullet (*Mullus barbatus*)

***Chapter III*: European anchovy (*Engraulis encrasicolus*)**

GENERAL DISCUSSION

Table of contents

Structure of the thesis

This PhD thesis is presented as a compendium of publications. As such, it is organized around three articles that have been prepared throughout the doctoral period of the author. Therefore, the main text of this thesis consists of a reprint of these papers, which are either published (2 articles) or still under review (1) in scientific journals.

Additionally, there is a general introduction that provides the overall approach of the research project and a general discussion where the main outcomes of the thesis are discussed as a whole. Finally, the references from all sections are listed together at the end of the thesis.

In order to meet the requirements to present the thesis as a compendium of publications, the complete references of the scientific articles and their quality indices are listed below:

Ferrer-Maza D., Lloret J., Muñoz M., Faliex E., Vila S., & Sasal P. (2014). Parasitism, condition and reproduction of the European hake (*Merluccius merluccius*) in the northwestern Mediterranean Sea. *ICES Journal of Marine Science*, 71(5): 1088–1099, DOI: 10.1093/icesjms/fst217. IF = 2.377; JR = Fisheries (Q1: 6/52), JR = Marine & Freshwater Biology (Q1: 23/102), JR = Oceanography (Q2: 16/61).

Ferrer-Maza D., Muñoz M., Lloret J., Faliex E., Vila S., & Sasal P. (2015). Health and reproduction of red mullet, *Mullus barbatus*, in the western Mediterranean Sea. *Hydrobiologia*, 753(1): 189-204, DOI: 10.1007/s10750-015-2205-5. IF = 2.275; JR = Marine & Freshwater Biology (Q1: 25/102).

Ferrer-Maza D., Lloret J., Muñoz M., Faliex E., Vila S., & Sasal P. (in peer review process). Links between parasitism, energy reserves and fecundity of European anchovy, *Engraulis encrasicolus*, in the northwestern Mediterranean Sea. *Conservation Physiology*.

The journals quality indices, Impact Factor (IF) and Journal Rank in Category (JR), were obtained from the 2014 Science Edition of the Journal Citation Report (JCR) produced by the Institute for Scientific Information (ISI).

Summary

Host-parasite coevolution suggests that fish are in dynamic equilibrium with their parasites and this has often led to parasitism being overlooked in fish health assessments. However, it is well-known that several fish parasites can become pathogenic and even fatal for fish during heavy infections. On the other hand, it is also widely accepted that parameters relating to the physical condition of fish and their reproduction provide essential data for estimating the productivity of exploited populations. Although parasitism might affect these parameters, research on this topic is generally scarce and, in the case of the Mediterranean Sea, almost non-existent.

This PhD thesis evaluates for the first time the links between parasitism, condition and reproduction of mature female individuals of three of the most captured fish species in the western Mediterranean Sea: European hake, *Merluccius merluccius*; red mullet, *Mullus barbatus*, and European anchovy, *Engraulis encrasicolus*. Indicators of fish energy reserves (total lipid content in liver, muscle and gonads) and reproductive capacity (fecundity and egg quality) were evaluated, as were the prevalence and intensity of infection by metazoan parasites.

The results of this thesis indicate that parasitism in these species is linked to their condition and reproduction, although the relationships do not always point in the same direction. In the case of *Merluccius merluccius*, the effect of anisakid nematodes on condition is mostly negative and occurs mainly when hake are allocating their energy reserves to gonadal development. In the case of *Mullus barbatus*, some digeneans negatively affect the energy reserves of fish, whereas some nematodes produce a rise in egg production but impair egg quality. The relationships between parasites and *Engraulis encrasicolus* are even more complex as positive and negative effects were found on the energy reserves and fecundity of this species.

By evaluating these relationships, this thesis also provides essential data on the health and reproduction of these three fish species, such as the spawning strategy, which we believe should be used to assess stocks and to carry out sustainable and profitable management of the fisheries. The results are also relevant from a human health perspective because they provide basic information regarding the presence and intensity of anisakid nematodes – a known health hazard which can cause a parasitic disease in people who consume infected fish.

Resum *(in Catalan)*

La teoria de la coevolució entre els paràsits i els seus hostes suggereix que els peixos i els seus paràsits coexisteixen en un estat d'equilibri dinàmic, la qual cosa implica que el parasitisme sovint és omès en la avaluació de la salut dels peixos. No obstant això, és un fet ben documentat que alguns paràsits dels peixos poden ser patogènics i fins i tot letals pels seus hostes en les infestacions més greus. Així mateix, és ben conegut que els paràmetres relatius a la condició física i la reproducció dels peixos proporcionen una informació essencial per estimar la productivitat de les poblacions de peixos explotades. Malgrat que el parasitisme podria afectar aquests paràmetres, la recerca en aquest camp és força limitada, i en el cas de la mar Mediterrània quasi inexistent.

La present tesi doctoral avalua, per primera vegada, els vincles entre parasitisme, condició i reproducció en femelles adultes de tres de les espècies més capturades a la Mediterrània occidental: el lluç europeu, *Merluccius merluccius*, el roger de fang, *Mullus barbatus* i el seitó, *Engraulis encrasicolus.* Amb aquesta finalitat, es varen avaluar els indicadors de les reserves energètiques (contingut total de lípids en el fetge, musculatura i gònades) i la capacitat reproductiva (fecunditat i qualitat de la posta), així com la prevalença i intensitat de la infestació per paràsits metazous.

Els resultats obtinguts indiquen que el parasitisme, la condició i la reproducció d'aquestes espècies estan vinculats, tot i que les relacions no sempre apunten en la mateixa direcció. Pel que fa al lluç, els efectes dels nematodes anisàkids sobre la condició són negatius i es manifesten principalment quan els individus estan destinant les seves reserves energètiques a desenvolupar les gònades. En el cas del roger, mentre que alguns digenis afecten negativament les seves reserves energètiques, alguns nematodes produeixen un augment en la producció d'ous i alhora una disminució en la qualitat d'aquests. Pel que fa al seitó, els efectes dels paràsits són encara més complexes ja que es varen trobar relacions tant positives com negatives entre el parasitisme i la condició o reproducció d'aquesta espècie.

Paral·lelament a l'anàlisi d'aquestes relacions, aquesta tesi també proporciona dades essencials sobre la salut i reproducció d'aquestes tres espècies, com per exemple l'estratègia reproductiva. Aquesta informació hauria de ser de gran utilitat en la avaluació dels stocks, així com per a dur a terme una gestió profitosa i sostenible dels recursos pesquers. Els resultats d'aquesta tesi també són rellevants des d'una perspectiva de salut humana ja que es proporcionen dades sobre la presència i intensitat dels anisàkids, paràsits que poden causar una parasitosi a les persones que consumeixen peix infestat per aquests nematodes.

Resumen *(in Spanish)*

La teoría de la coevolución entre parásito y huésped sugiere que los peces y sus parásitos coexisten en un estado de equilibrio dinámico, lo que implica que el parasitismo es a menudo pasado por alto en la evaluación de la salud de los peces. No obstante, es un hecho bien conocido que algunos parásitos de los peces pueden ser patogénicos e incluso letales para sus huéspedes en infestaciones graves. Así mismo, también está ampliamente aceptado que los parámetros relativos a la condición física y la reproducción de los peces proporcionan una información esencial para estimar la productividad de las poblaciones de peces explotadas. Aunque el parasitismo podría afectar estos parámetros, la investigación en este campo es bastante limitada, y en el caso del mar Mediterráneo casi inexistente.

La presente tesis doctoral evalúa, por primera vez, los vínculos entre parasitismo, condición y reproducción en hembras adultas de tres de las especies más capturadas en el Mediterráneo occidental: la merluza europea, *Merluccius merluccius*, el salmonete de fango, *Mullus barbatus* y el boquerón, *Engraulis encrasicolus*. A tal efecto, se evaluaron los indicadores de las reservas energéticas (contenido total de lípidos en el hígado, musculatura y gónadas) y la capacidad reproductiva (fecundidad y calidad de la puesta), así como la prevalencia e intensidad de la infestación por parásitos metazoarios.

Los resultados obtenidos indican que el parasitismo, la condición y la reproducción de estas especies están vinculados, aunque las relaciones no siempre apuntan en la misma dirección. En lo que concierne a la merluza, los efectos de los nematodos anisákidos sobre la condición son negativos y se manifiestan principalmente cuando los individuos están destinando sus reservas energéticas al desarrollo gonadal. En el caso del salmonete de fango, mientras que algunos digéneos afectan negativamente sus reservas energéticas, algunos nematodos producen un aumento en la producción de los huevos, a la vez que una disminución de la calidad de los mismos. Referente al boquerón, los efectos de los parásitos son aún más complejos ya que se encontraron relaciones tanto positivas como negativas entre el parasitismo y la condición o reproducción de esta especie.

Conjuntamente con el análisis de estas relaciones, la presente tesis también aporta datos esenciales sobre la salud y reproducción de estas tres especies, como por ejemplo la estrategia reproductiva. Dicha información debería ser de gran utilidad en la evaluación de los stocks, así como para llevar a cabo una gestión provechosa y sostenible de los recursos pesqueros. Los resultados de esta tesis también son relevantes desde una perspectiva de salud humana ya que se proporcionan datos sobre la presencia e intensidad de los anisákidos, parásitos que pueden causar una parasitosis a las personas que consumen pescado infestado por estos nematodos.

Résumé *(in French)*

La théorie de la coévolution hôte-parasite suggère que les poissons et leurs parasites coexistent dans un état d'équilibre dynamique, ce qui conduit souvent à négliger l'impact du parasitisme dans l'évaluation de la santé des poissons. Cependant, il est bien connu que certains parasites de poissons peuvent être pathogènes, au point qu'ils peuvent conduire à la mort de leurs hôtes dans les cas d'infestations les plus graves. Par ailleurs, il est également largement admis que les paramètres relatifs à la condition et à la reproduction des poissons fournissent des données essentielles pour estimer la productivité des stocks de poissons exploités. De manière surprenante, bien que le parasitisme puisse affecter ces paramètres, les recherches s'inscrivant dans ce domaine sont très limitées, et dans le cas de la mer Méditerranée presque inexistantes.

Cette thèse évalue, pour la première fois, les liens entre parasitisme, condition et reproduction chez des femelles adultes de trois espèces de poissons parmi les plus capturées en Méditerranée occidentale: le merlu européen, *Merluccius merluccius*, le rouget de vase, *Mullus barbatus* et l'anchois commun, *Engraulis encrasicolus*. Des indicateurs de réserves énergétiques (teneurs totales en lipides dans le foie, les muscles et les gonades) et de capacité de reproduction (fécondité et qualité de la ponte), ainsi que d'infestation par des parasites métazoaires (prévalence et intensité parasitaire) ont été évalués.

Les résultats indiquent que le parasitisme, la condition et la reproduction de ces espèces sont liées entre elles, bien que les relations impliquées ne pointent pas toujours dans la même direction. En ce qui concerne le merlu, il existe une relation négative entre les nématodes anisakidés et la condition des poissons mais principalement lorsque les merlus utilisent leurs réserves énergétiques pour le développement des gonades. Dans le cas du rouget,

tandis que certains digènes affectent négativement les réserves énergétiques de leur hôte, certains nématodes entraînent une augmentation de la production d'œufs, mais une diminution de leur qualité. Pour l'anchois, l'effet des macroparasites est encore plus complexe puisque nous montrons chez cette espèce aussi bien des relations positives que négatives entre parasitisme et condition ou reproduction.

En évaluant ces relations, cette thèse fournit également des données essentielles sur la santé et la reproduction de ces espèces de poissons, telles que leurs stratégies de reproduction, données qui devraient être utilisées dans l'évaluation des stocks pour effectuer une gestion durable et rentable des pêcheries. Les résultats de cette thèse sont également importants du point de vue de la santé humaine, car ils fournissent des données sur la présence et l'intensité des anisakidés, parasites qui peuvent causer des parasitoses chez l'homme et occasionner des désordres non négligeables chez les personnes qui consomment des poissons infestés par ces nématodes.

GENERAL INTRODUCTION

The general introduction provides the background of the research study, as well as the hypothesis statement, main objectives and a summary of the materials and methods used in this thesis.

General introduction

Background

Parasitism is usually defined as a non-mutual symbiotic relationship between species, where the parasite benefits at the expense of the host. In theory, therefore, parasitism should produce deleterious effects on fish health because the parasites drain energy from the fish that would otherwise be used to sustain the basic life history traits of fish, such as condition, growth or reproduction. However, it appears that through the coevolution of host and parasite, a dynamic equilibrium has arisen between fish and their parasites (Barret 1986). That would mean that parasites do not cause severe effects on their hosts because this would impair their chances of reaching the following stage in their life cycle or of reproducing.

This theoretical equilibrium has implied that parasitism is often overlooked in fish health assessment. However, it is well-known that fish parasites can induce changes in host behaviour and morphology (Lafferty & Morris 1996; Barber *et al.* 2000; Ward *et al.* 2002; Sasal & Thomas 2005), as well as can regulate host population dynamics and/or can influence community structure (Sindermann 1987; Mouritsen & Poulin 2002; Marcogliese 2005; Thompson *et al.* 2005; Longshaw *et al.* 2010). Finally, they can also become pathogenic and even fatal in heavy infections (Yamamoto *et al.* 1984; Poulin 2002; Rohde 2005; Planes *et al.* 2009; Woo & Buchmann 2012).

Host-parasite coevolution means that the two species undergo reciprocal adaptive genetic changes over time in response to the interaction between them (Thompson 1989), leading to a dynamic cycle in which parasite virulence and host resistance fluctuate. Moreover, parasite-host systems can not only coevolve to different levels of parasite virulence but also they can coevolve to commensalism and mutualism (Toft & Karter 1990).

Therefore, host-parasite coevolution endpoints are not as clear as it might seem, and it cannot be taken for granted that parasites are not causing any effects on the health of their hosts. In addition, alterations in the environment can result in disease because it can change the balance between parasites and hosts (Iwanowicz 2011). Consequently, and taking into account that new interactions between species arise as biological consequences of climate and/or other anthropogenic changes, the virulence of parasites is currently unknown and studying the effects of parasitism on fish health has become more important than ever before.

Moreover, in the present situation of an increasing over-exploitation of fish stocks as well as of depleted fish stocks, it is necessary to study the biology of the exploited species to provide reliable scientific information for improving fisheries management and to ensure the sustainability of this resource for the future. Indeed, we should bear in mind that people have never consumed as much fish or have never been so dependent on this sector for their well-being (FAO 2014).

In this sense, it is well-known that parameters relating to the condition of fish are essential for estimating the productivity of exploited populations (Shulman & Love 1999). In general, fish condition indicate the overall health of a fish and have great influence on growth, reproduction and survival (Lloret *et al.* 2014). In particular, the maternal condition has been identified as a key factor for egg production and quality in several fish species, e.g. Atlantic cod, *Gadus morhua* and haddock, *Melanogrammus aeglefinus* (Blanchard *et al.* 2003; Saborido-Rey *et al.* 2003; Alonso-Fernández *et al.* 2009); the Cape hakes *Merluccius paradoxus* and *M. capensis* (Grote *et al.* 2011) and pouting, *Trisopterus luscus* (Alonso-Fernández & Saborido-Rey 2011), among others.

Traditionally, fisheries biologists have estimated fish condition by using morphometric indices which are based on the relationships between length and mass (Bolger & Connolly 1989). However, these indices are not able to shed light on the underlying cause-and-effect relationships governing a fish's health and nutritional status (Arts & Kohler 2009). In contrast, biochemical measures, such as lipid content, are better indicators of fish condition because they reflect closer the energy reserves of fish and, by implication, the fish health (reviewed by Lloret *et al.* 2012a, 2014).

On the other hand, the reproductive biology of fishes is also one of the most important features in fish stock assessment and management. Actually, some characteristics of the fish reproduction, such as length at maturity or the spawning season, are essential when formulating stock regulatory measures. Particularly, the indicators of the reproductive capacity, such as fecundity, are very important for estimating the reproductive potential of fishes and hence the productivity of fish populations. That information is essential for implementing technical measures in order to maintain the stocks viability.

Although parasitism might affect the condition and reproductive capacity of fishes, research in this field is rather limited to freshwater fish species. There are some studies on the relationships between parasitism and condition (e.g. Tavares-Dias *et al.* 2000; Hoffnagle *et al.* 2006; Ondracková *et al.* 2010; Guidelli *et al.* 2011; Francová & Ondračková 2013) and also some that include reproductive variables (e.g. Tierney *et al.* 1996; Sasal & Pampoulie 2000; Sasal *et al.* 2001; Barber & Svensson 2003; Bagamian *et al.* 2004; Sasal 2006; Bean & Bonner 2009; Fogelman *et al.* 2009). However, most of these studies used simple morphometric indices of condition.

In any case, those studies evidenced that the effects of parasites are not always evident and straightforward. For instance, Tierney *et al.* (1996) studied the effects of the cestode *Schistocephalus solidus* on the body condition and reproductive status of stickleback, *Gasterosteus aculeatus*, and found that infected fish showed lower condition than uninfected ones depending on the season of the year considered. Another example is the study carried out by Bean & Bonner (2009) in which the effects of the cestode *Bothriocephalus acheilognathi* on the *Cyprinella lutrensis* condition and reproduction were investigated. Their findings indicated that the parasite has an impact on the condition of fish but parasitism does not affect the reproductive capacity of fish. On the contrary, Fogelman *et al.* (2009) found a significant impairment of the quantity and quality of the eggs of the females five-lined cardinalfish, *Cheilodipterus quinquelineatus* infected by the isopod *Anilocra apogonae*.

With regard to marine exploited species, information on the effects of parasites on the fish condition is limited to a few studies on *Engraulis encrasicolus* (Shchepkina 1980), *Engraulis japonicus* (Sajiki *et al.* 1992), *Gadus morhua* (Khan & Lacey 1986; Lysne *et al.* 2006), *Boreogadus saida* (Khan *et al.* 1997), *Sardinella* spp. (Ranjitsingh & Padmalatha 1997), *Mugil platanus* (Ranzani-Paiva & Silva-Souza 2004), *Solea solea* (Durieux *et al.* 2007) and *Oncorhynchus kisutch* (Schaufler *et al.* 2008). Concerning Mediterranean exploited species, the studies are limited to *Merluccius merluccius* (Guillaume *et al.* 1985), *Mullus barbatus* (Carreras-Aubets *et al.* 2011) and *Lithognathus mormyrus* (Sala-Bozano *et al.* 2012). Thus, there is a lack of information on the effects of parasites on the energy reserves and reproduction of exploited marine fishes worldwide, especially in the Mediterranean Sea.

Hypothesis

When fish become infected by metazoan parasites their condition and reproductive capacity are impaired because parasites drain energy from the fish.

Objectives

The main aim of this study is to assess the possible effects of parasitism on the condition and reproductive capacity of commercially exploited fish species in the Mediterranean Sea.

For this purpose, three cases of study were designed in the western Mediterranean Sea:

- European hake, *Merluccius merluccius* L. 1758
- Red mullet, *Mullus barbatus* L. 1758
- European anchovy, *Engraulis encrasicolus* L. 1758

To achieve this general aim, five specific objectives were established:

1. Evaluation of metazoan parasitism
    - 1.1. Examination of all specimens for metazoan parasites using a stereomicroscope.
    - 1.2. Collection, preservation and identification of parasites.
    - 1.3. Calculation of the quantitative descriptors of parasitic infection.
2. Evaluation of fish condition
    - 2.1. Determination of the total lipid content in liver, muscle and gonads by Soxhlet extraction.
    - 2.2. Calculation of the lipid-somatic indices which indicate the energy reserves of fish.
    - 2.3. Assessment of the lipid dynamics within the organism through the reproductive cycle.
3. Evaluation of the reproductive capacity
    - 3.1. Histology of the gonads of all specimens, calculation of the atresia intensity and classification into each ovarian developmental phase.

   - 3.2. Estimation of the fecundity by counting the eggs spawned per batch.
   - 3.3. Estimation of the egg quality by determination of the oocytes dry mass.
4. Statistical analysis of the relationships between variables
   - 4.1. Fisher's exact test and Mood's median test to analyse the parasitic infection.
   - 4.2. Mann-Whitney U test to compare infected and uninfected specimens.
   - 4.3. Spearman's Rank Correlation coefficient to study the relationships between the individual intensity of infection and the variables of condition and reproduction.
5. Discussion of the results
   - 5.1. Interpretation of the results of the statistical analyses.
   - 5.2. Discussion about the consequences for the health and productivity of the stocks.
   - 5.3. Discussion about the applicability of the results for stock assessment and management.

Materials and methods

Since this thesis is presented as a compendium of publications, the specific materials and methods used in the research are given in each of the publications. That is to say, in each chapter of the results section there is a subsection with a comprehensive explanation of the methodologies performed in each chapter. Here I summarize the overall materials and methods used along this study to provide an overall view of them.

In the present study, a total of 745 female individuals of three commercially exploited fish species were analysed. Specifically, the three selected species were (i) European hake, *Merluccius merluccius* L. 1758 (henceforth: hake)

(Fig.1), (ii) red mullet, *Mullus barbatus* L. 1758 (Fig. 2) and (iii) European anchovy, *Engraulis encrasicolus* L. 1758 (henceforth: anchovy) (Fig. 3).

Fig. 1. European hake, *Merluccius merluccius* L.1758.

Fig. 2. Red mullet, *Mullus barbatus* L. 1758.

Fig. 3. European anchovy, *Engraulis encrasicolus* L. 1758.

These three species were selected to perform the study because they are some of the most important target species of commercial fisheries in the Mediterranean Sea. According to the fisheries service of the Government of Catalonia, in 2014, anchovy was the fish species with highest landings (9.7 thousand tons) and with greatest total economic value (17.9 million euros) whereas hake was third in the ranking (1.5 thousand tons, 10.3 million euros). Red mullet was also reported as one of the main target species of the region in 2014 (492 tons, 2.6 million euros) (http://agricultura.gencat.cat/ca/ambits/pesca/).

Additionally, these three species are good biological models in terms of representativeness. Indeed, none of the three species belong to the same order, they differ in size and each present different life history traits. Moreover, their environments and feeding regimes are very different since hake is a demersal/piscivorous, red mullet is a benthic/detritivorous and anchovy is a pelagic/planktivorous (Table 1).

Table 1. Main characteristics of the three selected species.

	European hake (*M. merluccius*)	Red mullet (*M. barbatus*)	European anchovy (*E. encrasicolus*)
Order	Gadiformes	Perciformes	Clupeiformes
Family	Merlucciidae	Mullidae	Engraulidae
Environment	demersal	benthic	pelagic
Feeding regime	piscivorous	detritivorous	planktivorous
Common length	12-60 cm	10-20 cm	5-15 cm
Female length at maturity (L_m)*	34 cm	13 cm	11 cm
Minimum landing size	20 cm	11 cm	9 cm
Main fishery	bottom trawlers and longliners	bottom trawlers	purse seiners

*Mean of different references in the Mediterranean Sea gathered by Tsikliras & Stergiou (2014).

Hake and anchovy samples were caught in the Gulf of Lion (NW Mediterranean Sea) near Cap de Creus (Fig. I.1 and Fig. III.1 in the results section). According to UNEP/MAP/RAC/SPA (2013), the Gulf of Lion is recognised for its high cetacean richness; and the high abundance of these mammals, which are the definitive hosts of several parasitic nematodes, seems to be related with a high density of parasites (Rello *et al.* 2009). Hake as well as anchovy samples were obtained at the port of Roses, which is the most important fishing harbour in the region. Hake were captured with commercial bottom trawlers and longliners, whereas anchovy were caught by purse seiners.

Concerning red mullet, the samples were collected along the western coast of the Spanish Mediterranean Sea (Fig. II.1 in the results section). The vast majority of the samples were obtained from two MEDITS trawl surveys, thanks to a partnership with the Spanish Oceanographic Institute (IEO). The MEDITS survey programme (International bottom trawl survey in the Mediterranean) produces biological data on the demersal resources in terms of population distribution as well as demographic structure, on the continental shelves and along the upper slopes at a global scale in the Mediterranean Sea, through systematic bottom trawl surveys (Bertrand *et al.* 2002).

Our collaboration in MEDITS trawl surveys was to attend to the campaigns 2010 and 2011 carried out with the research vessel *Cornide de Saavedra*, in the western coast of the Spanish Mediterranean Sea (FAO-GFCM geographical sub-area GSA-06, *Northern Spain*) under the supervision of the coordinators Dr. Mariano García and Dr. Antonio Esteban (IEO). In addition to support the survey's own tasks (Fig. 4), all the female red mullet captured with the experimental trawls during the 22 days of surveys (in two consecutive years) were collected, dissected and examined for parasites on board.

Fig. 4. Picture of the scientific crew in the MEDITS06-11 (GSA06) trawl survey carried out in May-June 2011.

The entire sampling, i.e. samples of hake, red mulled and anchovy, was obtained during different periods between January 2010 and November 2012. Since the reproductive capacity of fish can only be assessed in mature specimens, the sampling was biased towards larger and thus adult individuals (Table 2).

Table 2. Total body length and total body mass of the fish sampled.

	European hake (*M. merluccius*)	Red mullet (*M. barbatus*)	European anchovy (*E. encrasicolus*)
Sample size (*n*)	139	335	271
Total body length (cm)	41.3 – 73.2	12.5 – 26.9	10.4 – 16.7
(mean ± *SD*)	52.0 ± 5.8	19.5 ± 2.5	13.8 ± 1.2
Total body mass (g)	539.0 – 2959.3	19.8 – 223.6	7.3 – 36.8
(mean ± *SD*)	1082.3 ± 414.7	90.4 ± 36.5	18.7 ± 5.6

As mentioned above, the methodologies performed in this study are detailed in each chapter of the results section. However, they are also schematically summarised in Figure 5.

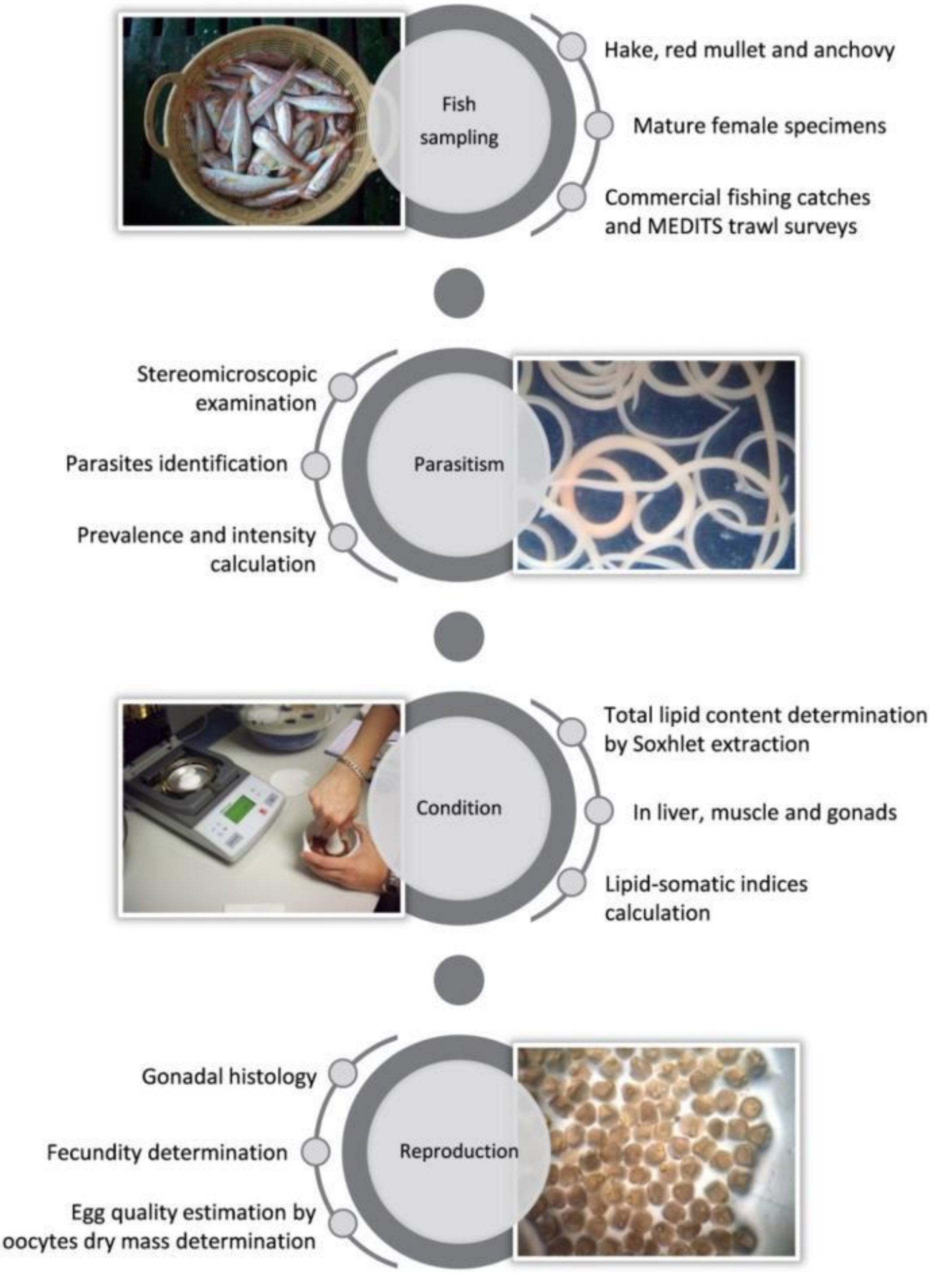

Fig. 5. Schematic summary of the methodologies used in this study.

Although the methods used along this study are practically the same for the three cases, the proportion of individuals used in each analysis differed between species (Table 3).

Table 3. Number of specimens used in each analysis.

	European hake *n* = 139	Red mullet *n* = 335	European anchovy *n* = 271
Parasitism evaluation	139	335	271
Lipid content in the liver	139	-	-
Lipid content in the muscle	-	156	210
Lipid content in the gonads	139	87	-
Gonadal histology	139	335	271
Fecundity determination	11	89	31
Egg quality estimation	9	89	-

It should be noted that the organs and tissues in which fishes store their lipids are different between species, i.e. the liver in hake (Lloret *et al.* 2008) or the muscle in red mullet (Lloret *et al.* 2007) and anchovy (Melo 1992). Therefore, the liver was selected for the determination of lipids stored in the body of hake and the muscle for the determination of lipids stored in red mullet and anchovy. The lipids in the gonads were considered as an indicator of the energy reserves used (or invested) in the reproductive process. Apart from that, the number of specimens used in each analysis also differed from several other reasons, e.g. the determination of lipids in anchovy's gonads was not possible because the gonadal masses were lower than the minimum required to perform the lipid extraction.

With regards to gonadal histology, we used traditional methodologies consisting in embedding a slice of tissue in paraffin, cutting it into very thin sections and perform the staining. However, it should be noted that in the present study the sections were processed with both hematoxylin-eosin and Mallory's trichrome stains (Fig. 6).

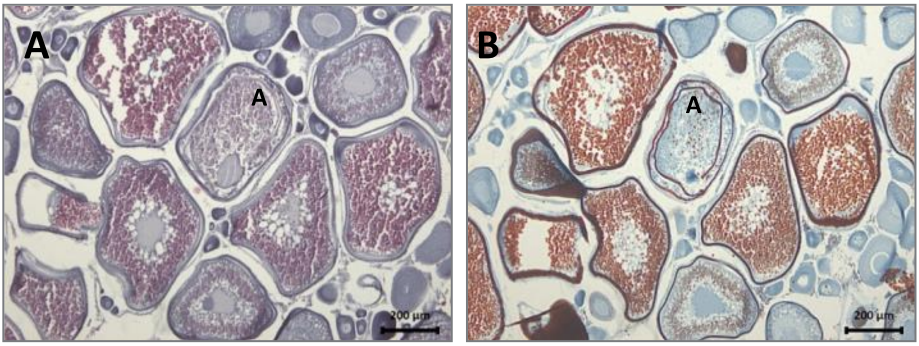

Fig. 6. Histological preparations of a hake's ovary processed with hematoxylin-eosin (A) or Mallory's trichrome (B) stains. The oocyte in the centre of the pictures corresponds to the same atretic oocyte.

In these pictures we can observe the same oocytes stained with hematoxylin-eosin (Fig. 6.A) or with Mallory's trichrome (Fig. 6.B). As it can be seen, the latter staining method greatly facilitates the detection of atretic oocytes as it highlights the zona radiata and its discontinuity.

Finally, another item that it is not explained in the articles is that a great part of the identification of the parasites was performed in the *CEntre de Formation et de Recherche sur les Environnements Méditerranéens* (CEFREM, UMR 5110), *Université de Perpignan Via Domitia* (France), under the supervision of Dr. Elisabeth Faliex. In 2012, I benefited from a three-month grant to stay in an international research centre through the FPI-MICINN 2010-2014 fellowship program (ref. EEBB-I-12-05174). During my stay, I learnt how to stain digeneans and I performed the molecular identification (PCR) of some nematodes. In this centre, I also had the opportunity to work with Dr. Rodney A. Bray (Natural History Museum, London) who kindly helped us with the identification of the digeneans.

RESULTS

The results section comprises three chapters that consist in three edited versions of the scientific articles, which compose the compendium of publications.

Chapter I:

European hake (*Merluccius merluccius*)

Ferrer-Maza D., Lloret J., Muñoz M., Faliex E., Vila S., & Sasal P. (2014). Parasitism, condition and reproduction of the European hake (*Merluccius merluccius*) in the northwestern Mediterranean Sea. *ICES Journal of Marine Science*, 71(5): 1088–1099, DOI: 10.1093/icesjms/fst217.

This is an edited version of the manuscript published in ICES Journal of Marine Science. The final publication is available at http://icesjms.oxfordjournals.org/content/71/5/1088.

Parasitism, condition and reproduction of the European hake (*Merluccius merluccius*) in the northwestern Mediterranean Sea

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Abstract

It is well known that parameters relating to physical condition and reproduction of fish provide essential data for estimating the productivity of exploited populations, as is the case with the European hake (*Merluccius merluccius*) in the northwestern Mediterranean Sea. Although parasitism might affect these parameters, research in this area is very scarce (and in the case of the Mediterranean almost non-existent). This study evaluates for the first time the potential link between parasitism, condition and reproduction of the European hake. Indicators of fish energy reserves (total lipid content in liver and gonads) and reproductive capacity (fecundity, egg quality and atresia) were evaluated, as were the prevalence and intensity of infection by metazoan parasites. The results indicate that the impact of anisakid nematodes is mostly negative and occurs mainly when hake are allocating their energy reserves to gonadal development. Although the results reveal a link between parasitism, condition and reproduction, we concluded that the northwestern Mediterranean hake population is in equilibrium with its metazoan parasites, which are not causing severe impairment to their physical condition or reproductive capacity.

Introduction

Host-parasite coevolution suggests that fish are in dynamic equilibrium with their parasites (Barret 1986) which means parasitism is often overlooked in fish health assessment. However, it is a well-known fact that several fish parasites induce changes in host behaviour and morphology (Sasal & Thomas 2005; Barber & Wright 2006) and some parasitic organisms can become pathogenic and even fatal in heavy infections (Poulin 2002; Rohde 2005; Woo & Buchmann 2012). Moreover, parasites can regulate host population dynamics and influence community structure (Sindermann 1987; Marcogliese 2005).

It is also well-known that parameters relating to the condition of fish are essential for estimating the productivity of exploited populations (reviewed by Shulman & Love 1999 and Lloret *et al.* 2012). Although parasitism may affect life history traits such as condition, reproduction and mortality, research in this field is rather scarce and mainly focused on freshwater fish species (Tavares-Dias *et al.* 2000; Hoffnagle *et al.* 2006; Guidelli *et al.* 2011; among others). Thus, there is a lack of information on the effects of parasites on the productivity of exploited marine fishes worldwide and, in particular, in the Mediterranean Sea.

The European hake (*Merluccius merluccius*, Linnaeus, 1758) (henceforth: hake) is a gadoid with a wide geographical distribution that comprises the Atlantic coast of Europe and western North Africa, the Mediterranean Sea, and the southern coast of the Black Sea. In the Western Mediterranean, hake is one of the most important target species of commercial fisheries (Oliver & Massutí 1995). Currently, all Mediterranean stocks are considered to be highly exploited and in some areas, such as the Gulf of Lion, hake stocks might have decreased beyond safe biological limits as there is a situation of increasing overexploitation (Lleonart *et al.* 2003; Colloca *et al.* 2013).

Owing to its wide distribution and high commercial value, hake has been broadly studied. Early works were aimed at understanding its reproductive biology as well as making the first attempts to estimate its reproductive potential (Hickling 1935; Tsimenidis & Papaconstantinou 1985; Sarano 1986; among others). Subsequently, other relevant studies (Murua *et al.* 1998, 2006; Murua & Motos, 2006) established that hake is a batch spawner with indeterminate fecundity. Thenceforth, the number of studies on its reproduction has increased, although most of them are focused on the Atlantic population (Korta *et al.* 2010; Mehault *et al.* 2010; El Habouz *et al.* 2011; among others).

In addition, most research on hake condition is based mainly on simple morphometric condition factors (Lloret *et al.* 2002; Ferraton *et al.* 2007; Hidalgo *et al.* 2008; Giacalone *et al.* 2010; Costa 2013) and only a few have evaluated lipid content with regard to fisheries ecology (Lloret *et al.* 2008; Domínguez-Petit & Saborido-Rey 2010; Domínguez-Petit *et al.* 2010) or for human nutritional purposes (Pérez-Villareal & Howgate 1987; Küçükgülmez *et al.* 2008).

Metazoan parasites of hake have been reported in both Mediterranean and Atlantic stocks. On the one hand, Gibson *et al.* (2003), compilers of the Host-Parasite Database of the Natural History Museum (London, UK), gathered over fifty references on helminth parasites (monogeneans, digeneans, cestodes, nematodes and acanthocephalans) found in hake. Since then, the research on helminths in hake (mainly nematodes) has been focused on genetic studies (Mattiucci *et al.* 2004; Farjallah *et al.* 2008; Ceballos-Mendiola *et al.* 2010). On the other hand, parasitic copepods of hake have also been frequently recorded (Grabda & Soliman 1975; Tirard *et al.* 1996; Raibaut *et al.* 1998; Gaglio *et al.* 2011).

Despite the wide variety of parasites found in hake and their suspected negative effects, little attention has been paid to the effects of these parasites on the condition and reproduction of hake. At present, there is no recent research available and the few published studies on the subject have always focused on a single species or taxonomic group of parasites (Smith & Williams 1967; Margolis 1970; Ramadan *et al.* 1981; Guillaume *et al.* 1985). Finally, to our knowledge, there has been no research so far into the possible relationship between parasitism and reproduction of hake.

The main goal of this study is to evaluate the links between metazoan parasites and the condition and reproduction of European hake in the NW Mediterranean Sea. The outcomes are discussed from a biological and ecological perspective, in order to provide useful ideas for improving stock assessment and management of this valuable exploited species.

Materials and methods

Fish sampling

A total of 139 females of *M. merluccius* were collected from January 2010 to March 2012 from commercial trawling and longline catches in the Gulf of Lion (NW Mediterranean Sea), between the *Cap de Creus* (Spain) and the Marseille coast (France), at depths ranging from 45 to 510 m (Fig. I.1). Samples were obtained at the port of Roses (Fig. I.1), which is one of the most important fishing harbours in the region.

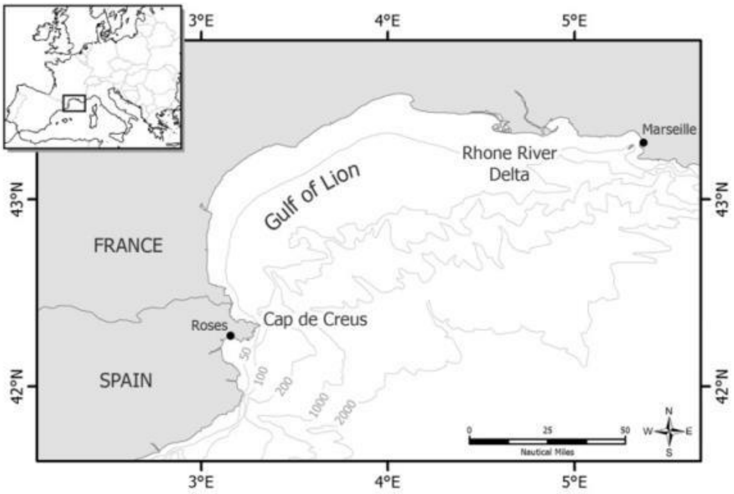


Fig. I.1. Map of the Gulf of Lion (northwestern Mediterranean), showing the port of Roses, where hake were sampled.

Only hake over 40 cm in length were collected to ensure that all samples were mature. The specimens were placed in individual plastic bags and transported on ice to the laboratory where they were immediately dissected. For each individual, total body length (L_T) was measured (± 0.1 cm), and total and eviscerated body weight (*TW* and *EW*), liver weight (*LW*) and gonads weight (*GW*) were recorded (± 0.1 g). The total body lengths of the 139 fish ranged from 41.3 to 73.2 cm (mean ± *SD* = 52.0 ± 5.8) and the total body weights ranged from 539.0 to 2959.3 g (mean ± *SD* = 1082.3 ± 414.7).

Once all the macroparasites were removed from each specimen, the whole liver and one ovary lobe were frozen at -20˚C for subsequent lipid content determination. The second ovary lobe was fixed in 4% buffered formaldehyde for histological processing.

Parasitism evaluation

All samples of hake were examined for metazoan parasites. First, a naked-eye examination was performed in order to remove all visible parasites from the body surface and the buccal cavity. Second, the entire viscera were removed from the body cavity, and the gills and internal organs (heart, oesophagus, stomach, intestine, spleen, gallbladder, liver and gonads) were examined using a stereomicroscope.

With regard to musculature, samples were examined from six individuals; the samples were obtained by filleting and crushing the tissue onto a transillumination platform. No metazoan parasites were found in these examinations nor were there any references in the literature to cestode or trematode larvae infecting European hake muscle. Furthermore, the prevalence of anisakid larvae in the viscera is clearly higher than in the muscle tissue (Valero *et al.* 2006). For these reasons, it was decided to regard the number of parasites in the musculature of hake as negligible.

All macroparasites were collected and washed with a saline solution (0.8% NaCl). They were first observed alive and then fixed in permanent preparations. Monogeneans, cestodes, nematodes, acanthocephalans and copepods were preserved in 70% ethanol, whereas digeneans were fixed in Bouin's solution under slight coverslip pressure. If necessary, and depending on the taxonomic group, the specimens were cleared in lactophenol or stained with borax carmine and mounted in Canada balsam.

Whenever possible, the parasites were morphologically identified to specific level following taxonomic keys and descriptions, such as Dawes (1947) for monogeneans; Williams (1958), Gaevskaja & Aljoshkina (1995) and Bray *et al.* (2008) for digeneans; Khalil *et al.* (1994) and Kuchta *et al.* (2008) for cestodes; Petter & Maillard (1987, 1988) and Køie (2001) for nematodes; Amin (1987) and Kvach (2006) for acanthocephalans; and Scott & Scott (1913) and Kabata (1992) for copepods.

Since nematode larvae belonging to the *Anisakis* genus were difficult to identify to specific level, they were provisionally assigned to two clades

(*Anisakis* larvae *Type I* and *Anisakis* larvae *Type II*) on the basis of their ventriculus length and the presence or absence of a mucron (Murata *et al.* 2011). In addition, a molecular verification on a subsample of 23 worms was performed after amplification and sequencing of a 629 bp fragment of the mitochondrial Cytochrome Oxidase 2 (*cox 2*) gene, according to the protocol described by Mattiucci *et al.* (2011).

The classification 'anisakid nematodes' included all nematodes belonging to the Anisakidae family, i.e. *Anisakis pegreffii*, *Anisakis physeteris*, *Hysterothylacium aduncum* and *Hysterothylacium fabri*, plus the anisakids in early larval stage designated as 'unidentified larvae'. In the present study, the term 'ectoparasites' is used to denote the parasite species found on the surface of the hake specimens, and 'endoparasites' to denote those species found within the body.

As described by Bush *et al.* (1997), the prevalence of parasites (*P*) was calculated as the proportion of fish infected with a particular parasite species (or taxonomic group) and the individual intensity of infection as the number of individuals of a particular species in a single infected host. The mean intensity was calculated as the average number of parasites of a particular species found in the infected hosts. The median intensity and its 95% confidence interval (*CI*) were also calculated.

Condition

For each individual, the total lipid content (% wet weight) was determined following the Soxhlet method described by Shahidi (2001), which was successfully tested for Mediterranean hake in a previous study (Lloret *et al.* 2008). Following the procedure described in Lloret *et al.* (2008), a lipid hepatosomatic index (*LHSI*) and a lipid gonadosomatic index (*LGSI*) were calculated as $LHSI = (ABSL/EW)100$ and $LGSI = (ABSG/EW)100$, where *ABSL* and *ABSG* are the absolute lipid content in liver and gonads, respectively, which were obtained by multiplying the respective lipid contents (% wet weight) by the wet weight of either the liver or the gonads.

The *LHSI* was considered as an indicator of hake condition because the liver constitutes the major organ of lipid storage in this species (Lloret *et al.* 2008), as is the case in gadoids in general (Marshall *et al.* 1999). The *LGSI* was used as a proxy of the energy reserves available for reproduction.

Reproduction

The gonadosomatic index (*GSI*), which is the relative weight of ovaries, was calculated as $GSI = (GW/EW)100$. It is assumed that *GSI* increases when spawning takes place, thus this index provides information on the fish reproductive cycle.

Hake ovaries were fixed and sliced transversely in their middle area. The resulting slices were embedded in paraffin, cut into 8-10 μm sections and stained with both Mallory's trichrome and hematoxylin-eosin stains. The ovarian development phases (*regenerating*, *developing*, *spawning capable*, *actively spawning* and *regressing*) were determined following Brown-Peterson *et al.* (2011).

A Chi-square test for independence indicated no significant association between year and ovarian development phase, $\chi^2(8, n = 138) = 4.5, p = 0.81$. Therefore, since the proportion of females in each particular phase did not differ among the three years of sampling, the data was combined and analysed as if it were a single group of samples.

The prevalence of atresia (*PA*) was defined as the proportion of females with observed α-atretic oocytes, and the relative intensity of atresia (*IA*) was calculated for each female as the number of α-atretic oocytes divided by the total number of vitellogenic oocytes (α-atretic and normal). Three different fields from different histological slides of each specimen were analysed.

Ovaries containing oocytes in migratory nucleus or hydrated stages and without recent post-ovulatory follicles (POFs), i.e. the empty follicular envelopes that appear following ovulation, were selected to assess the fecundity of these females ($n = 11$). As in Murua *et al.* (2006) and Recasens *et al.* (2008), homogeneity in oocyte distribution within hake ovaries was assumed.

Slices from the central area of the 11 previously-selected ovaries were weighed (± 0.1 mg). Then, the oocytes were separated using a washing process and sorted by size through several sieves (from 400 to 1000 µm) to facilitate counting and measurement of oocytes, which was carried out using a computer-aided image analysis system (*Image-Pro® Plus 5.1*; www.mediacy.com).

When oocyte size distribution followed a two-component mixture model, an algorithm of the *mixtools* package (Benaglia *et al.* 2009) for *R* software (www.r-project.org) was applied. This statistical procedure was used to describe quantitatively the properties of the overlapping mixtures and to calculate the number of oocytes belonging to the next batch. Thus, batch fecundity (*BF*) was defined by the number of eggs spawned per batch, and the relative batch fecundity (*BFrel*) was calculated as the value of batch fecundity per gram of eviscerated female body weight.

The dry mass of hydrated oocytes was used as an indicator of egg quality. Samples of 50 hydrated oocytes (with two replicates) were removed from nine ovaries. The sample was weighed (± 0.1 mg) after drying for 24 h at 110°C. The mean dry weight of hydrated oocytes (*ODW*) was calculated by dividing the dry weight by the number of oocytes per replicate.

Statistical analyses

The aggregated nature of parasite distributions leads to the concentration of a high proportion of parasites in a few host individuals. As argued by Rózsa *et al.* (2000), it is useful to report the confidence interval for the median intensity of infection. For this reason, the 95% *CI* was calculated, by the BCa method with 2000 bootstrap replications, using the free *Quantitative Parasitology 3.0* software (www.zoologia.hu/qp/qp.html).

This software, which was developed to manage the particularly left-biased frequency distribution of parasites, was also used to compare the prevalences (Fisher's exact test) and the median intensities (Mood's median test) for each parasite taxon (Monogenea, Digenea, Cestoda, Nematoda, Acanthocephala and Copepoda) throughout the different seasons and ovarian development phases. Bonferroni's correction was used to counteract the problem of multiple comparisons.

Once the normality of the data was tested and rejected, several non-parametric tests were performed to assess the possible effects of parasitism on hake condition (*LHSI* and *LGSI*) and reproduction (*IA*, *BF*, *BFrel* and *ODW*). The Mann-Whitney U test was used to analyse possible differences between infected and uninfected hake specimens. The Spearman's Rank Correlation coefficient was used to verify the possible relationships between the condition and reproduction parameters and the individual intensity of the infection by parasites.

Both analyses were performed for each species and taxon throughout the different ovarian development phases. In order to detect any possible synergistic effects, the correlations were also analysed with the total number of (i) individual parasites, (ii) species, (iii) ectoparasite species, and (iv) endoparasite species. The analyses performed also took into account the site of infection of anisakid nematodes (mesenteries, liver, gonads or digestive tract, i.e. stomach and intestines). The level of statistical significance adopted was $p < 0.05$.

Results

Condition and reproduction

The *LHSI* presented the highest mean value in September (1.11 ± 0.66) and the lowest in August (0.39 ± 0.19), while the *LGSI* presented the highest mean value in January (0.34 ± 0.32) and the lowest in May (0.01 ± 0.00). The *GSI* presented a clear seasonal pattern. High values were observed during the winter, with the highest monthly mean in January (5.56 ± 5.19) and another noticeable peak in July (3.09 ± 1.99). The lowest monthly mean (0.53 ± 0.13) was recorded in May.

Females in different reproductive phases appeared throughout the year and were found to be spawning-capable practically all year round (Fig. I.2).

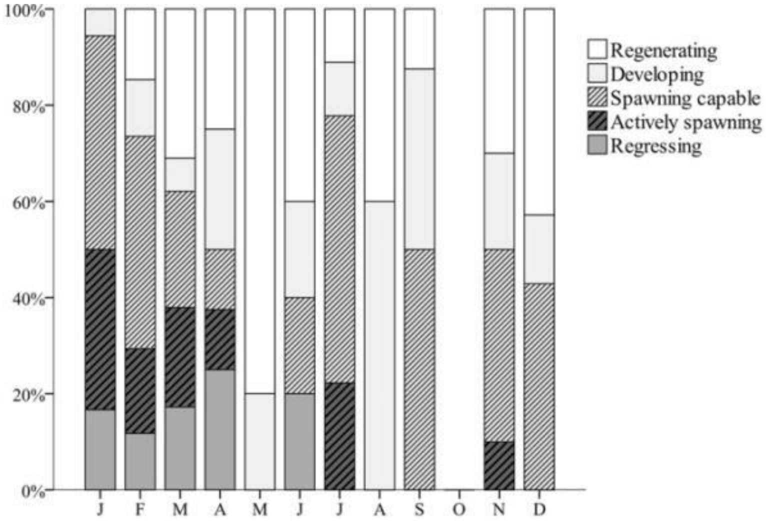


Fig. I.2. Monthly variations in the relative frequency of the ovarian development phases in hake (from January 2010 to March 2012).

In addition, and as shown in Figure I.3, the variations of *LHSI* and *LGSI* mean values were related to the ovarian development phase, i.e. the *LHSI* decreased with increasing *LGSI*. This opposing pattern was reported for each season except summer. Thus, it appears that the indices recorded are highly dependent on the ovarian development phase. Accordingly, the analyses of the effects of parasitism on condition and reproduction were carried out in relation to the status of gonadal maturation.

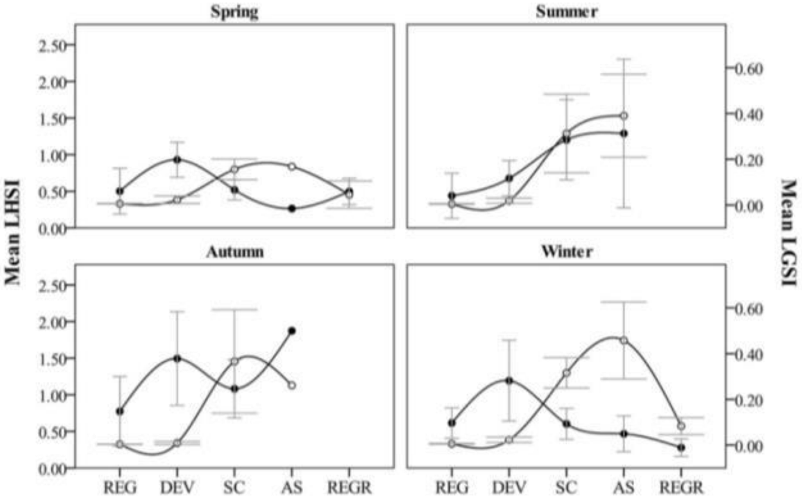


Fig. I.3. Seasonal mean *LHSI* (●) and *LGSI* (○) during the different ovarian development phases (REG, regenerating; DEV, developing; SC, spawning capable; AS, actively spawning; REGR, regressing). The bars represent ± standard deviations.

Prevalence and relative intensity of atresia were also closely related to the ovarian development phase. Females in regenerating or developing phases did not present any sign of α-atresia, while females in spawning capable (*PA* = 31.3%; *IA* = 5.94 ± 10.55%), actively spawning (*PA* = 13.6%; *IA* = 57.86 ± 36.51%) and regressing (*PA* = 60.0%; *IA* = 97.57 ± 7.28%) phases, showed different levels of α-atresia.

The batch fecundity and the relative batch fecundity were calculated for six females with oocytes in the migratory nucleus stage and for five females with hydrated oocytes. Figure I.4 shows the oocyte size-frequency distributions shown by each group. A Student's t-test did not show any significant differences between the two groups regarding *BF* or *BFrel*. Thus, the results were combined and the 11 females were considered as a single group of females with oocytes in advanced stage of maturation.

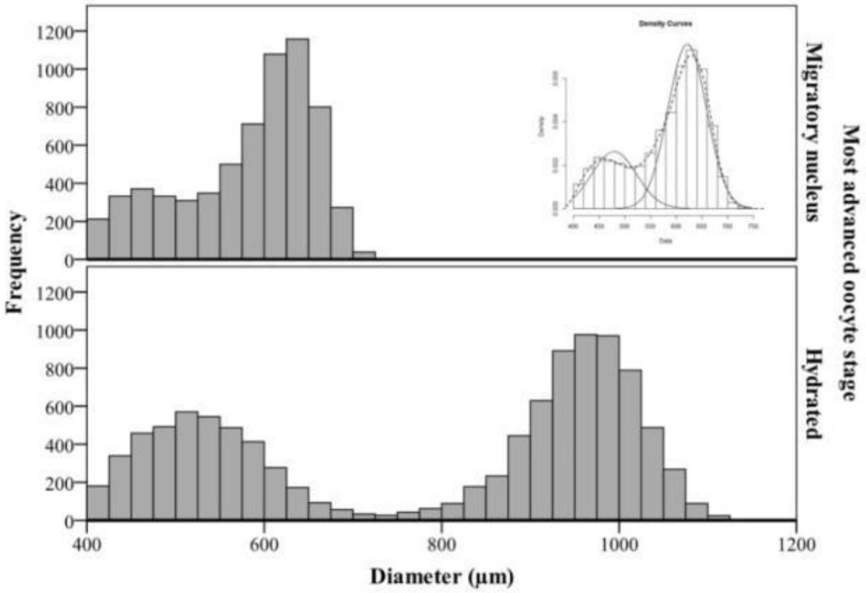


Fig. I.4. Oocyte size-frequency distributions (25 µm diameter class). Each distribution corresponds to an individual specimen [above, female with oocytes in migratory nucleus stage (L_T = 46.1 cm); below, female with hydrated oocytes (L_T = 67.2 cm)]. Since females in the migratory nucleus stage showed a two-component overlapping mixture distribution (small chart), oocytes with a 95% probability of belonging to the second component (larger diameter group) were considered as being from the next batch.

The *BF* ranged from 35470 to 268223 eggs (mean = 137077 ± 81919) and there was a correlation with the variables of total length (Spearman's rho; $\rho = 0.62$, $n = 11$, $p = 0.043$), eviscerated weight ($\rho = 0.62$, $n = 11$, $p = 0.043$), *LHSI* ($\rho = 0.75$, $n = 11$, $p = 0.008$) and *LGSI* ($\rho = 0.73$, $n = 11$, $p = 0.011$). Meanwhile, the *BFrel* ranged from 50 to 274 eggs·g^{-1} (eviscerated weight) (mean = 127 ± 68) and was strongly associated with *LGSI* ($\rho = 0.88$, $n = 11$, $p < 0.001$).

Finally, the hydrated oocyte dry weight ranged from 0.0310 to 0.0596 mg (mean = 0.0485 ± 0.0089) and did not present significant correlations with any of the analysed variables.

Parasitism

Without exception, all of the hake specimens analysed were infected with at least one parasite species. A total of 2054 metazoan parasites belonging to nineteen species were identified (Table I.1).

The ectoparasites were represented by five species: one monogenean and four copepods; and the endoparasites included fourteen species: four digeneans, two cestodes, six nematodes and two acanthocephalans. Since the identification of these species is based on adult features, parasite larvae such as metacestodes, as well as nematodes in the early larval stages, were not identified to the species level.

Nematodes species belonging to the *Anisakis* (Dujardin, 1845) genus have fairly similar morphology and therefore, identifying different species based only on morphological features is difficult. However, in this study, a differentiation between two morphotypes (*Type I* and *Type II* larvae) was possible.

A subsequent molecular analysis performed on a subsample of each morphotype revealed that the *Type I* larvae were *Anisakis pegreffii* (Campana-Rouget and Biocca, 1955), and the *Type II* larvae were *Anisakis physeteris* (Baylis, 1923). Since these results were in line with the results of a detailed review on hosts and distribution of anisakid nematodes (Mattiucci & Nascetti 2008), this outcome was inferred to the remaining *Type I* and *Type II* larvae.

Table I.1. Taxonomic composition, number of infected hosts, prevalence and intensity of metazoan parasites found in *Merluccius merluccius* from the Gulf of Lion (NW Mediterranean Sea). The parasite development stage, the site of infection and the ovarian development phase of hosts are also shown.

Parasite species	Stage	Site	Ovarian development phase	Infected hosts	P% (*n*=139)	Intensity Min-Max	Intensity Mean ± *SD*	Intensity 95% *CI*
Monogenea								
Anthocotyle merluccii (van Beneden & Hesse, 1863)	*A*	*G*	*REG, DEV, SC, AS, REGR*	26	18.71	(1-4)	1.54 ± 0.76	1.31 - 1.87
Digenea								
Aporocotyle spinosicanalis (Williams, 1958)	*A*	*H*	*AS*	1	0.72	(2-2)	2	-
Hemiurus communis (Odhner, 1905)	*A*	*S*	*AS*	1	0.72	(7-7)	7	-
Hemipera magnaprostatica (Gaevskaja & Aljoshkina, 1995)	*A*	*S*	*REG, DEV, AS*	5	3.60	(1-2)	1.20 ± 0.45	1.00 - 1.40
Lecithochirium musculus (Looss, 1907)	*A*	*S*	*DEV, SC*	2	1.44	(1-2)	1.50 ± 0.71	1.00 - 1.50
Cestoda								
Clestobothrium crassiceps (Rudolphi, 1819)	*A*	*I*	*REG, DEV, SC, AS, REGR*	107	76.98	(1-44)	4.87 ± 5.32	4.14 - 6.37
Tetraphyllidean metacestodes	*P*	*I*	*REG, DEV, SC, AS*	16	11.51	(1~100) *	13.63 ± 25.20	5.75 - 34.00
Nematoda								
Anisakis pegreffii (Campana-Rouget & Biocca, 1955)	*L3*	*I, L, M*	*REG, DEV, SC, AS, REGR*	115	82.73	(1-68)	7.78 ± 10.06	6.28 - 9.99
Anisakis physeteris (Baylis, 1923)	*L3*	*L, M*	*REG, DEV, SC, AS, REGR*	49	35.25	(1-8)	2.31 ± 1.84	1.88 - 2.92
Capillaria gracili (Bellingham, 1840)	*A*	*I*	*REG, DEV, SC, AS, REGR*	23	16.55	(1-17)	3.39 ± 4.46	2.04 - 5.83
Hysterothylacium aduncum (Rudolphi, 1802)	*L3, L4, A*	*I*	*REG, DEV, SC, AS, REGR*	24	17.27	(1-11)	2.42 ± 2.78	1.62 - 3.98
Hysterothylacium fabri (Rudolphi, 1819)	*L3, L4*	*I*	*REG, SC, AS, REGR*	6	4.32	(1-1)	1	-
Unidentified larvae	*L*	*I, S*	*REG, DEV, SC, AS, REGR*	12	8.63	(1-8)	1.58 ± 2.02	1.00 - 2.75
Acanthocephala								
Acanthocephaloides propinquus (Dujardin, 1845)	*A*	*S*	*SC, AS*	2	1.44	(1-1)	1	-
Echinorhynchus sp.	*A*	*S*	*SC*	2	1.44	(1-2)	1.50 ± 0.71	1.00 - 1.50
Copepoda								
Chondracanthus merluccii (Holten, 1802)	*A*	*B, G*	*REG, SC, REGR*	5	3.60	(1-4)	1.60 ± 1.34	1.00 - 2.20
Clavella stellata (Krøyer, 1838)	*A*	*G, Sk*	*REG, DEV, AS*	6	4.32	(1-2)	1.17 ± 0.41	1.00 - 1.33
Parabrachiella insidiosa (Heller, 1865)	*A*	*G*	*REG, DEV, SC, AS, REGR*	29	20.86	(1-6)	1.76 ± 1.15	1.45 - 2.31
Parabrachiella merluccii (Bassett-Smith, 1896)	*A*	*G*	*REG, DEV, SC*	14	10.07	(1-2)	1.21 ± 0.43	1.00 - 1.43

n, sample size. Stage: *A*, adult; *L*, early-stages larvae; *L3*, third-stage larvae; *L4*, fourth-stage larvae; *P*, plerocercoid larvae. Site: *B*, buccal cavity; *G*, gills; *H*, heart; *I*, intestines; *L*, liver; *M*, mesenteries; *S*, stomach; *Sk*, skin. Ovarian development phase of hosts: *REG*, regenerating; *DEV*, developing; *SC*, spawning capable; *AS*, actively spawning; *REGR*, regressing.

* Up to 100 metacestodes were counted from one single host, but the real intensity might be higher.

Nematodes were the dominant group ($P = 91.37\%$), followed by cestodes (79.86%), copepods (33.81%) and monogeneans (18.71%). Digeneans (5.76%) and acanthocephalans (2.88%) were detected more sporadically.

The Fisher's exact tests showed no significant differences between prevalences (species and taxa) from one season to another or through the different ovarian development phases. Likewise, the Mood's median test showed no significant difference in the median intensities (species and taxa) for season or ovarian development phases. Thus, neither season nor ovarian development phase affect the parasite load in hake.

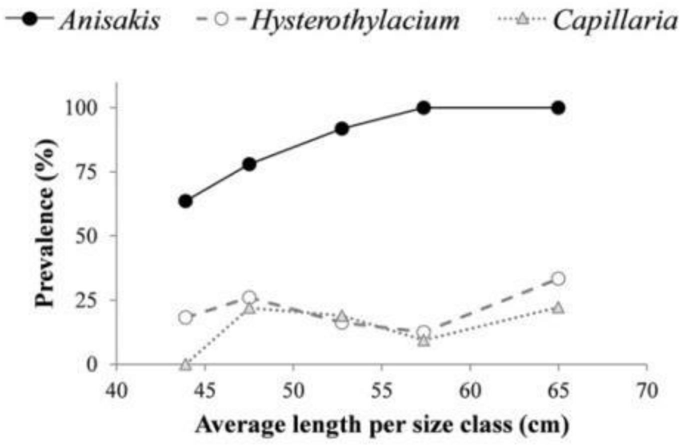


Fig. I.5. Prevalence of each genus of nematode in hake according to size class. The number of hosts examined per size class was: 11 specimens < 45 cm (average 43.9), 50 specimens between 45 and 50 cm (average 47.5), 37 specimens between 50 and 55 cm (average 52.8), 32 specimens between 55 and 60 cm (average 57.4), and 9 specimens > 60 cm (average 65.0).

As shown in Figure I.5, the prevalence of the infection by nematodes belonging to the *Anisakis* genus increased with host size (average length per size class) reaching prevalences of 100% for size classes above 55 cm. The relationship between total length and individual intensity of infection by nematodes (genus *Anisakis*, *Hysterothylacium* and *Capillaria*) was also investigated using Spearman's Rank Correlation. There was a strong positive correlation between total length and individual intensity of *Anisakis* ($\rho = 0.56$, $n = 139$, $p < 0.001$), with high intensities of infection associated with fish of greater length.

Parasite effects

The significant results ($p < 0.05$) of the Mann-Whitney U test and the Spearman's Rank Correlation are shown in Table I.2.

Hake in the regenerating phase that were infected with the copepods, *Clavella stellata* and *Parabrachiella insidiosa*, showed lower median values of *LGSI* and *LHSI*, respectively (Mann-Whitney U test). In addition, a lower median value of *LGSI* was also found in hake infected by *P. insidiosa* that were in spawning capable phase, although in this phase there was no correlation with the individual intensity of infection (Spearman's Rank Correlation, $p > 0.05$).

In both regenerating and developing ovary phases, hake infected by the monogenean, *Anthocotyle merluccii*, showed higher values of *LHSI* and a positive correlation between *LHSI* and individual intensity of infection by this monogenean.

Hake in the developing phase that were infected with anisakid nematodes in the mesenteries displayed lower median values of *LHSI* and *LGSI* compared with uninfected specimens. But when these same parasites were found in the liver during the developing phase, only *LGSI* was significantly lower.

Also in the developing phase, *LGSI* was negatively correlated to the intensity of *Anisakis pegreffii* (the most prevalent nematode), to the total number of parasite species and to the sub-category 'total number of endoparasite species'. We also found a negative correlation between *LGSI* and the intensity of *A. pegreffii* for hake in the spawning capable phase.

In the same phase, there were differences in the intensity of atresia (*IA*) between hake that were infected or uninfected with anisakids in the mesenteries. Although the median value was almost the same in both groups (Table I.2), the Mann-Whitney U Test revealed differences in the data distribution: the maximum value of *IA* in the uninfected group was 0.44% and in the infected group it was 30.59%.

When hake was actively spawning, the hydrated oocyte dry weight (*ODW*) of specimens infected with anisakids in the liver was higher than in uninfected hake; there was also a strong positive correlation between *ODW* and anisakids in the liver. The intensity of infection by anisakids in the digestive tract was also positively correlated with *ODW* and *LGSI*, although *LGSI* presented a negative correlation with the total number of ectoparasite species. Finally, there was a positive correlation between the intensity of infection by the cestode, *Clestobothrium crassiceps*, and *IA* for hake in regressing phase.

Table I.2. Results of Mann-Whitney U test used to verify the existence of differences between infected and uninfected hake and Spearman's Rank Correlation coefficient (ρ) used to evaluate possible relationships among the condition and reproduction variables and the individual intensity of parasitism. Only significant results ($p < 0.05$) are presented.

Ovarian development phase (*n*)	Parasite		Mann-Whitney U Test						Spearman's Rank Correlation		
		Variable	*n* Uninf.	*n* Inf.	*Md* Uninf.	*Md* Inf.	*U*	p^a	*n*	ρ	*p*
Regenerating (32)	*Anthocotyle merluccii*	*LHSI*	29	3	0.480	1.134	5	0.006**	32	0.450	0.010
	Clavella stellata	*LGSI*	30	2	0.005	0.002	3	0.024	32	-0.380	0.034
	Parabrachiella insidiosa	*LHSI*	28	4	0.547	0.169	20	0.034	32	-0.370	0.035
Developing (21)	*Anthocotyle merluccii*	*LHSI*	17	4	0.704	1.284	11	0.040	21	0.441	0.045
	Anisakis pegreffii	*LGSI*	5	16	0.027	0.012	13	0.025	21	-0.509	0.018
	Anisakid nematodes in the mesenteries	*LHSI*	6	15	1.455	0.704	13	0.011	-	-	-
	Anisakid nematodes in the mesenteries	*LGSI*	6	15	0.027	0.011	19	0.045	21	-0.440	0.046
	Anisakid nematodes in the liver	*LGSI*	11	10	0.027	0.010	25	0.036	-	-	-
	Total number of species	*LGSI*	-	-	-	-	-	-	21	-0.586	0.005**
	Total number of endoparasites species	*LGSI*	-	-	-	-	-	-	21	-0.461	0.036
Spawning capable (48)	*Anisakis pegreffii*	*LGSI*	-	-	-	-	-	-	48	-0.314	0.030
	Anisakid nematodes in the mesenteries	*IA* (%)	12	36	0.000	0.000	143	0.034	-	-	-
	Parabrachiella insidiosa	*LGSI*	35	13	0.313	0.162	142	0.047	-	-	-
Actively spawning (22)	Anisakid nematodes in the liver	*ODW* (mg)	6	3	0.048	0.057	0	0.024	9	0.807	0.009**
	Anisakid nematodes in the digestive tract	*LGSI*	-	-	-	-	-	-	22	0.585	0.004**
	Anisakid nematodes in the digestive tract	*ODW* (mg)	-	-	-	-	-	-	9	0.771	0.015
	Total number of ectoparasites species	*LGSI*	-	-	-	-	-	-	22	-0.428	0.047
Regressing (15)	*Clestobothrium crassiceps*	*IA* (%)	-	-	-	-	-	-	15	0.567	0.027

n, subsample size; Uninf., uninfected fish; Inf., infected fish; *Md*, variable median. Variables: *LHSI*, lipid hepatosomatic index; *LGSI*, lipid gonadosomatic index; *IA*, intensity of atresia; *ODW*, hydrated oocyte dry weight. Anisakid nematodes encompasses: *Anisakis pegreffii*, *Anisakis physeteris*, *Hysterothylacium aduncum*, *Hysterothylacium fabri* and anisakids in early larval stage classified as unidentified larvae.

[a] Asymptotic significances (2-tailed) are displayed for Mann-Whitney U tests with sample size above 10 in all groups, otherwise, exact significances [2*(1-tailed Sig.)] are given.

** Level of statistical significance $p < 0.01$

Discussion

With regard to the ectoparasites, our results indicate that their impact on the condition and reproduction of hake depends on the species of parasite concerned. Hake infected with the monogenean, *Anthocotyle merluccii*, appeared to be in better condition (i.e. they had more energy reserves in the liver) than specimens that were not infected with this parasite. One possible explanation might be that when hake are in regenerating and developing phases, they prioritize the accumulation of energy in the liver rather than allocating it to the parasitic immune response, resulting in specimens with higher energy reserves in the liver having higher intensities of infection.

In contrast, the results showed that hake infected by the parasitic copepods, *Clavella stellata* and *Parabrachiella insidiosa*, had lower energy reserves in the liver and gonads than specimens that were not infected with these parasites. The adult females of these two species, which belong to the Lernaeopodidae family, are permanently anchored to the host by a strong attachment organ that can cause proliferative cell changes. In response to this infection, the hosts may allocate part of their stored energy to repairing the damage inflicted by these parasites.

The results also showed a negative correlation between the number of ectoparasite species (all species together) and the energy reserves in the gonads of hake in the actively spawning phase; however, no correlation was found when each species of parasite was analysed separately. This might indicate that there was a synergetic effect of the ectoparasite species.

In the literature, there are contradictory results regarding the effects of specific ectoparasites on hake condition. On the one hand, Gaglio *et al.* (2011) studied the prevalence of the copepod, *C. stellata*, in hake and found that smaller hake had more parasites than larger ones, concluding that the copepod caused little damage to the hake population due to the immunological status of the larger hake. On the other hand, Guillaume *et al.* (1985) studied the influence of the blood-sucking copepod, *Lernaeocera branchialis*, on the hake's erythrocyte constants and found different types of anaemia depending on the intensity of infection. The monogenean, *A. merluccii*, reported in this study, remains attached to the gills of their hosts and feeds on mucus and epithelial cells, while the copepod, *L. branchialis*, sucks blood on the gills of its host. Hence, the impact of ectoparasites on hake might also depend on the nutritional requirements of the parasite group it belongs to.

With regard to the endoparasites, our results indicate that their impact on hake condition and reproduction is mostly negative, whichever parasite species is considered. In the developing phase, the negative correlations between energy reserves in gonads and both the total number of species and the total number of endoparasite species indicates that there might also be a synergetic effect.

During gonad development in hake, anisakid nematodes in the mesenteries appeared to affect negatively the energy reserves in the liver and, specifically, the nematode *A. pegreffii* affected negatively the energy reserves in the gonads; in addition, a high intensity of infection by *A. pegreffii* may affect the gonadal energy reserves when hake reaches the spawning capable phase. In this sense, severe damage in liver that was heavily infected with anisakid nematodes has been reported (Margolis 1970 cited by Levsen & Berland 2012; Ramadan *et al.* 1981). Probably, the individual intensity of infection plays an important role in terms of the adverse effects of parasitism.

Furthermore, during the spawning capable phase, the presence of anisakid nematodes in the mesenteries was related to high values of the intensity of atresia. Such a relationship may be due to a trade-off aimed at partially compensating for some of the energy taken by the parasites.

Conversely, hake in the actively spawning phase with higher intensities of anisakid nematodes in the liver and in the digestive tract have better egg quality, as shown by a higher oocyte dry weight, as well as an increase in the gonad energy reserves. We can hypothesize that this paradoxical result may be due to the fact that fish become infected when actively feeding and, therefore, hake that feed more have higher energy reserves but also a greater chance of becoming infected by nematodes. However, this result must be interpreted with caution because fish in the actively spawning phase either have hydrated oocytes ready to be released or have just released a batch and, therefore, individual energy reserves in gonads are highly variable.

Finally, at the end of the individual spawning season, i.e. regressing phase, atresia intensity was also positively correlated to the intensity of infection by the cestode, *Clestobothrium crassiceps*, but it should be noted that the proportion of atretic oocytes in the regressing phase is highly variable and dependent on the particular time at which the specimen is captured. Indeed, females in regressing phase are at various different stages in the process of resorption of unreleased oocytes, i.e. recycling the energy used in gamete development and making it available, thus improving body condition.

There is a considerable amount of literature on nematodes because (i) they are the dominant group parasitizing hake and (ii) some anisakid nematodes may cause human diseases. Disregarding discrepancies that may be due to variation in methodologies, anisakid nematodes prevalences and intensities reported in our study are within the average ranges reported in previous studies (Abollo *et al.* 2001; Mattiucci *et al.* 2004; Valero *et al.* 2006; Angelucci *et al.* 2011). However, it is noteworthy that the maximum intensity of infection by anisakids in Atlantic waters is clearly higher than it is in the NW Mediterranean Sea, reaching intensities of up to 3400 larvae in a single infected hake (Ceballos-Mendiola *et al.* 2010) compared with a maximum of 72 larvae found in our study.

In this sense, another result that should be noted is the relationship between prevalence and intensity of nematodes and the size of hake. It seems that larger hake harboured more *Anisakis* nematodes than smaller ones. By contrast, there is no similar relationship with other nematodes, not even with other genera of the Anisakidae family, such as *Hysterothylacium*.

This may be related to the life cycles of nematodes. Hake are among a number of fish that can become the definitive host for *Hysterothylacium*, which will reproduce, eventually die and be expelled from the hake's body. In contrast, hake are intermediary hosts for *Anisakis*. These larvae remain embedded in the tissue of the hake at the highly resistant L3 larval stage, where they wait until the hake is preyed upon (or dies and is fed upon) by the final host, such as cetaceans. Over the course of their lives, hake may accumulate more of these L3 *Anisakis* larvae, which are more difficult to eliminate than other nematodes. This would explain how more of these larvae were found in larger hake. Valero *et al.* (2006) also found higher values of *Anisakis* spp. prevalence in larger hake. These findings have important implications for human health risk assessment since *Anisakis* larvae can cause a clinical disease in humans known as anisakidosis.

Concerning hake reproduction, the results of this study indicate that Mediterranean hake has a protracted spawning season which continues practically all year round with a spawning peak in winter and another short peak in summer, which is consistent with results obtained in different Mediterranean areas (Recasens *et al.* 2008; Al-Absawy 2010). The joint analysis of energy reserves in liver and gonads showed a fluctuation in the amount of lipids between tissues depending on the ovarian development phase. In other words, gonads develop in detriment to the accumulated lipidic energy in the liver.

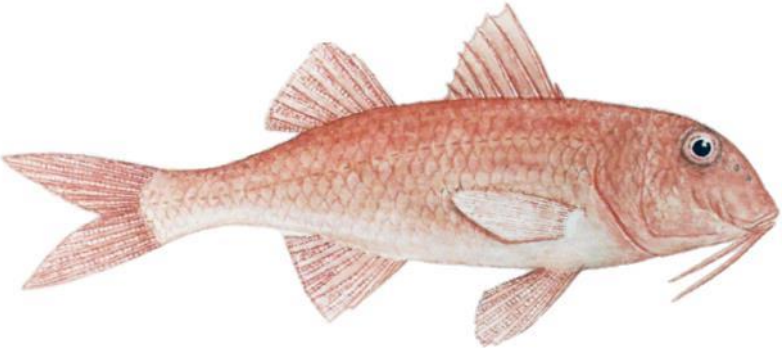
This reproductive strategy, known as *capital breeding*, has been reported for other gadoids (Marshall *et al.* 1999), but interestingly, in summer, it seems that hake change their strategy and behave like an *income breeder*, i.e. the energy allocated to reproduction comes from concurrent feeding. It should be noted that Domínguez-Petit & Saborido-Rey (2010) demonstrated that European hake in the North Atlantic always develop the gonads when there is an energy intake surplus rather than doing so at the expense of accumulated energy.

The reason for this discrepancy between the North Atlantic and the Mediterranean might be that hake is such a plastic species that it can adapt its breeding strategy to the particular biotic and abiotic factors that characterize each geographical area. The Mediterranean is a poorer sea, in terms of food, but in summer there are many more resources available since the peak of abundance of most prey populations occurs in this season (Bozzano *et al.* 1997), thus leading to a change in the reproductive strategy of the species.

The values of fecundity are consistent with previous studies performed in the Mediterranean (Recasens *et al.* 2008), in the North Atlantic (Murua *et al.* 1998, 2006; Korta *et al.* 2010) and in the eastern central Atlantic (El Habouz *et al.* 2011). Batch fecundity increases proportionally with the size of hake, and it should be noted that correlations between batch fecundity and energy reserves, i.e. *LHSI* and *LGSI*, are still stronger than the correlations with hake size. Analogous findings reported by Domínguez-Petit & Saborido-Rey (2010) led the authors to hypothesize that larger females have higher fecundity because they have a greater capacity for obtaining energy than smaller individuals.

Prevalence and relative intensity of atresia follow the expected values for species with indeterminate fecundity, such as in the hake stock of the North Atlantic (Murua & Motos 2006). Finally, the results of the hydrated oocyte dry weight, which were used as an indicator of egg quality, showed values that were decidedly similar to an earlier study carried out on the North Atlantic population (Mehault *et al.* 2010).

Overall, the results seem to be consistent with the hypothesis that there is a dynamic equilibrium between hosts and parasites (Barret 1986), because potent effects of metazoan parasites on hake condition and reproduction were not detected within the infection intensities observed. Furthermore, no new parasites infecting hake, which could generate a new immune response and an eventual decline in physical condition or reproductive capacity in this species, have been found to date. In this sense, and taking into account new species interactions arising from the biological consequences of global climate and anthropogenic change, we consider it is important to continue monitoring parasitism in commercial species such as hake, as well as the possible additive or synergistic effects on hake condition and reproduction resulting from an eventual increase of the parasite load in the future.



Chapter II:

Red mullet (*Mullus barbatus*)

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Health and reproduction of red mullet, *Mullus barbatus*, in the western Mediterranean Sea

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Abstract

The reproductive and general health of exploited fish stocks is an essential element of sustainable and profitable fisheries. The main purpose of this study is to assess the relationships between reproduction and two important parameters of fish health (parasitism and energy reserves) in female specimens of red mullet, *Mullus barbatus*, from the western Mediterranean Sea. We present new data for this species on (i) the prevalence and intensity of infection by metazoan parasites; (ii) the total lipid content in muscle and gonads as a measure of condition and (iii) fecundity and egg quality as a measure of their reproductive capacity. The results show that *M. barbatus* is a batch spawner with an income breeding strategy, an asynchronous development of oocytes and indeterminate fecundity. The results also indicate that the three most abundant and prevalent parasites significantly affect the condition and reproduction of *M. barbatus*. Specifically, the digenean, *Opecoeloides furcatus*, causes a reduction in the female's energy reserves while the nematodes, *Hysterothylacium fabri* and *H. aduncum*, produce a rise in egg production but impair egg quality. These implications of the relationships between parasitism, fish health and fish reproduction should be taken into consideration in the assessment and management of exploited species.

Introduction

Parasitism is usually defined as a non-mutual symbiotic relationship between species, where the parasite benefits at the expense of the host. In theory, therefore, parasitism should produce deleterious effects on fish health because the parasites drain energy from the fish that would otherwise be used to sustain the fish's condition, growth or reproduction. However, it appears that through the coevolution of host and parasite, a dynamic equilibrium has arisen between fish and their parasites (Barret 1986). Indeed, in most cases, parasites do not cause severe effects on their hosts because this would impair their chances of reaching the following stage of their life cycle or of reproducing.

Lipid storage and dynamics within the organism are an important aspect of fish health (Adams 1999). The energy reserves of fish are a highly significant indicator of their condition and may have important implications in research into fish life-history traits and, consequently, for stock productivity and fisheries in general (reviewed by Lloret *et al.* 2012a, 2014). In particular, the maternal condition has been identified as an important factor in determining egg production and egg condition in a number of fish species, e.g. Atlantic cod, *Gadus morhua* L. 1758, and haddock, *Melanogrammus aeglefinus* L. 1758, (Saborido-Rey *et al.* 2003; Alonso-Fernández *et al.* 2009).

However, little attention has been paid to the effects of parasitism on maternal condition or, consequently, on reproductive capacity. There are some studies on the relationships between parasitism and condition (e.g. Ondracková *et al.* 2010; Guidelli *et al.* 2011; Francová & Ondračková 2013) and also some that include reproductive variables (e.g. Sasal *et al.* 2001; Bagamian *et al.* 2004; Bean & Bonner 2009). However, most of these studies used simple morphometric indices of condition and have focused on freshwater fish species or on the effects of a single species of parasite.

With regard to marine exploited species, information on the effects of parasites on the energy reserves is limited to a few studies on northwestern Atlantic cod, *Gadus morhua*, (Khan & Lacey 1986), young-of-year common sole, *Solea solea* L. 1758, in the northeastern Atlantic Ocean (Durieux *et al.* 2007), anchovy, *Engraulis encrasicolus* L. 1758, in the Black Sea (Shchepkina 1980) and Japanese anchovy, *E. japonicus* Temminck & Schlegel 1846, (Sajiki *et al.* 1992). To our knowledge, the only study into the relationships between parasitism, energy reserves and reproductive capacity of an exploited marine species is our previous work on the European hake, *Merluccius merluccius* L. 1758, in the northwestern Mediterranean Sea (Ferrer-Maza *et al.* 2014). In short, little is known about the effects of parasites on the condition and the productivity of marine fishes.

Red mullet, *Mullus barbatus* L. 1758, is a demersal species that inhabits sandy and muddy bottoms of the continental shelf of the Mediterranean Sea, including the Black Sea, and also the eastern Atlantic from Scandinavia to Senegal (Fischer *et al.* 1987). This species is an important component of the Mediterranean demersal resources exploited by bottom trawling and small-scale fisheries (Fischer *et al.* 1987; Tserpes *et al.* 2002). The stocks of *M. barbatus* are highly vulnerable to recruitment changes (Tserpes *et al.* 2002) and all stocks in the Mediterranean Sea are currently considered overexploited (FAO 2012).

Despite the importance of energy reserves in estimating the productivity of exploited populations, the lipid content of *M. barbatus* has rarely been considered in the context of fishery ecology (Lloret *et al.* 2005, 2007; Roncarati *et al.* 2012).

The available knowledge on the reproductive biology of *M. barbatus* is also very scarce and based mainly on macroscopic observations or simple morphometric indices (Desbrosses 1935). There have been only a few attempts to estimate its fecundity (Tirasin *et al.* 2007; Aydın & Karadurmuş

2013) and a single histological study describing the different stages of oocyte development (Kokokiris *et al.* 2014). Therefore, little information exists on the spawning strategy or the oocyte recruitment pattern.

The metazoan parasites of *M. barbatus* have, in contrast, been widely reported, mostly in studies aimed at identifying a single species or group of parasites (e.g. Martínez-Vicaria *et al.* 2000; Ramdane *et al.* 2013), but also in the reporting of a list of the metazoan parasite fauna of this species (Carreras-Aubets *et al.* 2011, 2012).

The main purpose of this study is to assess the relationships between reproduction and two important parameters of fish health (i.e. parasitism and energy reserves) with regard to *M. barbatus* in the western Mediterranean Sea. By considering these relationships, this study also sheds light on several essential but undocumented aspects of the reproduction of this species, such as the oocyte recruitment pattern or the spawning strategy. The outcomes are discussed from a biological and ecological perspective in order to provide useful information for improving stock assessment and management of this economically valuable species.

Materials and methods

Fish sampling

A total of 335 mature female specimens of *M. barbatus* were collected from January 2010 to May 2012, on a quarterly basis in order to obtain representatives from each ovarian developmental phase. However, most of the sampling events (89%) were concentrated in the second quarter, i.e. the spawning period of the species, because this is when the reproductive capacity of females (fecundity and egg quality) can be measured. Moreover, as the females presumably need to allocate more of their energy to egg production during this period, they should be more sensitive to an eventual effect of parasites on their overall condition.

Specimens were caught via bottom trawls (from MEDITS trawl survey and commercial trawlers) at depths ranging from 30 to 250 m along the western coast of the Spanish Mediterranean Sea (FAO-GFCM geographical sub-area GSA-06, *Northern Spain*) (Fig. II.1).

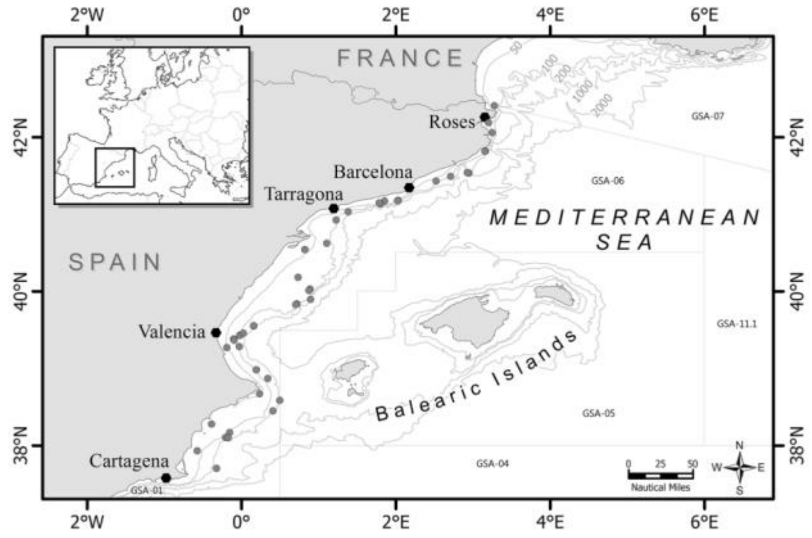


Fig. II.1. Map of the western Mediterranean Sea showing the locations where *Mullus barbatus* were caught (FAO-GFCM geographical sub-area GSA-06, *Northern Spain*).

For each individual, the total body length (L_{T}) was recorded (± 0.1 cm), as well as total body mass (M_{T}), eviscerated body mass (M_{E}) and gonad mass (M_{G}) (± 0.1 g). The total lengths of the specimens sampled ranged from 12.5 to 26.9 cm (mean ± *SD* = 19.5 ± 2.5).

All the muscle tissue from the left side of each specimen and one ovary lobe were removed and frozen at −20°C for subsequent lipid content determination, while the second ovary lobe was fixed in 4% buffered formaldehyde for histological processing and subsequent fecundity estimation.

Parasitism evaluation

All the specimens were examined for metazoan parasites prior to the removal of muscle and gonads for lipid content determination and histology. The entire viscera were removed from the body cavity, and the gills and internal organs (heart, oesophagus, stomach, intestine, spleen, gallbladder, liver, and gonads) were examined using a stereomicroscope.

The macroparasites were collected and washed with a saline solution (0.8% NaCl). They were first observed alive and then fixed in permanent preparations. Cestodes, nematodes and isopods were preserved in 70% ethanol, whereas digeneans were fixed in Bouin's solution under slight coverslip pressure. If necessary, and depending on the taxonomic group, the specimens were cleared in lactophenol or stained with borax carmine and mounted in Canada balsam.

Parasites were morphologically identified to the lowest taxonomic level possible following the available keys and descriptions, such as Gibson *et al.* (2002), Jones *et al.* (2005) and Bray *et al.* (2008) for digeneans or Naidenova & Nikolaeva (1968), Petter *et al.* (1984) and Petter & Maillard (1987, 1988) for nematodes. Since the identification of each species of parasite is based on adult features, some parasite larvae could not be identified to the species level. These included immature digeneans, L3 larvae of *Contracaecum* sp., Tetraphyllidean plerocercoids and the praniza larvae of the gnathiid isopods (*Gnathia* sp.).

As described by Bush *et al.* (1997), the prevalence of parasites (P_P) was calculated as the proportion of fish infected with a particular parasite species and the individual intensity of the infection as the number of individuals of a particular species in a single infected host. The mean intensity was calculated as the average number of parasites of a particular species found in the infected hosts. The median intensity and its 95% confidence interval (*CI*) were also calculated.

Energy reserves determination

A subsample of muscles ($n = 156$) and ovaries ($n = 87$) large enough to perform the lipid extraction (minimum required = 4 g of tissue) were selected to assess the condition (indicated by energy reserves) of *M. barbatus*. Females whose gonads were large enough to perform lipid extraction appeared only in May and June (spawning season). In that season, corresponding to a sensitive critical period of the fish life cycle, i.e. reproduction, more analyses of muscular lipid reserves were performed in order to optimize the detection of potential effects of parasitism on the condition of *M. barbatus*.

In muscle as well as in gonads, the total lipid content (% wet mass) was determined following the Soxhlet method described by Shahidi (2001). A lipid musculo-somatic index (I_{LM}) and a lipid gonado-somatic index (I_{LG}) were calculated as $I_{\mathrm{LM}} = 100\,ABS_{\mathrm{M}}\,M_{\mathrm{E}}^{-1}$ and $I_{\mathrm{LG}} = 100\,ABS_{\mathrm{G}}\,M_{\mathrm{E}}^{-1}$, where ABS_{M} and ABS_{G} are the absolute lipid content in muscle and gonads, respectively, which were obtained by multiplying the respective lipid contents (% wet mass) by the wet mass of either the muscle or the gonads. The muscle wet mass (M_{M}) was estimated using the equation $M_{\mathrm{M}} = 0.64\,M_{\mathrm{E}} - 0.9$ ($R^2 = 0.99$, $n = 98$, $p < 0.0005$) given by Lloret *et al.* (2007) for *M. barbatus* in the northwestern Mediterranean.

The I_{LM} was considered as an indicator of *M. barbatus* condition because the muscle tissue constitutes the main source of lipid reserves in this species (Lloret *et al.* 2007), whereas the I_{LG} was used as an indicator of the energy reserves that were being used in the development of the gonads.

Reproductive cycle description and atresia evaluation

The gonado-somatic index (I_{G}), which is the relation between the gonad mass (M_{G}) and the eviscerated mass (M_{E}) of the females, was calculated as $I_{\mathrm{G}} = 100\,M_{\mathrm{G}}\,M_{\mathrm{E}}^{-1}$. The mass of ovaries increases with vitellogenesis and hydration of eggs, thus this index provides information on the fish reproductive cycle.

Furthermore, one ovary lobe from each *M. barbatus* ovary was fixed and sliced transversely in its midsection. The resulting slices were embedded in paraffin, cut into 8–10 µm sections, and stained with both hematoxylin-eosin and Mallory's trichrome stains. The latter staining method highlights the zona radiata and its continuity and facilitates the detection of degenerating oocytes which will not be spawned, i.e. atretic oocytes (Muñoz *et al.* 2010).

To describe the developmental stage of the oocytes, we used the terminology employed by (Lowerre-Barbieri *et al.* 2011): primary growth (PG), cortical alveolar (CA), vitellogenesis (VIT1 and VIT2), maturation (OM), hydrated oocytes (H) and atresia. On the other hand, we used the presence of specific histological markers to classify our specimens into four ovarian developmental phases, following (Brown-Peterson *et al.* 2011): *regenerating* (REG) - sexually mature but reproductively inactive; d*eveloping* (DEV) - gametes begin to develop; *spawning capable* (SC) - advanced, developed gametes ready for the spawning season; and *regressing* (REGR) - massive atresia which indicates the end of the reproductive cycle.

The prevalence of atresia (P_A) was defined as the proportion of females with observed α-atretic oocytes, and the relative intensity of atresia (I_A) was calculated for each female as the number of α-atretic oocytes divided by the total number of vitellogenic oocytes (α-atretic and normal). Three different fields from different histological slides of each specimen were analysed.

Fecundity and egg quality estimations

Ovaries containing hydrated oocytes, but which did not have recent post-ovulatory follicles (POFs), were selected to assess the fecundity (n = 89). Slices from the central area of the ovaries were weighed (± 0.1 mg) and the oocytes were separated using a washing process, as described by Lowerre-

Barbieri & Barbieri (1993), and sorted by size through several sieves (from 400 to 600 µm). The oocytes were counted and their diameters were measured using a computer-aided image analysis system (Image-Pro® Plus 5.1, Media Cybernetics, Inc., Bethesda, MD, USA).

In addition to obtaining the oocyte diameter-frequency distributions from the above procedure, we wanted to investigate this species' spawning pattern (total spawner vs. batch spawner) and its oocyte recruitment pattern (synchronous vs. asynchronous oocyte development and determinate vs. indeterminate fecundity). To this end, 10 ovaries, identified as being from specimens in the spawning capable phase in the previous histological analysis, were randomly selected and used to measure the mean diameter of oocytes in the different stages of development. Oocytes in primary growth (PG), cortical alveolar (CA), vitellogenesis (VIT1 and VIT2) and maturation (OM) stages (n = 379) were measured individually in histological sections, whereas the hydrated oocytes (H) (n = 247) were measured using the aforementioned image analysis system, since they usually show an irregular shape in the histological preparations.

Investigation of the spawning pattern showed that *M. barbatus* is a batch spawner. Thus, in order to assess the reproductive capacity, we also estimated the batch fecundity (F_{B}), defined as the number of eggs spawned per batch, and the relative batch fecundity (F_{Brel}), defined as the value of batch fecundity per gram of eviscerated female body mass.

Finally, the dry mass of hydrated oocytes was used as an indicator of egg quality. Samples of 200 oocytes in late maturation were removed from the ovaries mentioned above (n = 89) and weighed (± 0.1 mg) after drying for 24 h at 110°C. The mean dry mass of oocytes (M_{DO}) was calculated by dividing the sample's dry mass by the number of oocytes in the sample.

Data analysis

The aggregated nature of parasite distributions leads to the concentration of a high proportion of parasites in a few host individuals. As argued by Rózsa *et al.* (2000), it is useful to report the confidence interval (*CI*) for the median intensity of infection. For this reason, a 95% *CI* was calculated, by the *BCa* method with 2000 bootstrap replications, using the free *Quantitative Parasitology 3.0* software (Reiczigel & Rózsa 2005).

This software, which was developed to manage the particularly left-biased frequency distribution of parasites, was also used to compare the prevalences (Fisher's exact test) and the median intensities (Mood's median test) for each parasite species through the different ovarian developmental phases of the hosts. A false discovery rate (FDR) approach was used to counteract the problem of multiple comparisons (Benjamini & Hochberg 1995; Verhoeven *et al.* 2005).

A general linear model (GLM) was used to determine the effects of total body length (L_{T}), year, latitude and depth of capture, on the condition and reproduction response variables, i.e. lipid musculo-somatic index (I_{LM}), lipid gonado-somatic index (I_{LG}), relative batch fecundity (F_{Brel}) and the dry mass of oocytes (M_{DO}). In order to satisfy normality assumptions, I_{LM} was natural-log-transformed. Explanatory variables were removed by backward elimination and only those which contributed significantly to the model were kept. As shown in Table II.1, some of the explanatory variables had significant effects on I_{LM}, I_{LG} and F_{Brel} although no effect on M_{DO} was found. To eliminate this influence and focus on the effects of parasitism, all subsequent analyses were performed with the adjusted values of these response variables.

Table II.1. Results of the analysis of variance for the response variables [natural-log-transformed lipid musculo-somatic index (I_{LM}), lipid gonado-somatic index (I_{LG}) and relative batch fecundity (F_{Brel})], and the effects of the explanatory variables [year, total length (L_T), latitude and depth of capture]. The deviance explained by each variable is also shown. No significant interactions between explanatory variables were found.

Response variable	Explanatory variables	Df	MS	*F*	*P*	DE (%)
I_{LM}	LATITUDE					-
	DEPTH					-
	YEAR	2	1.985	6.552	0.002	7.9
	L_T	1	1.969	6.502	0.012	4.1
	Corrected model	3	2.134	7.047	0.000	12.2
	Error	152	0.303			
I_{LG}	LATITUDE					-
	DEPTH					-
	YEAR	2	0.255	10.406	0.000	20.0
	L_T	1	0.219	8.904	0.004	9.7
	Corrected model	3	0.277	11.282	0.000	29.0
	Error	83	0.025			
F_{Brel}	LATITUDE					-
	YEAR					-
	L_T	1	29005.964	7.946	0.006	8.5
	DEPTH	1	27065.881	7.415	0.008	7.9
	Corrected model	2	20022.160	5.485	0.006	11.3
	Error	86	3650.366			

Df: degrees of freedom; MS: mean square; DE: deviance explained

Once the normality of the data was tested through the different ovarian developmental phases, and rejected, several non-parametric tests were performed to assess the possible effects of parasitism on *M. barbatus* condition (adjusted I_{LM} and adjusted I_{LG}) and reproduction (adjusted F_{Brel} and M_{DO}).

The Mann-Whitney U test was used to analyse possible differences between infected and uninfected *M. barbatus* specimens. The Spearman's Rank Correlation coefficient was used to assess the possible relationships between the condition and reproduction parameters and the individual intensity of infection by parasites. These two analyses were performed for the specialist parasites, as well as for the generalist parasites provided the latter appeared in at least 10 specimens, i.e. with a prevalence of infection above 3% (Table II.2). The level of statistical significance adopted was $p < 0.05$.

Results

Of the 335 specimens of *M. barbatus* examined, 276 (82.4%) were infected with at least one metazoan parasite species, with an individual intensity of infection that ranged from 1 to 81 parasites (mean ± $SD = 8.38 \pm 10.41$). A total of 2313 parasites belonging to at least 13 taxonomic groups were identified: six digeneans, five nematodes, one cestode and one isopod (Table II.2). The real number of different species might be higher than reported as the groups of larvae classified as “immature digeneans”, *Contracaecum* sp., Tetraphyllidean plerocercoids and *Gnathia* sp. may include several species.

The most prevalent species was the nematode *Hysterothylacium fabri* (Rudolphi 1819), with a prevalence of 63.9% and a mean intensity of 3.95 ± 5.94 parasites. The species with the highest intensity of infection was the digenean *Opecoeloides furcatus* (Bremser in Rudolphi 1819), with a prevalence of 55.5% and a mean intensity of 6.97 ± 8.66 parasites (Table II.2).

Table II.2. Taxonomic composition, number of infected hosts, prevalence (P_P) and intensity of metazoan parasites found in *Mullus barbatus* from the western Mediterranean Sea. The parasite developmental stage, the site of infection and the ovarian developmental phase of hosts are also shown.

Parasite species	Stage	Site	Ovarian developmental phase	Infected hosts	P_P (n=335)	Intensity		
						Min-Max	Mean ± s.d.	Median 95% *CI*
Digenea								
Derogenes latus (Janiszewska, 1953)	*A*	*D*	*DEV, SC*	6	1.79	(1-7)	2.50 ± 2.51	1.00 - 4.50
Lasiotocus mulli (Stossich, 1883)*	*A*	*D*	*REG, REGR*	2	0.60	(1-4)	2.50 ± 2.12	1.00 - 2.50
Lecithocladium excisum (Rudolphi, 1819) Lühe, 1901	*A*	*D*	*DEV, SC*	2	0.60	(2-3)	2.50 ± 0.71	2.00 - 3.00
Opecoeloides furcatus (Bremser in Rudolphi, 1819)*	*A*	*D*	*REG, DEV, SC, REGR*	186	55.52	(1-53)	6.97 ± 8.66	5.88 - 8.41
Proctotrema bacilliovatum (Odhner, 1911)*	*A*	*D*	*DEV, SC, REGR*	4	1.19	(1-7)	3.25 ± 2.63	1.50 - 5.75
Unidentified immature specimens	*L*	*D*	*SC, REGR*	6	1.79	(1-15)	3.50 ± 5.65	1.00 - 10.33
Nematoda								
Ascarophis mullusi (Naidenova & Nikolaeva, 1968)*	*A*	*D*	*DEV, SC, REGR*	4	1.19	(1-5)	2.25 ± 1.89	1.00 - 4.00
Contracaecum sp.	*L3*	*D, M*	*SC, REGR*	2	0.60	(1-1)	1	-
Cucullanus longicollis (Stossich, 1899)	*A*	*D*	*SC, REGR*	13	3.88	(1-6)	1.85 ± 1.41	1.31 - 2.85
Hysterothylacium aduncum (Rudolphi, 1802)	*L3, L4, A*	*D, M*	*REG, DEV, SC, REGR*	29	8.66	(1-4)	1.55 ± 0.78	1.28 - 1.83
Hysterothylacium fabri (Rudolphi, 1819)	*L3, L4*	*D, L, M*	*REG, DEV, SC, REGR*	214	63.88	(1-42)	3.95 ± 5.94	3.28 - 4.81
Cestoda								
Tetraphyllidean plerocercoids	*P*	*D*	*REG, DEV, SC*	6	1.79	(1-3)	1.50 ± 0.84	1.00 - 2.17
Isopoda								
Gnathia sp. (praniza larvae)	*L*	*G*	*SC, REGR*	9	2.69	(1-7)	2.67 ± 1.94	1.67 - 4.11

n, sample size. Stage: *A*, adult; *L*, immature larvae; *L3*, third-stage larvae; *L4*, fourth-stage larvae; *P*, plerocercoid larvae. Site: *D*, digestive tract; *G*, gills; *L*, liver; *M*, mesenteries. Ovarian developmental phase of hosts: *REG*, regenerating; *DEV*, developing; *SC*, spawning capable; *REGR*, regressing.
*Indicates a specialist parasite of mullets (*Mullus* spp.).

The Fisher's exact test showed differences in the prevalence of *O. furcatus* through the different ovarian developmental phases of hosts, being significantly higher ($p = 0.002$) in specimens in spawning capable phase ($P_P = 61.8\%$) than in specimens in regenerating phase ($P_P = 22.2\%$).

No significant differences during different ovarian developmental phases were found in the prevalences of the other parasites. Neither were there any significant differences among the median intensities of any parasite species during the different ovarian developmental phases (Mood's median test, $p > 0.05$).

In relation to condition, the I_{LM} values of the 156 specimens analysed for lipids in the muscle ranged from 0.21 to 3.78 (mean ± $SD = 1.03 \pm 0.63$), whereas the I_{LG} values of the 87 specimens analysed for lipids in the ovaries ranged from 0.25 to 1.40 (mean ± $SD = 0.93 \pm 1.83$).

A Spearman's Rank Correlation test revealed a positive correlation ($r_s = 0.31$, $n = 87$, $p = 0.004$) between these two variables. All the specimens with ovaries large enough to perform the lipid extraction were in spawning capable phase.

The I_{LM} was analysed through the different ovarian developmental phases (Fig. II.2) and a Kruskal-Wallis Test revealed that the distribution of I_{LM} was the same during each ovarian developmental phase.

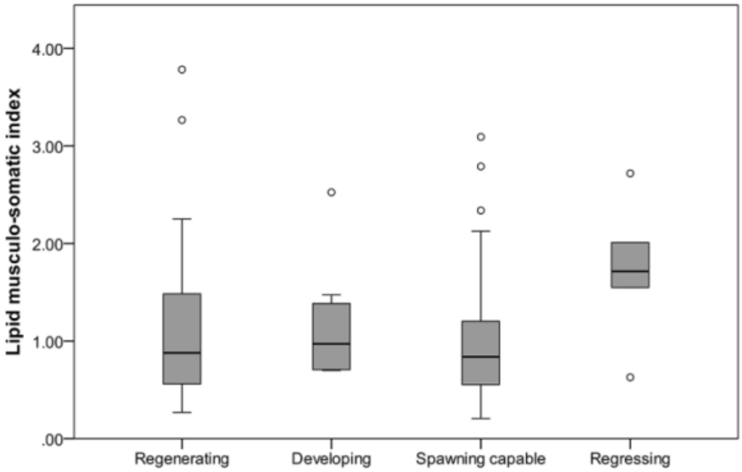


Fig. II.2. Boxplots of lipid musculo-somatic index (I_{LM}) through the different ovarian developmental phases of female *Mullus barbatus*. Regenerating (*n* = 17), developing (*n* = 11), spawning capable (*n* = 123), regressing (*n* = 5).

The histological analyses of gonads showed that *M. barbatus* maintained a reserve of primary growth (PG) oocytes, which is the first stage of oocyte growth and covers the chromatin nucleolar and the perinucleolar stages. The secondary growth of oocytes begins with the cortical alveolar (CA) stage, which was identified by the formation of the chorion and the presence of small oil droplets and cortical alveoli in the periphery of the cytoplasm.

The development of oocytes progressed through several substages of vitellogenesis (VIT1 and VIT2), characterised by the presence of yolk globules and large oil droplets. The oocyte maturation (OM) stage was recognised by the fusion of the yolk material, the formation of a single large oil droplet and the migration of the germinal vesicle towards the plasma membrane. Finally, the last maturing event was the hydration (H) substage which is an uptake of fluid by the oocyte that leads to ovulation.

As oocytes developed, their size increased gradually, with each stage sharing overlapping diameter ranges with the previous and subsequent stages, except in the case of the hydrated oocytes which had much larger diameters (Fig. II.3).

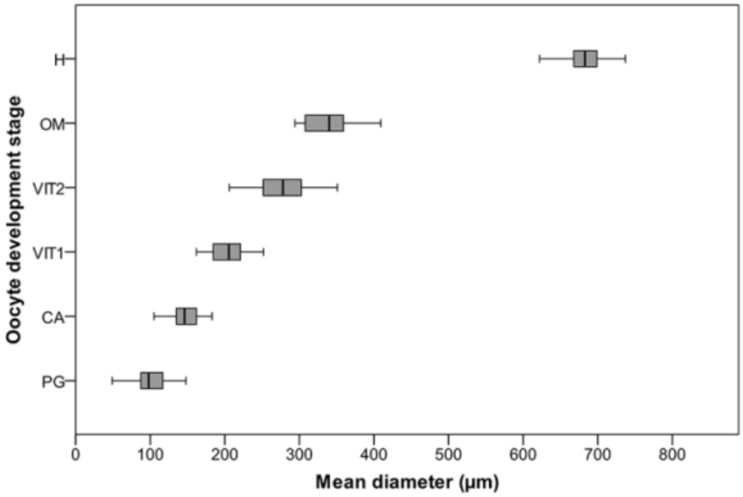


Fig. II.3. Boxplots of the stage-specific oocyte diameter belonging to 10 randomly selected *Mullus barbatus* specimens in spawning capable phase. PG: Primary growth stage (at the perinucleolar stage), mean diameter (μm): 99 ± 24 (*n* = 88); CA: Cortical alveolar stage, 148 ± 18 (*n* = 94); VIT1: Primary stage of vitellogenesis, 204 ± 23 (*n* = 76); VIT2: Secondary stage of vitellogenesis, 279 ± 36 (*n* = 95); OM: Maturation stage, 340 ± 33 (*n* = 26); H: Hydration stage, 684 ± 23 (*n* = 247).

The gonado-somatic index (I_G), presented a clear seasonal pattern. The highest values were observed in the second quarter, with the highest monthly means in May (11.23 ± 3.62) and June (10.97 ± 2.03), while for the rest of the year, the I_G values remained low, with the lowest monthly mean in October (0.88 ± 0.24).

Indeed, the histological examination of the ovaries showed that, overall, females spent the first and fourth quarter in either the regenerating phase (with only PG oocytes present) or the developing phase (with new CA and VIT1 oocytes present) (Fig. II.4). All the females in the spawning capable phase (with PG, CA, VIT1, VIT2, OM and H oocytes present) appeared in the second and third quarter. Females in the regressing phase, which showed massive atresia, were captured in the second quarter, i.e. throughout the spawning season.

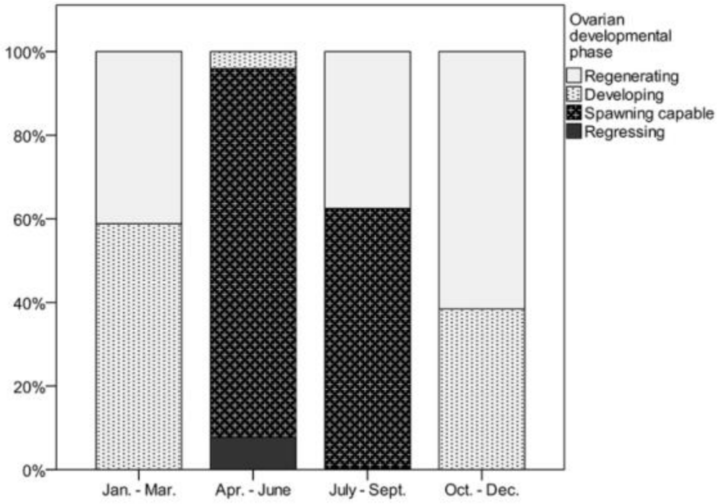


Fig. II.4. Quarterly variations in the relative frequency of the ovarian developmental phases in *Mullus barbatus* (from January 2010 to May 2012).

Ovaries with atretic oocytes were not detected among the individuals in either the regenerating or the developing phases, but females in the spawning capable phase (P_A = 11.61%) presented a moderate relative intensity of atresia (I_A = 12.15 ± 10.09%) and, without exception, all the individuals in the regressing phase (P_A = 100%) showed high levels of atresia ($75\% < I_A \leq 100\%$).

Most females in the spawning capable phase showed a bimodal oocyte diameter-frequency distribution with a first component (smaller diameters) containing oocytes in different stages of development, with a gap at around 550 µm, and a separate second component (larger diameters) containing only oocytes in advanced stage of maturation (Fig. II.5). This type of oocyte size distribution is prevalent in batch spawner species, therefore those oocytes that were above 550 µm in size were considered as belonging to the next batch.

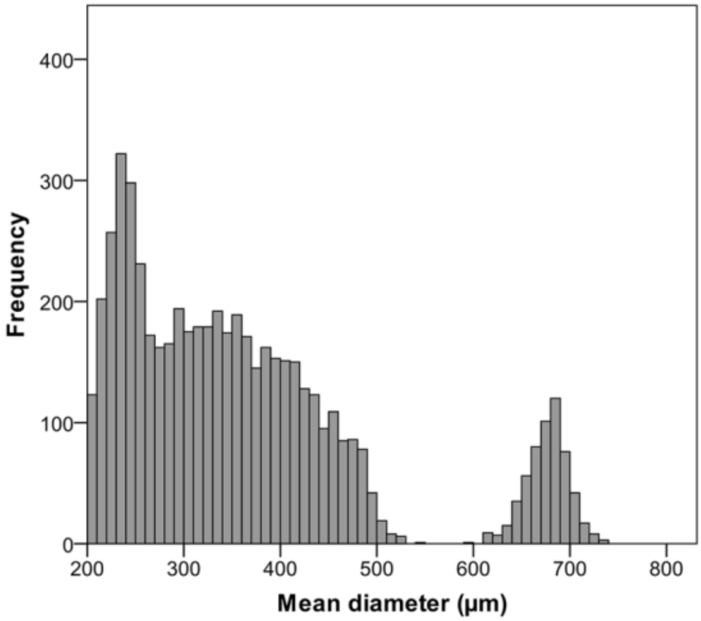
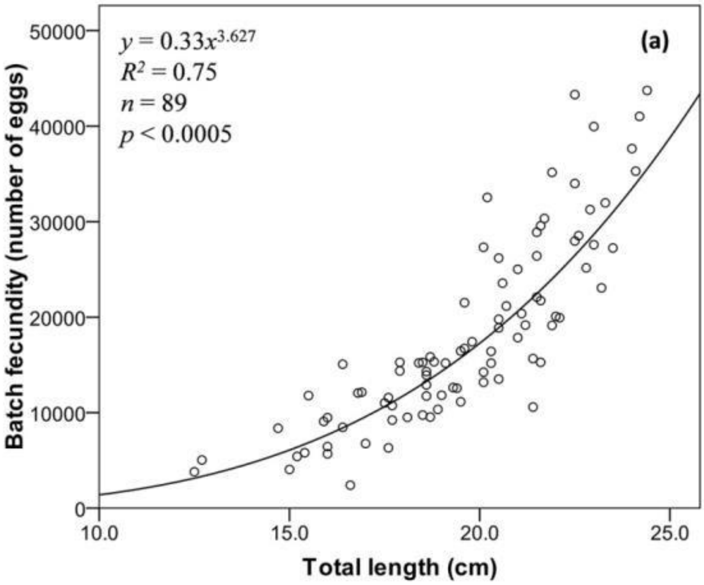


Fig. II.5. Oocyte diameter-frequency distribution (over 200 µm) shown by most of the female *Mullus barbatus* with hydrated oocytes. This example corresponds to one female with $L_T = 21.4$ cm and $F_B = 15664$ eggs.

The batch fecundity (F_B) ranged from 2408 to 43736 eggs (mean ± $SD = 18163 \pm 9778$) and was positively related to the size of *M. barbatus* (Fig. II.6). The total length-batch fecundity points fitted a power function regression with the following equation $F_B = 0.33 \cdot L_T^{3.627}$ ($R^2 = 0.75$, $n = 89$, $p < 0.0005$), while the eviscerated mass-batch fecundity points fitted better to a linear regression, $F_B = 272.3 \cdot M_E - 2554.9$ ($R^2 = 0.76$, $n = 89$, $p < 0.0005$).



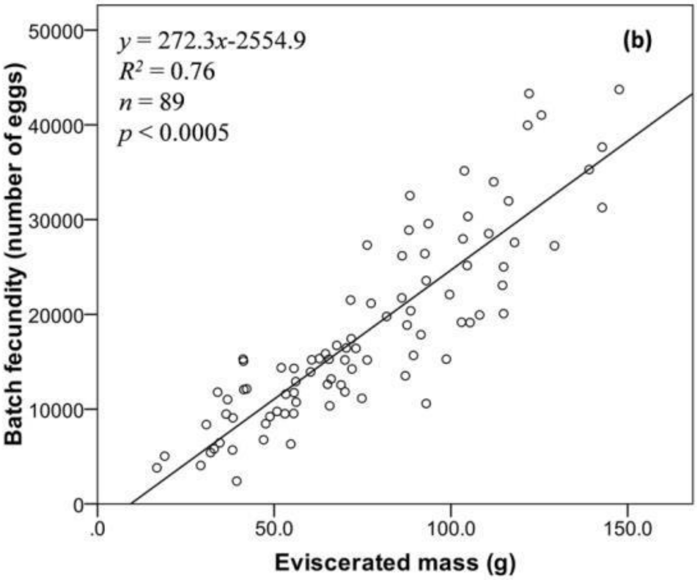


Fig. II.6. Relation between batch fecundity and (a) total length (power function regression), or (b) eviscerated mass (linear regression) of female *Mullus barbatus*.

The F_{Brel} ranged from 61 to 371 eggs·g^{-1} (eviscerated mass) (mean ± SD = 234 ± 63). Finally, the M_{DO} ranged from 0.007 to 0.016 mg (mean ± SD = 0.012 ± 0.002) and did not present any correlation with the analysed variables.

No significant effect of parasitism was found on either the condition or the reproduction of red mullet in regenerating, developing or regressing phases. However, fish in spawning capable phase showed significant differences in their condition and reproduction that were related to parasitism variables (Table II.3).

Individuals that were infected by the digenean *Opecoeloides furcatus* displayed lower median values of I_{LM} compared with uninfected specimens (Mann-Whitney U Test, Table II.3). There was also a negative correlation between the individual intensity of infection by this parasite and the individual value of I_{LM} (Spearman's Rank Correlation, Table II.3).

With regard to the reproduction variables, specimens infected by the nematode *Hysterothylacium aduncum* (Rudolphi, 1802) showed a higher median value of F_{Brel} and a positive correlation between the individual intensity of infection by this nematode and the individual value of F_{Brel}. Similar relationships were found between F_{Brel} and infection by *Hysterothylacium fabri*, i.e. individuals infected by this nematode displayed higher values of F_{Brel}. However, the individuals infected by *H. fabri* showed lower values of M_{DO} than the uninfected specimens.

We found no significant effects caused by the rest of the parasites that were analysed, i.e. *Lasiotocus mulli* (Stossich, 1883), *Proctotrema bacilliovatum* (Odhner, 1911), *Ascarophis mullusi* (Naidenova & Nikolaeva, 1968) and *Cucullanus longicollis* (Stossich, 1899).

Table II.3. Results of Mann-Whitney U test used to verify the existence of differences between infected and uninfected *Mullus barbatus* and Spearman's Rank Correlation coefficient (*rs*) used to evaluate possible relationships among the condition and reproduction variables and the individual intensity of parasitism. Only significant results ($p < 0.05$) are presented.

Ovarian developmental phase (*n*)	Parasite	Variable	Mann-Whitney U Test						Spearman's Rank Correlation		
			n Uninf.	*n* Inf.	*Md* Uninf.	*Md* Inf.	*U*	p^a	*n*	*rs*	*p*
Spawning capable (267)	*Opecoeloides furcatus*	I_{LM}	39	84	-0.116	-0.169	1126	0.005	123	-0.278	0.002
	Hysterothylacium aduncum	B_{Frel}	82	7	232	250	155	0.044	89	0.211	0.047
	Hysterothylacium fabri	B_{Frel}	28	61	228	238	569	0.012	89	0.300	0.004
	Hysterothylacium fabri	M_{DO}	28	61	0.013	0.011	546	0.006	-	-	-

n, subsample size; Uninf., uninfected fish; Inf., infected fish; *Md*, variable median. Variables: I_{LM}, adjusted log-transformed lipid musculo-somatic index; B_{Frel}, adjusted relative batch fecundity; M_{DO}, dry mass of oocytes.

[a] Asymptotic significances (2-tailed) are displayed for Mann-Whitney U tests with sample size above 10 in all groups, otherwise, exact significances [2*(1-tailed Sig.)] are given.

Discussion

This study provides important new data on aspects of health and reproduction of red mullet, an exploited marine species, with particular focus on the links between parasitism, condition and reproductive capacity. We found that red mullet is a batch spawner with an income breeding strategy, an asynchronous development of oocytes and indeterminate fecundity. We also found that the parasites, *Opecoeloides furcatus* and *Hysterothylacium* spp., affect the condition and reproduction of this species.

The results of the histological observation of ovaries, coupled with the gonado-somatic index analysis show that the spawning period of *M. barbatus* collected in the western Mediterranean Sea takes place in spring-summer, with a peak of spawning activity in May and June, which coincides with data provided by Tsikliras *et al.* (2010). The fact that females in the regressing phase appeared throughout the spawning season may indicate that, although the spawning season for the species extends for several months, the individual spawning period might be much shorter.

The oocyte diameter-frequency distributions showed, for the first time, an asynchronous development of oocytes and that *M. barbatus* is a batch spawner. The stage-specific oocyte diameter showed a gradual increase and a slight overlapping between the diameters of cortical alveolar and vitellogenic oocytes. The absence of a hiatus separating the diameter of yolked oocytes from the one of unyolked oocytes indicates an indeterminate fecundity, i.e. the annual fecundity is not fixed before the onset of spawning and unyolked oocytes continue to mature and to be spawned during the spawning season (Murua *et al.* 2003).

Despite the lack of published data on the oocyte recruitment pattern of this species, Anastasopoulou and Saborido-Rey (2011) showed that the recruitment of oocytes from primary growth to secondary growth stages continues throughout an individual's spawning period, indicating an indeterminate strategy which is consistent with our observations. In addition, the high levels of atresia observed in the regressing phase of our specimens also indicated an indeterminate fecundity, since females have to resorb the over production of secondary growth oocytes (Murua & Saborido-Rey 2003; Murua & Motos 2006).

The results of this study indicate that the total length and the eviscerated mass of *M. barbatus* originating from the western Mediterranean Sea are good predictors of its batch fecundity, as also reported in Turkish waters, such as the Aegean and Levantine Sea (Tirasin *et al.* 2007) or the eastern Black Sea (Aydın & Karadurmuş 2013). It was observed that the number of eggs increases as a power of the length of the fish and linearly with its mass. It can therefore be assumed that large specimens have a higher reproductive capacity than smaller ones. This result provides further support for the hypothesis that, for many fishes, the removal of large individuals can adversely affect the reproductive potential of species (Birkeland & Dayton 2005; Lloret *et al.* 2012b). Concerning egg quality, as measured by the hydrated oocyte dry mass, no previous studies were found with which to compare the results, thus we consider that future studies should take this variable into account.

The results of this study concur with the comprehensive study by Carreras-Aubets *et al.* (2012) and indicate that although *M. barbatus* can harbour a wide range of helminths, the most prevalent and abundant parasites are the digenean *Opecoeloides furcatus* and the nematode *Hysterothylacium fabri*.

Our analysis of the parasitism throughout the different ovarian developmental phases of the host showed that there were no changes in the intensities of infection of any of the parasites through the reproductive cycle (although differences were found in the prevalence of *O. furcatus*, which was higher in specimens in the spawning capable phase than those in the regenerating phase).

The fact that there is no reduction in the number of parasites in fish in the spawning capable phase compared to other phases would indicate that red mullet continues to feed during reproduction since, in most cases, fish become infected when actively feeding. If this is so, it would imply an income breeding strategy. In relation to this, while it is true that some authors found that *M. barbatus* curtails feeding during the spawning season in spring-summer (Vassilopoulou & Papaconstantinou 1993; Chérif *et al.* 2011), others have argued that the levels of vacuity (empty stomachs) are very low throughout the year (Esposito *et al.* 2014) or that *M. barbatus* actually feed intensely during the reproductive period (Bizsel 1987).

The results of this study show that there is no decrease in the lipids stored in the muscle as the gonads develop, indicating that reproduction is fuelled by direct food intake. Moreover, the positive correlation found between lipid content in muscle and in the gonads reinforces the hypothesis that *M. barbatus* does not mobilize lipids from the muscle to the gonads. Similarly, Lloret *et al.* (2007) analysed the total lipid content in muscle and gonads of pre-spawning females of *M. barbatus* and also found that, although *M. barbatus* allocates lipid reserves to the gonad during the reproductive period, there was no depletion of the muscle lipids. These results provide further evidence that *M. barbatus* is an income breeder rather than a capital breeder.

The evidence that *M. barbatus* continue feeding throughout the spawning season coupled with their income breeding strategy is consistent with their indeterminate fecundity. That is to say, the number of eggs is not fixed before the onset of the spawning season but, instead, is adjusted to food intake, without reliance on stored energy (Rijnsdorp & Witthames 2005). However, it should be noted that some authors have pointed out that the boundaries between capital/income breeding and determinate/indeterminate fecundity are not as clear as had previously been assumed (Saborido-Rey *et al.* 2010; Ganias 2013).

For example, Domínguez-Petit *et al.* (2010) argued that for the European hake, *Merluccius merluccius*, in the northeastern Atlantic, gonadal development was at the expense of food intake, whereas Ferrer-Maza *et al.* (2014) has shown that, in the northwestern Mediterranean, this species is a capital breeder most of the year except in summer, when the abundance of its prey is at its peak in the Mediterranean. Thus, spawning strategies may be flexible within species and may depend on the availability of food. There is a well-documented distinction between capital breeders and income breeders, but there are also species that use a variety of mixed-breeding strategies (Houston *et al.* 2006; McBride *et al.* 2013). It seems probable that some species of fish may be capable of adopting either a capital or income strategy, or a blend of both in response to internal or external conditions.

The study of the relationships between parasitism, condition and reproduction of fish has highlighted that the specimens of *M. barbatus* in the spawning capable phase infected by the digenean *Opecoeloides furcatus* showed a lower lipid musculo-somatic index than uninfected specimens. *Opecoeloides furcatus*, like most digeneans, is a parasite of the digestive tract where it feeds on mucus, epithelial cells and probably some of the host's gut content. This result might indicate a negative effect of *O. furcatus* on the energy reserves of *M. barbatus*, especially in the spawning season when fish are dividing their energy consumption between maintaining fitness and reproduction.

With regard to the latter, parasitism seems to affect reproduction by causing quantitative and qualitative changes in the spawning. Although specimens infected by the nematodes *Hysterothylacium aduncum* and *H. fabri* showed higher fecundity than uninfected ones, it was found – at least in the case of those infected by *H. fabri* – that there was a lower mass of hydrated oocytes. Accordingly, it can be hypothesized that nematodes belonging to the genus *Hysterothylacium* cause a reduction in the quality of the eggs, but in response, female *M. barbatus* try to compensate for this by producing a higher number of eggs.

As mentioned in the introduction, a limited number of studies have dealt with the effects of parasitism on the energy reserves or the reproduction of marine species. Fogelman *et al.* (2009) found that female five-lined cardinalfish, *Cheilodipterus quinquelineatus* Cuvier 1828, infected by the isopod *Anilocra apogonae* Bruce 1987, had fewer and smaller eggs than uninfected fish. Sasal *et al.* (2001) also found that the females of the gobiid fish *Gobius bucchichii* Steindachner 1870, infected by the acanthocephalan *Acanthocephaloides propinquus* Dujardin 1845, showed a reduction in egg production.

With regard to exploited fish, there are contradictory results about the effects of nematodes on energy reserves. For example, while Shchepkina (1980) found that the anchovy, *Engraulis encrasicolus*, infected by *Contracaecum* sp. showed lower lipid content, Sajiki *et al.* (1992) found that *E. japonicus* infected by *Anisakis* sp. did not experience a depletion in the total lipid content, but there was an increase in the free fatty acids in their viscera. Finally, Ferrer-Maza *et al.* (2014) have recently revealed that the anisakid nematodes, especially *Anisakis pegreffii* Campana-Rouget & Biocca 1955, negatively affect the condition of the European hake, *Merluccius merluccius*, mainly when these fish are allocating their energy reserves to gonadal development, while infections by other parasites do not affect them significantly.

Along with those of previous studies, our results have demonstrated that parasitism, condition and reproduction are closely linked. The physical health of exploited stocks, which depends on the health and reproductive capacity of the individuals, is an essential element of sustainable and profitable fisheries (Lloret *et al.* 2012a). Therefore, the results of this study, especially those concerning the effects of parasites on condition and reproduction, may have an important impact on the development of management strategies and research involving other species, whether wild or captive-bred.

In summary, the present study not only provides basic knowledge on the reproduction, condition and parasitism of *Mullus barbatus* in the western Mediterranean Sea, but also some important implications of their relationships. The results lead us to suggest that *M. barbatus* should be classified as an iteroparous batch spawner species with an income breeding strategy, an asynchronous development of oocytes and indeterminate fecundity. This study also showed that larger females have a higher fecundity, confirming that older spawners contribute heavily to reproduction. We have also concluded that although *M. barbatus* can harbour a wide range of helminths, only the most abundant and prevalent ones (i.e. the digenean *Opecoeloides furcatus* and the nematodes *Hysterothylacium* spp.) significantly affect the condition and reproduction of *M. barbatus* during the spawning season. Furthermore, the effects are complex: the digenean *O. furcatus* causes a reduction in the energy reserves of fish, whereas infection by the nematodes *Hysterothylacium* spp. results in a rise in egg production but impaired egg quality. This shows that the relationships between parasitism, condition and reproduction are not always evident in income breeder fish. We consider, therefore, that further research regarding the role of the energy reserves and the nutrient acquisition would be of great help in understanding the energy cost of reproduction and parasitism.

Chapter III:

European anchovy (*Engraulis encrasicolus*)

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Links between parasitism, energy reserves and fecundity of European anchovy, *Engraulis encrasicolus*, in the northwestern Mediterranean Sea

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Abstract

The European anchovy, *Engraulis encrasicolus* L. 1758, is one of the most sought-after target species in the NW Mediterranean Sea. However, this stock currently consists of small individuals and landings are reported to have decreased considerably. The main purpose of this study is to assess, for the first time, the interrelationships between size, fecundity, energy reserves and parasitism in female anchovy, in order to analyse the implications for the health of NW Mediterranean anchovy stocks arising from the current shortage of large individuals. Our results have revealed that smaller individuals show lower fecundity, lower lipid content and a higher intensity of certain parasites. Since it is known that smaller individuals now predominate in the population, our findings indicate that the health of the northwestern Mediterranean anchovy stock is currently impaired. Consequently, as a precautionary measure, we consider that the minimum landing size (MLS) for anchovy in the Mediterranean Sea should be increased in order to facilitate the recovery of the stock.

Introduction

The European anchovy, *Engraulis encrasicolus*, is a small planktivorous pelagic fish with a wide distribution range comprising the Atlantic coast of Europe and western Africa, the Mediterranean Sea and the Black Sea (Fischer *et al.* 1987). The global catch of European anchovy in 2012 was 489297 tonnes, which is 20% less than in 2003 (FAO 2014). In the western Mediterranean Sea, anchovy remains one of the most sought-after target species by commercial trawlers or purse seiners, although landings of this species have been steadily declining since 2000 (Palomera *et al.* 2007). Indeed, although the abundance of anchovy in the NW Mediterranean Sea remains relatively high, its biomass and the mean size of individuals have diminished dramatically (Van Beveren *et al.* 2014).

Different hypotheses have been proposed to explain the decrease in anchovy landings. It has been pointed out that the extension of the fishing season has led to recruitment overfishing which may be affecting the NW Mediterranean anchovy stock (Lleonart & Maynou 2003). Another factor is that the size-at-first-maturity for anchovy is greater than the minimum legal landing size established for this species in the Mediterranean Sea (Palomera *et al.* 2003; Tsikliras & Stergiou 2014) and this may be affecting its reproductive potential. In addition, changes in environmental conditions, such as decreasing river flows and increasing water temperatures, have also been identified as possible factors in the decline in anchovy landings (Lloret *et al.* 2004; Martín *et al.* 2012). Recently, special attention has been paid to the changes in the size distribution and condition of anchovy and their potential links with its presently low biomass in the NW Mediterranean Sea (as recorded by Van Beveren *et al.* 2014).

Certainly, fish condition parameters are essential for estimating the health and productivity of exploited populations and energy reserves, in particular, are a highly significant indicator of their condition (reviewed by Lloret *et al.*

2012, 2014). Therefore, studying the energy reserves of anchovy and the lipid storage dynamics throughout its reproductive cycle could shed some light on the status of anchovy stocks in the NW Mediterranean Sea.

On the other hand, parasitism has also been identified as a factor affecting the condition and reproduction of several fish species (e.g. Barber & Svensson 2003; Bagamian *et al.* 2004; Bean & Bonner 2009). However, only a few studies have analysed the relationships between parasitism, energy reserves and reproduction in commercially exploited fish species in the Mediterranean (Ferrer-Maza *et al.* 2014, 2015). There is, to our knowledge, only one other study concerning the European anchovy, carried out in the Black Sea, which has looked into the effects of parasites (in this case, nematodes) on the lipid composition of the European anchovy (Shchepkina 1985).

Nevertheless, given that there are a number of well-known cases of collapse in anchovy fisheries and considering the social and economic upheaval such collapses can cause (Pita *et al.* 2014), the health of anchovy stocks is well worth studying.

Consequently, the aim of this study was to evaluate the health of the NW Mediterranean anchovy stock based on three indicators (energy reserves, fecundity and parasitism), and to discuss the possible implications of the shortage of large individuals in this stock. We analysed the interrelationships among size, fecundity and two important health indicators such as lipid content and metazoan parasitism, in female anchovy throughout their reproductive cycle. These interrelationships are discussed from biological and ecological perspectives in order to provide information that can be used to improve the management of this stock, which is highly valued but in poor condition.

Materials and methods

Fish sampling

A total of 271 female specimens of European anchovy, *Engraulis encrasicolus* L. 1758, were collected on a monthly basis from January 2011 to November 2012. The samples were obtained from commercial trawlers and purse seiners at the port of Roses, which is one of the most important commercial fishing harbours in the region (Fig. III.1). The specimens were caught near Cap de Creus (NW Mediterranean Sea) and its adjacent waters (no further than 25 miles from the shoreline between 42°33'-41°40'N and 3°03'-2°48'E) (Fig. III.1).

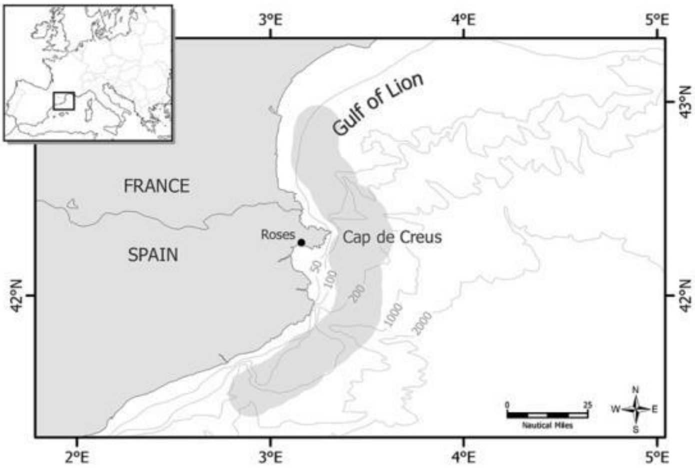

Fig. III.1. Map of the Cap de Creus (northwestern Mediterranean Sea), showing the port of Roses, where anchovy were sampled. The shadowed area represents approximately the zone of capture.

The specimens were transported on ice to the laboratory where they were immediately dissected. For each individual, the total body length (L_T) (± 0.1 cm), total body mass (M_T) (± 0.01 g), eviscerated body mass (M_E) (± 0.01 g), muscle mass (M_M) (± 0.01 g) and gonad mass (M_G) (± 0.1 mg) were recorded.

All the muscle tissue of each specimen was removed and frozen at −20°C for a subsequent determination of lipid content, while the ovaries were fixed in 4% buffered formaldehyde for histological processing and fecundity estimation. The total lengths (L_T) of all specimens ranged from 10.4 to 16.7 cm (mean ± $SD = 13.8 \pm 1.2$, $n = 271$); among these were nine immature specimens ranging from 10.4 to 12.7 cm (11.43 ± 0.63, $n = 9$).

Reproductive phase classification

The gonado-somatic index (I_G), which is the relationship between gonad mass (M_G) and eviscerated body mass (M_E) of the females, was calculated as $I_G = 100\ M_G\ M_E^{-1}$.

Subsequently, one ovary lobe from each specimen was fixed and sliced transversely in its midsection. The resulting slices were embedded in paraffin, cut into 8–10 µm sections, and stained with both hematoxylin-eosin and Mallory's trichrome stains. The latter staining method highlights the zona radiata and its continuity and facilitates the detection of atretic oocytes (Muñoz *et al.* 2010), i.e. degenerating oocytes which will not be spawned.

The prevalence of atresia (P_A) was calculated as the proportion of females with observed α-atretic oocytes; the relative intensity of atresia (I_A) was calculated for each specimen as the number of α-atretic oocytes divided by the total number of vitellogenic oocytes (α-atretic and normal).

Following Brown-Peterson *et al.* (2011), all the specimens were classified into six ovarian developmental phases depending upon the presence of specific histological markers. These phases (and the percentage of specimens in each phase) are as follows: *immature* (3.3%) – fish have not reached the sexual maturity (never spawned); *regenerating* (19.9%) – sexually mature but reproductively inactive; *developing* (3.3%) – fish with gametes that are beginning to develop; *spawning capable* (25.5%) – advanced, developed gametes ready for the spawning season; *actively* *spawning* (30.6%) – oocytes in migratory nucleus stage, hydration or ovulation; and *regressing* (17.4%) – massive atresia which indicates the end of the reproductive cycle.

Fecundity estimation

The fecundity estimation was performed on 31 specimens following the oocyte size-frequency method described by Hunter *et al.* (1985). The females selected were from the actively spawning group and had oocytes in migratory nucleus stage, i.e. the most advanced stage before hydration and release. To ensure that these specimens had not recently released part of their mature oocytes, we used the postovulatory follicle degeneration key (VII stages) proposed by Alday *et al.* (2010), and any females with recently spawned follicles (stages I to IV) were not selected.

Subsequently, slices from the central area of the 31 selected ovaries were weighed (± 0.1 mg) and the oocytes were separated using a washing process, as described by Lowerre-Barbieri & Barbieri (1993), and sorted by size through several sieves (from 200 to 600 µm). The oocytes were counted and their diameters were measured using a computer-aided image analysis system (Image-Pro® Plus 5.1, Media Cybernetics, Inc., Bethesda, MD, USA).

Since the oocyte size distribution followed a two-component mixture model, we applied an algorithm of the *mixtools* package (Benaglia *et al.* 2009) for *R* software (www.r-project.org). This statistical procedure, which had been successfully employed in a previous study (Ferrer-Maza *et al.* 2014), was used to describe quantitatively the properties of the overlapping mixtures and to calculate the number of oocytes belonging to the next batch.

Since *E. encrasicolus* is a multiple spawner with indeterminate fecundity (Ganias *et al.* 2014), the reproductive capacity of the individuals was estimated according to the batch fecundity (F_B), defined as the number of eggs spawned per batch, and the relative batch fecundity (F_{Brel}), defined as the value of batch fecundity per gram of eviscerated female body mass.

Energy reserves determination

To assess the energy reserves of anchovy, first, a visual assessment of their mesenteric fat was carried out following Lingen & Hutchings (2005). All the specimens were allocated to one of the five mesenteric fat stages defined according to the amount of fat associated with their intestine (ranging from *no visible fat* to *intestine completely covered by fat*).

Then, a subsample of muscles ($n = 210$) was selected to perform the lipid extraction and evaluation. The total lipid content (% wet mass) in muscle was determined following the Soxhlet method described by Shahidi (2001). A lipid musculosomatic index (I_{LM}) was calculated as $I_{\mathrm{LM}} = 100\,ABS_{\mathrm{M}}\,M_{\mathrm{E}}^{-1}$, where ABS_{M} is the absolute lipid content in muscle, which was obtained by multiplying the lipid content (% wet mass) by the wet mass of the muscle.

The I_{LM} and the mesenteric fat stage were considered as indicators of anchovy condition because the muscle tissue and the mesenterial fat constitute the primary and secondary lipid repositories respectively in anchovy (Melo 1992).

Parasitism evaluation

All the specimens were examined for metazoan parasites prior to the removal of muscle and gonads for lipid content determination and histology. The entire viscera were removed from the body cavity, and the internal organs were examined using a stereomicroscope. A large subsample of individuals (the first 75 samples) was also examined for metazoan parasites in the musculature. The samples of muscle were examined after filleting and flattening the tissue onto a trans-illumination platform. As no metazoan parasites were found in these examinations, it was decided to regard the number of parasites in the musculature as negligible.

When found, the parasites were collected and washed with a saline solution (0.8% NaCl). They were first observed alive and then fixed in permanent preparations. Nematodes were preserved in 70% ethanol and cleared in Amann's lactophenol, whereas digeneans were fixed in Bouin's solution under slight coverslip pressure and then stained with Grenacher's alcoholic borax carmine solution and mounted in Canada balsam.

Parasites were morphologically identified to the lowest possible taxonomic level following the available keys and descriptions, such as Petter & Maillard (1988) and Moravec (2007) for nematodes or Gibson *et al.* (2002), Jones *et al.* (2005) and Bray *et al.* (2008) for digeneans.

Since the identification of the parasite species is normally based on adult features, some of the parasite larvae found in this study could not be identified to the species level. Therefore, the real number of different species might be higher than reported since there may be several different species within the groups classified as Hemiuridae metacercariae, Didymozoidae metacercariae, *Anisakis* sp. (*Type I* larvae), *Spinitectus* sp., unidentified nematode larvae and Tetraphyllidean plerocercoids. However, while this may be important in certain types of biodiversity studies, in our case, we have assumed that each group we have classified consists of parasites with very similar life cycles and morphology and that the members of each group probably have the same consequences on the condition of their hosts.

Following Bush *et al.* (1997), the prevalence of parasites (P_P) was calculated as the proportion of fish infected with a given parasite species and the individual intensity of the infection was calculated as the number of individuals of a particular species in a single infected host. The mean intensity was calculated as the average number of parasites of a given species found in the infected hosts. The median intensity and its 95% confidence interval (*CI*) were also calculated.

Data analysis

The aggregated distribution of parasites leads to the concentration of a high proportion of individuals of a particular parasite in a few specimens of the host. As argued by Rózsa *et al.* (2000), it is useful to report the confidence interval (*CI*) for the median intensity of infection. For this reason, the median 95% *CI* was calculated using the free *Quantitative Parasitology 3.0* software (Reiczigel and Rózsa, 2005).

This software, which was developed to manage the particularly left-biased frequency distribution of parasites, was also used to compare the prevalences (Fisher's exact test) and the median intensities (Mood's median test) for each parasite species throughout the different ovarian developmental phases of the hosts.

As normality could not be achieved by any method, several non-parametric tests (Mann-Whitney U test [U] and Spearman's Rank Correlation coefficient [r_S]) were performed to assess the possible relationships between parasitism and energy reserves (I_{LM}) and/or fecundity (F_{Brel}). The level of statistical significance adopted was $p < 0.05$ and a false discovery rate (FDR) approach was used to counteract the problem of multiple comparisons (Benjamini & Hochberg 1995; Verhoeven *et al.* 2005).

Results

Reproductive cycle

Higher values of I_{G} were observed in specimens captured from April until August, with the highest monthly mean in June (mean ± SD = 5.72 ± 2.59, n = 23). Indeed, as shown in Figure III.2, the histological examination of the ovaries showed that females spent the winter in the regenerating phase until early spring when specimens in the developing phase began to appear. Then, in late spring and summer, the spawning period took place as females in spawning capable or actively spawning phases were observed. Finally, individuals in the regressing phase appeared mainly in summer and autumn.

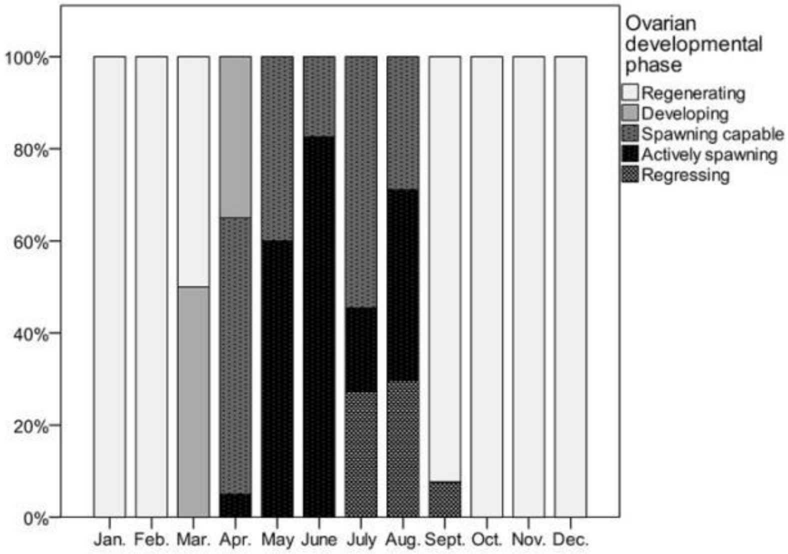

Fig. III.2. Monthly variations in the relative frequency of ovarian developmental phases in anchovy (from January 2011 to November 2012).

Ovaries with atretic oocytes were not detected among individuals in regenerating phase, but different intensities of atresia (I_A) were found in specimens during other phases: (i) developing ($P_A = 22.2\%$, $I_A = 18.5\%$), (ii) spawning capable ($P_A = 10.1\%$, $I_A = 13.7\%$) and (iii) actively spawning ($P_A = 8.4\%$, $I_A = 28.2\%$). Finally, all the individuals in the regressing phase ($P_A = 100\%$) showed massive atresia ($75\% < I_A \leq 100\%$).

Fecundity

The batch fecundity (F_B) and the relative batch fecundity (F_{Brel}) were calculated for 31 females that were in the actively spawning phase and had oocytes in the migratory nucleus stage. The oocyte-diameter frequency of these females showed a bimodal distribution (Fig. III.3), with a first component (smaller diameters) containing oocytes in different stages of development (mainly in cortical alveolar and vitellogenic stages) and a second component (larger diameters) containing only oocytes in advanced stage of maturation, which were considered as being the batch that was about to be released.

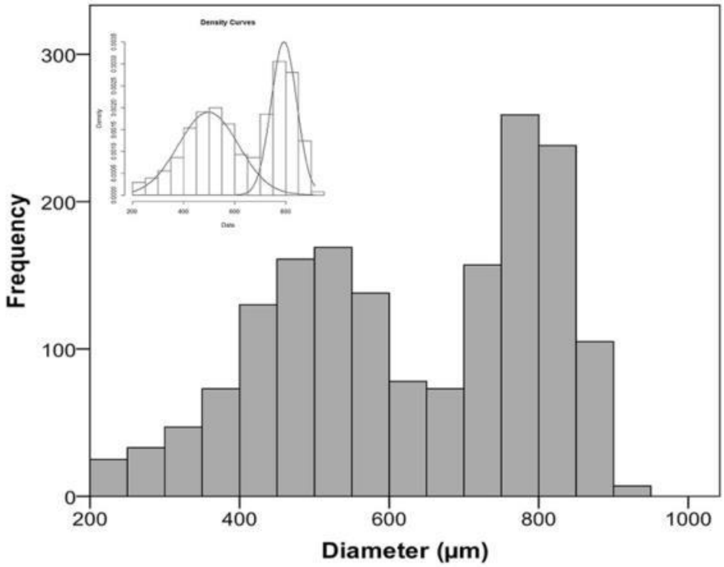

Fig. III.3. Oocyte diameter frequency distribution (for oocytes over 200 µm) representing most of the female anchovy specimens with oocytes in migratory nucleus stage. This example corresponds to one female with $L_T = 14.9$ cm and $F_B = 10672$ eggs. The small chart shows the two different statistical components of the overlapping mixture distribution. Oocytes with 95% probabilities of belonging to the second component (larger diameter group) were considered as being part of the next batch.

The F_B ranged from 981 to 21750 eggs (11998 ± 5397, $n = 31$) and was positively related to the size of the specimen. The total length-batch fecundity points fitted a power function regression with the following equation: $F_B = 5.708 \cdot 10^{-8} \cdot L_T^{9.640}$ ($r^2 = 0.66$, $n = 31$, $p < 0.001$). The F_{Brel} ranged from 96 to 983 eggs (587 ± 227, $n = 31$) and was also positively related to total length: $F_{Brel} = 1.400 \cdot 10^{-5} \cdot L_T^{6.490}$ ($r^2 = 0.49$, $n = 31$, $p < 0.001$).

Energy reserves

In relation to condition, the lipid musculosomatic index (I_{LM}) values ranged from 0.31 to 7.58 (1.97 ± 1.65, $n = 210$). The relationship between I_{LM} and the total length showed a significant positive correlation ($r_s = 0.38$, $n = 210$, $p < 0.001$). Despite the moderate regression coefficient, it can be observed in Figure III.4 that the larger specimens (over 12.5 cm approx.) had a high variability in their I_{LM}, but all the smaller specimens (up to 12.5 cm.) had low values of I_{LM}.

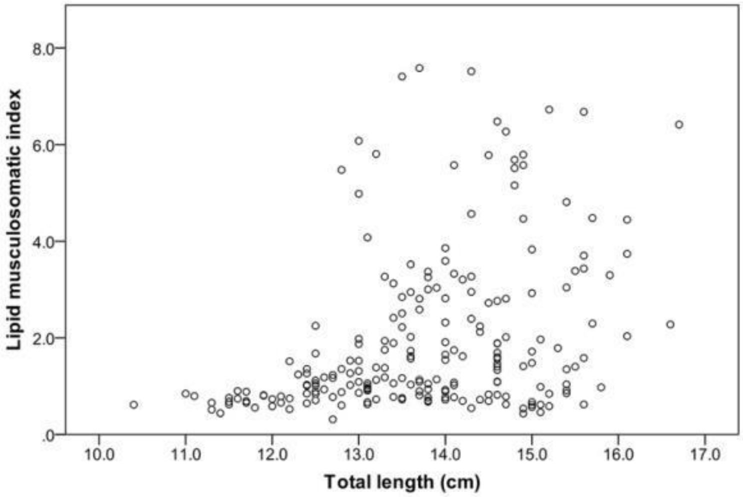

Fig. III.4. Scatterplot of lipid musculosomatic index (I_{LM}) of anchovy in relation to total body length ($n = 210$).

There was also a positive correlation ($r_s = 0.56$, $n = 210$, $p < 0.001$) between I_{LM} and the mesenteric fat stages. Indeed, as shown in Figure III.5, the mean I_{LM} increased as mesenteric fat increased through the five fat stages described previously.

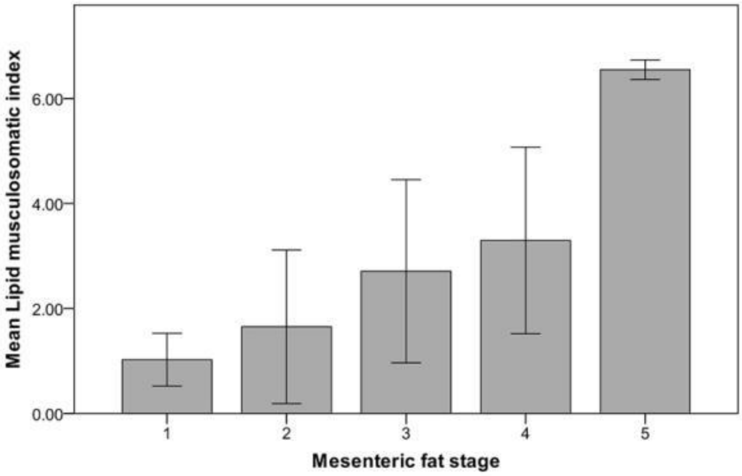

Fig. III.5. Mean lipid musculosomatic index (I_{LM}) of anchovy in relation to mesenteric fat stages. The bars represent ± standard deviations.

On the other hand, a Kruskal-Wallis test found significant differences ($H = 31.25$, $n = 210$, $p < 0.001$) in the I_{LM} during the different ovarian developmental phases of specimens (Fig. III.6).

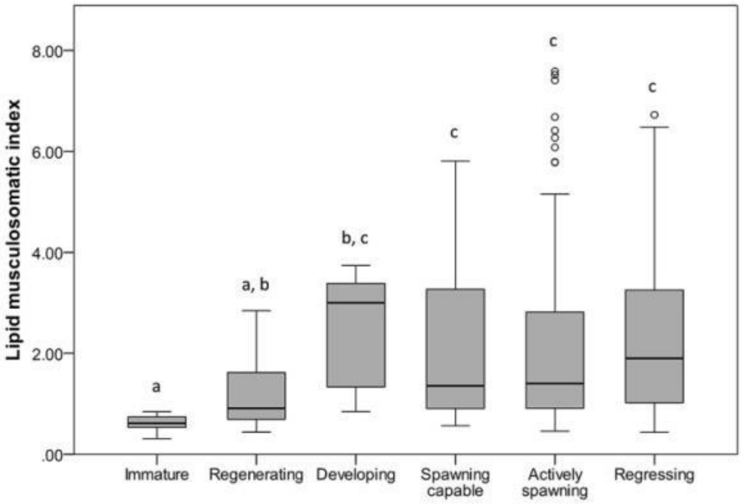

Fig. III.6. Box-and-whisker plots of lipid musculosomatic index (I_{LM}) in relation to the different ovarian developmental phases of anchovy. Significant differences among groups (Kruskal-Wallis test, $p < 0.05$) are indicated with different letters.

Immature and regenerating specimens showed lower I_{LM} than specimens in the other four phases, among which there were no significant differences in their I_{LM} values.

Finally, there was no relationship between I_{LM} and fecundity since no correlation was found between I_{LM} and F_{B} ($r_s = 0.13$, $n = 26$, $p = 0.518$) or between I_{LM} and F_{Brel} ($r_s = 0.11$, $n = 26$, $p = 0.591$).

Parasitism

Of all the dissected anchovy specimens, 62.7% were infected with at least one metazoan parasite taxa, with a median intensity that ranged from four to five parasites (95% *CI*). Over 5000 parasites were classified into eight helminth taxa: three digeneans, four nematodes and one cestode (Table III.1).

The hemiurid metacercariae (Digenea) were the most prevalent parasites, with a prevalence of 36.16%. However, the parasites with the highest intensity of infection were the tetraphyllidean plerocercoids (Cestoda), with a mean intensity of 197.52 ± 378.57. It should be noted that the only parasite species found at the adult stage, the digenean *Aphanurus stossichi*, was also relatively prevalent ($P_{\mathrm{P}} = 23.62\%$), as well as the *L3* larvae of the nematode *Hysterothylacium aduncum* ($P_{\mathrm{P}} = 18.08\%$).

Table III.1. Taxonomic composition, number of infected hosts, prevalence (P_P) and intensities of metazoan parasites found in the European anchovy, *Engraulis encrasicolus*, from Cap de Creus (NW Mediterranean Sea). The parasite developmental stage, the site of infection and the ovarian developmental phase of hosts are also given.

Parasite species	Stage	Site	Ovarian developmental phase	Infected hosts	P_P (n = 271)	Intensity		
						Min-Max	Mean ± SD	Median 95% CI
Digenea								
Aphanurus stossichi (Monticelli, 1891)	*A*	*S*	*IM, REG, SC, AS, REGR*	64	23.62	(1-25)	3.31 ± 3.50	(2-3)
Hemiuridae	*M*	*P*	*IM, REG, DEV, SC, AS, REGR*	98	36.16	(1-100)	9.79 ± 15.04	(3-4)
Didymozoidae	*M*	*P*	*DEV, SC, AS*	10	3.69	(1-7)	2.60 ± 2.17	(1-6)
Nematoda								
Anisakis sp. (*Type I* larvae)	*L3*	*I*	*REG, SC, AS, REGR*	11	4.06	(1-2)	1.09 ± 0.30	(1-1)
Hysterothylacium aduncum (Rudolphi, 1802)	*L3*	*I*	*IM, REG, DEV, SC, AS, REGR*	49	18.08	(1-4)	1.45 ± 0.74	(1-1)
Spinitectus sp.	*L*	*I*	*REG, AS*	3	1.11	(1-1)	1	1
Unidentified larvae	*L*	*I*	*REG, SC, AS*	21	7.75	(1-3)	1.29 ± 0.64	(1-1)
Cestoda								
Tetraphyllidean	*P*	*I*	*REG, SC, AS, REGR*	23	8.49	(1-1000[a])	197.52 ± 378.57	(2-100)

n, sample size. Stage: *A*, adult; *M*, metacercariae; *L*, immature larvae; *L3*, third-stage larvae; *P*, plerocercoid larvae. Preferred site: *S*, stomach; *P*, pyloric caeca; *I*, intestines. Ovarian developmental phase of hosts: *IM*, immature; *REG*, regenerating; *DEV*, developing; *SC*, spawning capable; *AS*, actively spawning; *REGR*, regressing.

[a] Up to 1000 plerocercoids were counted for four individual hosts, but the real intensities might be higher.

In three cases, the digenean *A. stossichi*, the hemiurid metacercariae and the nematode *H. aduncum*, the Fisher's exact test showed significant differences ($p < 0.001$) between prevalences during the different ovarian developmental phases of their hosts (Fig. III.7).

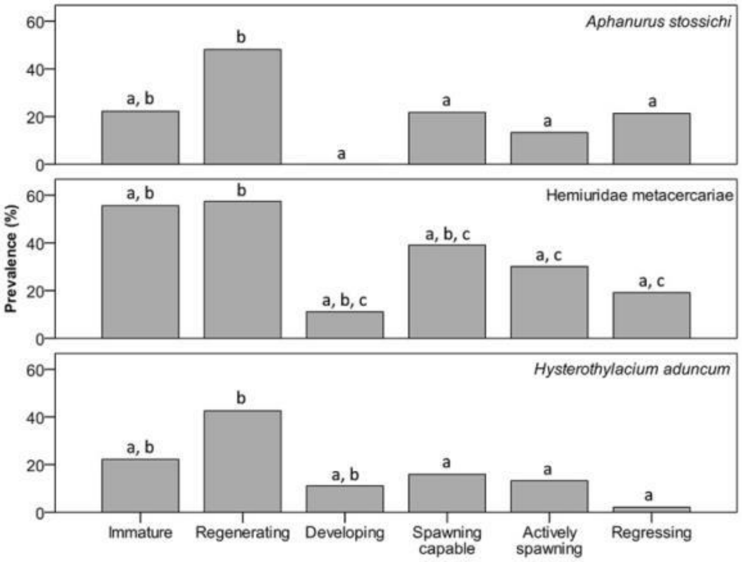

Fig. III.7. Prevalences (P_P) of parasites in relation to the different ovarian developmental phases of anchovy. Significant differences among groups (Fisher's exact test, $p < 0.05$) are indicated with different letters.

Overall, immature anchovy specimens and those in regenerating phase showed higher parasite prevalences than specimens in the other developmental phases. Despite the significant differences in parasite prevalences, the Mood's median test showed no difference among the median intensities of any parasite species during the different ovarian developmental phases of their hosts.

There was, however, a negative relationship between anchovy length and individual intensity of infection for the aforementioned three parasites (i) *A. stossichi* ($r_s = -0.40$, $n = 271$, $p < 0.001$), (ii) the hemiurid metacercariae ($r_s = -0.18$, $n = 271$, $p = 0.004$), and (iii) *H. aduncum* ($r_s = -0.21$, $n = 271$,

$p = 0.002$). In contrast, this relationship was positive in the case of the didymozoid metacercariae ($r_s = 0.20$, $n = 271$, $p = 0.002$).

Parasitism effects on energy reserves and fecundity

Anchovy in regenerating, spawning capable, actively spawning and regressing phases showed significant differences in their energy reserves (I_{LM}) and fecundity (B_{Frel}) related to parasitism (Table III.2).

Specimens in regenerating phase and infected by the digenean *A. stossichi* displayed lower median values of I_{LM} compared with uninfected specimens (Mann-Whitney U Test, Table III.2). There was also a negative correlation between individual intensities of infection by this digenean and individual values of I_{LM} (Spearman's Rank Correlation, Table III.2). Similar results were found for the anchovy in spawning capable, actively spawning and regressing phases infected by the hemiurid metacercariae. Similar negative correlations were found for individuals in the actively spawning phase infected by the unidentified nematode larvae as well as for the specimens in regressing phase infected by *A. stossichi*. Negative relationships were also found between fecundity and infection by *A. stossichi* or *H. aduncum*.

Conversely, anchovy infected by the didymozoid metacercariae showed higher median values of B_{Frel} than uninfected specimens and a positive correlation between individual intensities of infection and individual values of B_{Frel} was found. Finally, the specimens in regressing phase infected by *Anisakis* sp. also showed a positive correlation between individual intensities of infection and individual values of I_{LM}, although the Mann-Whitney U Test did not find significant differences between specimens infected and uninfected by this nematode.

Table III.2. Results of Mann-Whitney U test used to verify the existence of differences between infected and uninfected *Engraulis encrasicolus* and Spearman's Rank Correlation coefficient (r_s) used to evaluate possible relationships between the individual intensities of parasitism and the energy reserves and fecundity of fish. Only significant results ($p < 0.05$) are presented.

Ovarian developmental phase (*n*)	Parasite	Variable	Mann-Whitney U Test						Spearman's Rank Correlation		
			n Uninf.	*n* Inf.	*Md* Uninf.	*Md* Inf.	*U*	P^a	*n*	r_s	*P*
Regenerating (54)	*Aphanurus stossichi*	I_{LM}	23	23	1.090	0.758	175	0.049	46	-0.318	0.031
Spawning capable (69)	Hemiuridae (metacercariae)	I_{LM}	33	24	2.276	1.044	267	0.037	57	-0.271	0.041
Actively spawning (83)	Hemiuridae (metacercariae)	I_{LM}	43	22	1.524	1.168	326	0.042	65	-0.262	0.035
	Unidentified nematode larvae	I_{LM}	54	11	1.547	1.057	177	0.036	65	-0.268	0.031
	Aphanurus stossichi	B_{Frel}	28	3	594.0	416.0	8	0.023	31	-0.419	0.019
	Didymozoidae (metacercariae)	B_{Frel}	24	7	543.5	701.0	129	0.033	31	0.384	0.033
	Hysterothylacium aduncum	B_{Frel}	28	3	594.0	244.0	10	0.032	31	-0.390	0.030
Regressing (47)	*Aphanurus stossichi*	I_{LM}	20	10	2.355	1.017	30	0.002	30	-0.585	0.001
	Hemiuridae (metacercariae)	I_{LM}	23	7	2.035	1.353	40	0.047	30	-0.397	0.030
	Anisakis sp.	I_{LM}	-	-	-	-	-	-	30	0.366	0.047

n, subsample size; Uninf., uninfected fish; Inf., infected fish; *Md*, variable median. Variables: I_{LM}, lipid musculosomatic index; B_{Frel}, relative batch fecundity.

[a] Asymptotic significances (2-tailed) are displayed for Mann-Whitney U tests with sample size above 10 in both groups, otherwise, exact significances [2*(1-tailed Sig.)] are given.

As for the remaining parasites (*Spinitectus* sp. and the tetraphyllidean plerocercoids), no significant effect was found on either the energy reserves or the fecundity of the host. Nor was any significant effect of parasitism found on the energy reserves of immature or developing anchovy (whose fecundity cannot be evaluated).

Discussion

Overall, our results revealed that smaller individuals show lower fecundity, lower lipid content and a higher intensity of certain parasites. Since it has been shown that smaller individuals now predominate in the population (Van Beveren *et al.* 2014), our findings indicate that the health of the northwestern Mediterranean anchovy stock is currently impaired. Indeed, we detected two important problems. First, there has been a reduction in the reproductive capacity and the energy reserves of this stock and second, there is an increase in the intensity of some parasites which, in some cases (e.g. certain digeneans and nematodes), were shown to adversely affect the energy reserves of the host.

With regard to reproduction, the results of this study show that the spawning season of the European anchovy in the NW Mediterranean Sea takes place between April and August, which is consistent with what has been reported by other authors for other regions of the Mediterranean Sea (Tsikliras *et al.* 2010).

The values of fecundity reported in this study are also coherent with other studies carried out with the European anchovy in the Adriatic Sea (Casavola *et al.* 1996) or in the northeast Atlantic Ocean (Sanz & Uriarte 1989; Motos 1996). The batch fecundity was positively related with total length, which means that larger individuals have a higher reproductive capacity than smaller ones, as is commonly the case with several NW Mediterranean commercial fish species (Muñoz *et al.* 2005; Ferrer-Maza *et al.* 2014, 2015; Villegas-Hernández *et al.* 2014, 2015a; b).

In the present study, the size of all the immature anchovy specimens we examined exceeded 9 cm, which is currently the minimum landing size (MLS) for anchovy in the Mediterranean Sea. As suggested by Tsikliras & Stergiou (2014) an increase in the MLS would be more appropriate in order to ensure the recruitment of this species. Indeed, a hypothetical increase in the MLS would not only allow juveniles to reach sexual maturity but would also increase the fecundity of females and, therefore, the stock's productivity. Furthermore, the absence of a relationship between batch fecundity and energy reserves seems to indicate that the size of anchovy has a greater influence than the condition of the fish on the reproductive potential of this species.

In relation to energy reserves, the results showed that because the stock currently consists of mostly small individuals (Van Beveren *et al.* 2014), the energy reserves of individual anchovy and of the whole population are low. We identified two different groups according to the size of the individuals: those larger than 12.5 cm showed strong variations in lipid content in the muscle, while all individuals below this size had low energy reserves. These findings are in accordance with the aforementioned work by Van Beveren *et al.* (2014) who produced a comprehensive study using an extensive and reliable data time series (1992-2012) that was compiled from scientific surveys. They found that although the abundance of anchovy had remained relatively high, its biomass and mean size had declined dramatically.

According to the authors, the median length of the whole data set remained fairly constant at around 12.5 cm, with a peak in 2005 and a subsequent decline with the lowest sizes being recorded during the period 2009-2012. Furthermore, during this later period, 2009-2012, the analysis of the relative condition factor also indicated that the condition of the anchovy was poor. It seems, therefore, that size and condition are linked and that there is a shift in the energy reserves of anchovy at median lengths of around 12.5 cm.

It should also be noted that these changes in energy reserves are not attributable to the reproductive status of the specimens since our results showed that there was no depletion of lipids that correlated with the spawning season, i.e. from developing to regressing phases, which is probably because anchovy in the Mediterranean Sea continue feeding during their spawning season (Costalago *et al.* 2012; Nikolioudakis *et al.* 2014).

Consequently, it can be assumed that for the good of the stock, the median size of landing for anchovy should be over 12.5 cm as this would mean that there are enough lipids in the stock for vital processes such as growing, maintaining fitness during reproduction, etc.

An accessory result concerning energy reserves is the clear relationship between lipid content in muscle and the mesenteric fat stage using the method proposed by Lingen & Hutchings (2005). We therefore consider that the mesenteric fat stage is a good indicator of energy reserves in European anchovy in the NW Mediterranean Sea, and we support the incorporation of this cheap, quick and easy method for estimating anchovy energy reserves into other pelagic fish surveys.

Our results on parasitism showed that the metazoan parasite fauna of anchovy in the NW Mediterranean is dominated by digeneans and nematodes, most of them in larval stages. Although some parasites (*A. stossichi*, the hemiurid metacercariae and *H. aduncum*) were more prevalent in immature and regenerating specimens, no difference in the median intensity of infection was found during the different ovarian developmental phases. It appears, therefore, that the reproductive status of females during the spawning season does not significantly affect the parasite load.

However, individual intensities of parasitism showed that smaller specimens of anchovy harboured more parasites (*A. stossichi*, the hemiurid metacercariae and *H. aduncum*) than larger specimens. Conversely, our results also showed that higher intensities of didymozoid metacercariae were found in larger anchovy.

There is little research available concerning most of the various parasites that infect anchovy in the Mediterranean Sea, with the exception of nematode infection, on which there is a wide range of research. Indeed, because anisakid nematodes are involved in a disease that affects humans, i.e. anisakidosis, there is a considerable amount of literature concerning infections by these nematodes in anchovy (e.g. Rello *et al.* 2009; Gutiérrez-Galindo *et al.* 2010; De Liberato *et al.* 2013; Serracca *et al.* 2014).

Such studies have reported values of prevalence ranging from 0% to 25%, which is in accordance with our results (*H. aduncum* $P_P = 18.08\%$ and *Anisakis* sp. $P_P = 4.06\%$). However, one study carried out in the Adriatic Sea (Mladineo *et al.* 2012) found a 76% prevalence of anchovy infected by *Anisakis pegreffii*, which is much higher than any of the prevalences reported in others parts of the Mediterranean Sea.

In contrast to our study, Mladineo *et al.* (2012) reported a positive relationship between anchovy length and the intensity of infection by that parasite. However, Rello *et al.* (2009) and De Liberato *et al.* (2013) found no significant correlation between length and intensity of infection. In any case, there appears to be significant differences between different areas of the Mediterranean in the prevalence and intensity of anisakid nematodes which depends on the abundance of their intermediate and/or definitive hosts (Rello *et al.* 2009).

With regard to the effects of parasites on energy reserves, the results of this study indicate that the digenean *A. stossichi*, the hemiurid metacercariae and some unidentified nematode larvae can negatively affect the energy reserves of anchovy in regenerating, spawning capable, actively spawning or regressing phases. This conclusion is reinforced by our previous result which showed that there was no depletion of lipids that correlated with the spawning season (developing, spawning capable, actively spawning or regressing phases). Therefore, we can hypothesize that the observed differences in the energy reserves are due to the effect of parasitism rather than that of reproduction.

Although most of the effects were negative, we also found a positive relationship between *Anisakis* sp. and energy reserves. However, only one of the two tests performed was significant, which means that this positive relationship is not as strong as the negative ones.

Similar results were found by Shchepkina (1985), who analysed the lipid concentration in the liver, and the white and red muscles of anchovy in the Black Sea and found that specimens that were heavily infected by nematodes showed lower lipid concentration (especially triglycerides) in their tissues than lightly infected specimens. Nevertheless, it should be noted that other biotic and abiotic factors, particularly food availability, can also influence anchovy condition. For example, Brosset *et al.* (2015) demonstrated that the concentration of mesozooplankton has a significant positive influence on anchovy condition in the Gulf of Lion.

As far as the effects of parasitism on fecundity are concerned, it appears that *A. stossichi* and *H. aduncum* have a negative effect on female egg production while the didymozoid metacercariae has a positive effect. However, these results must be interpreted with caution. It is more likely that these differences in fecundity are attributable to anchovy length rather than parasitism.

Indeed, as previously reported, anchovy length is a good indicator of the individual's fecundity as there is a significant positive relationship between the two variables. Furthermore, when anchovy length versus individual intensity of infection was analysed, smaller anchovy were found to have more *A. stossichi* and *H. aduncum*, while larger anchovy had more didymozoid metacercariae. Therefore, we consider that parasitism does not significantly affect the fecundity of female anchovy and that the variations we found were due to the influence of body length.

Taken together, our results reveal that the current prevalence of smaller individuals in the NW Mediterranean anchovy stock has several consequences for the health of the stock as a whole, such as lower fecundity, lower lipid content and higher intensity of certain parasites. In order to allow the population to recover, we strongly suggest an increase in the minimum landing size for anchovy in the Mediterranean Sea. This would lead to a number of benefits: (i) more juveniles could reach sexual maturity, (ii) larger females could make a stronger contribution to the reproductive potential, (iii) there would be an increase in the amount of lipids and (iv) there would be a lower intensity of parasitism (Fig. III.8).

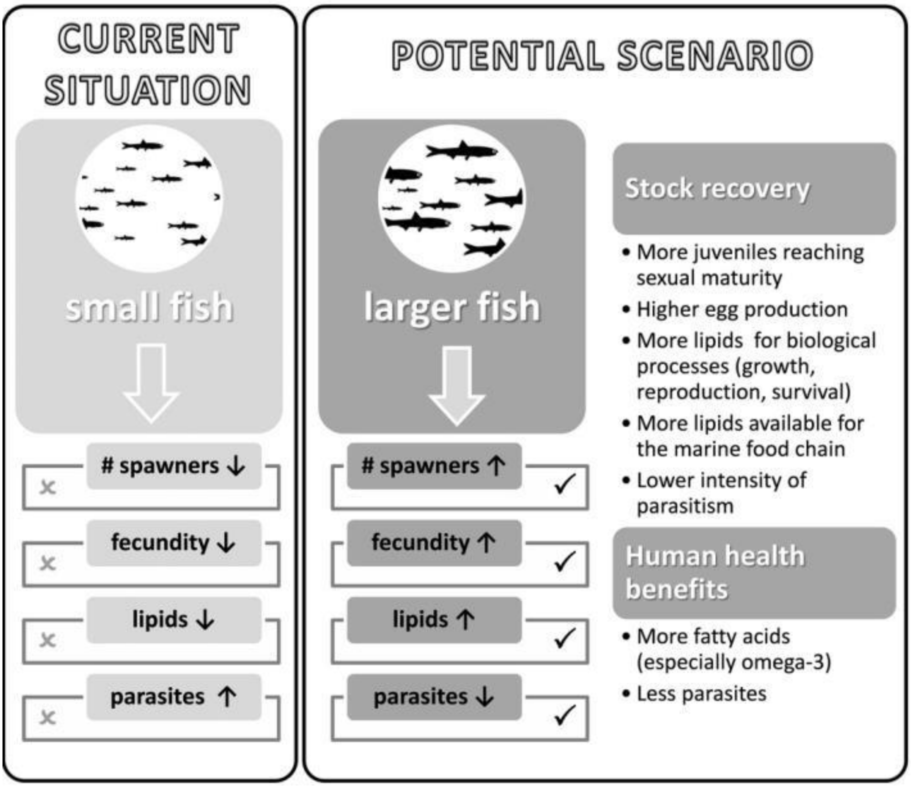

Fig. III.8. Implications for the health of the stock in a hypothetical scenario of an increase in the minimum landing size (MLS) for European anchovy in the Mediterranean Sea.

We consider that all of these potential benefits should be taken into consideration in the management of the European anchovy, not only because of the economic and ecological implications of further deterioration in anchovy stocks but also for the implications on human health: bigger individuals provide consumers with greater nutritional benefits (more fish oil) and fewer health risks (less parasites).

GENERAL DISCUSSION

The purpose of the general discussion is to integrate the main outcomes of the three articles covered in this thesis and to discuss the applicability of the results.

General discussion

The present study was designed to determine the possible effects of parasitism on the condition and reproduction of three commercially exploited fish species in the Mediterranean Sea. In contrast to most of the previous works on the subject, the present research (i) have focused on exploited marine fish species, (ii) have considered not only the effects of a single species of parasite but the whole metazoan parasite fauna of the hosts, (iii) have used biochemical indicators of fish condition instead of simple morphometric indices, and (iv) have analysed the effects of parasitism on the reproductive capacity of fish.

Overall, the results indicate that parasitism can be either positively or negatively related to the energy reserves and the reproductive capacity of fish. Therefore, that refutes the general hypothesis established in this PhD thesis that supposed a negative effect of parasitism on fish condition and reproduction. The most obvious finding to emerge from the analysis is that although parasitism, condition and reproduction are linked, their relationships do not always point in the same direction. Therefore, although the most common assumption stipulates a negative effect of parasites on fish health, results from this thesis contribute to the idea that this is not always the case.

Table 4 summarises the significant relationships (Mann-Whitney U test or Spearman’s Rank Correlation coefficient) found in the three species between parasitism and the energy reserves (total lipid content in liver or muscle and gonads), the relative fecundity (eggs per gram of eviscerated body mass) and the egg quality (dry mass of hydrated oocytes).

Table 4. Schematic summary of the significant relationships (Mann-Whitney U test or Spearman's Rank Correlation coefficient) found between parasitism and the energy reserves or reproduction variables of the three species analysed.

Parasite species	European hake		Red mullet			European anchovy	
	energy reserves	energy gonads	energy reserves	fecundity	egg quality	energy reserves	fecundity
Monogenea							
Anthocotyle merluccii	+						
Digenea							
Aphanurus stossichi						-	-
Didymozoidae metacercariae							+
Hemiuridae metacercariae						-	
Opecoeloides furcatus			-				
Cestoda							
Tetraphyllidean metacestodes							
Nematoda							
Anisakis pegreffii		-					
Anisakis sp. (Type I larvae)						+	
Hysterothylacium aduncum				+			-
Hysterothylacium fabri				+	-		
Unidentified larvae						-	
Copepoda							
Clavella stellata		-					
Parabrachiella insidiosa	-	-					

+ positive relationship; - negative relationship

First, the effect of parasitism on fish lipid reserves and reproductive capacity depends on both the species of parasite considered and the fish studied. As it can be seen in Table 4, the energy reserves in the liver of hake are negatively related with the copepod *Parabrachiella insidiosa* but positively related with the monogenean *Anthocotyle merluccii*. However, the lipid content in hake's gonads is negatively related with the nematode *Anisakis pegreffii* and the copepods *Clavella stellata* and *P. insidiosa*.

Concerning red mullet, the energy reserves in the muscle are negatively related with the digenean *Opecoeloides furcatus*. On the other hand, the fecundity of red mullet is positively related with the nematodes *Hysterothylacium aduncum* and *H. fabri* although the quality of their eggs is negatively related with the latter.

With regard to anchovy, the energy reserves in the muscle are negatively related with the digenean *Aphanurus stossichi*, the Hemiuridae metacercariae and the unidentified nematode larvae. On the contrary, the energy reserves are positively related with the nematode *Anisakis* sp. Finally, the batch fecundity of anchovy is negatively related with *A. stossichi* and *H. aduncum*, but positively related with the Didymozoidae metacercariae.

Therefore, we found both positive and negative relationships between parasitism and condition or reproduction in the three analysed fish species. As we found similar heterogenic effects of parasites in the three studied species, neither the environments nor the feeding regimes of the fish studied appear to have a significant impact on these relationships.

Regarding fish condition, most of the significant results indicate a negative effect of parasitism on the energy reserves of specimens. In the case of hake, as seen in chapter I, the effects of parasites seem to be more evident when hake is allocating its energy to reproduction. A similar effect was also found in the case of red mullet (chapter II), in which the energy reserves were affected by parasitism in the spawning season. In the case of anchovy (chapter III), parasitism can affect the energy reserves all year round, as we found negative relationships for all the ovarian developmental phases.

However, although most of the signals indicate a negative effect of parasites on the fish condition, in some cases, i.e. hake infected by the monogenean *Anthocotyle merluccii* and anchovy infected by the nematodes *Anisakis* sp. the relationships were positive. In the case of anchovy, this result might indicate that specimens that are intensely feeding are in better condition but also they harbour more *Anisakis* sp. because nematodes are acquired by consumption. Similar positive relationships between endoparasites and fish condition have been previously reported in some fish species (e.g. Aquino Moreira *et al.* 2010; Costa-Dias *et al.* 2010).

In the case of hake, though, the monogenean *A. merluccii* is an ectoparasite that infects hake by attaching to hake's gills with suckers provided with hooks, i.e. is not acquired by consumption. However, it could be hypothesised that food-rich environments for hake are also propitious to infection by specialist parasites of hake, such as *A. merluccii*. Consequently, hake inhabiting those environments would be in better condition but also would have more chances to become infected. In any case, the main weakness of this study, as usually happens with studies on wild-caught fishes, is that we did not know the timing of infection. It is plausible to think that the potential effects of parasites on the host condition do not appear immediately after infection. Therefore, without knowing how long hake have been infected with these parasites, we cannot insure that *A. merluccii* will not cause negative effects on hake's condition. Actually, previous studies have demonstrated a negative effect of some ectoparasites on fish condition (e.g. Guillaume *et al.* 1985; Khan & Lacey 1986).

Indeed, the different effects on fish condition between endoparasites and ectoparasites have been previously reported. For example, Guidelli *et al.* (2011) analysed the condition of four species of *Leporinus* spp. and found positive relationships between condition and some endoparasites; and negative relationships between condition and some ectoparasites. They suggested that the different infection strategies of these two categories of parasites could have influence on the potential effects as the performance of the immune system differs between the organs and tissues of host.

With regard to the effects of parasites on fish reproduction, the relationships found in this thesis are rather complex. First of all, it should be noted that the reproductive capacity of fishes can be assessed by the quantity of eggs spawned (fecundity) but also by the quality (size and weight) of the eggs. In addition to the uncertain importance relative of those two variables, we found difficult to compare our results with other authors because of the scarcity of previous studies on the relationships between parasitism and reproductive capacity.

In this thesis, we have showed that some parasites may positively affect fecundity of some fish species. However, as it is the case for red mullet, if the number of eggs released per batch is positively impacted by parasitism, the quality of these eggs is impaired. As discussed in chapter II, this result may mean that some parasites can cause a reduction in the quality of the host's eggs, but in response, red mullet would try to compensate this impact by producing a higher number of eggs. The negative effects of parasitism on the quality of eggs have been demonstrated in some fish species, such as *Gasterosteus aculeatus* (Heins & Baker 2003) or *Cheilodipterus quinquelineatus* (Fogelman *et al.* 2009). Therefore, despite the increase in the fecundity of red mullet, parasitism would not affect positively the productivity of fish, since viability during the early life stages of fish depends mainly on egg quality, which is associated with the quantity of nutrients stored in the oocytes (Brooks *et al.* 1997).

In this work, it was not possible to assess the anchovy eggs quality because the oocyte dry masses were too low to measure them with an appropriate level of accuracy. Therefore, we do not know if the observed increase of anchovy's fecundity associated with infection by Didymozoidae metacercariae was also accompanied by a reduction in egg quality, as it was highlighted for red mullet. However, it has to be pointed out that we found negative relationships between anchovy's fecundity and the infection by two other parasites (*A. stossichi* and *H. aduncum*). Therefore, as it seems to happen with energy reserves, anchovy's fecundity is also positively and negatively related to parasitism depending on the parasite considered. Didymozoidae metacercariae as well as the digenean *A. stossichi* and the nematode *H. aduncum* are all endoparasites but they were found in different stages of their life cycle. For example, Didymozoidae and *A. stossichi* are both digeneans, but whereas the former was found in an early larval stage (metacercariae), the latter was found in its adult stage. Therefore, the impact of parasites might depend not only on the organ or tissue infected but also on the nutritional requirements of each life cycle stage in which parasites are found. Again, these results contribute to reveal the complex relationships that are established between parasitism, condition and reproduction of fish.

Finally, regarding hake, we did not found any statistically significant effect of parasitism on the fecundity or quality of the eggs probably because European hake is a species with a high plasticity in the reproductive characteristics (Korta *et al.* 2010). Moreover, the reproductive capacity of hake has only been analysed in a few individuals because hake do not have a clearly definite spawning season and this makes it difficult to capture the actively spawning and without POFs females required for this analysis.

Despite the significant relationships between parasitism and fish reproduction found in this thesis, we should bear in mind that the reproductive capacity of fishes may also vary spatially and/or temporally in response to other particular environmental conditions, such as water temperature or food availability (Witthames *et al.* 1995; Villegas-Hernández *et al.* 2015a; b). Moreover, since fish condition have also been identified as a key factor in female egg production (Kjesbu *et al.* 1991), our results must be interpreted with caution. Probably, the effects of parasitism on the reproductive capacity are not evident because either the size or the condition of fishes has greater influence on egg production.

In addition to the main question of this study aiming to determine the effects of parasitism on the condition and reproduction of three commercially exploited fish species, other relevant results were found. The assessment of the condition and reproduction and the analysis of their relationships indicate that, the northwestern Mediterranean *Merluccius merluccius* has a protracted spawning season which continues practically all year round with a spawning peak in winter and another short peak in summer, which is consistent with results obtained in different Mediterranean areas (Recasens *et al.* 2008; Al-Absawy 2010). Moreover, the development of gonads in Mediterranean hake is detrimental to the accumulation of lipidic resources in the liver except in summer when the energy allocated to reproduction comes from concurrent feeding. This hypothesis could be corroborated by the highest abundance of hake's prey in the Mediterranean Sea during this period. It should be noted though that Domínguez-Petit & Saborido-Rey (2010) showed that European hake in the North Atlantic always develop the gonads when there is an energy intake surplus rather than doing so at the expense of accumulated energy. This discrepancy provides further support to the idea that the European hake is a species with a high plasticity in the reproductive characteristics.

Concerning red mullet, we showed that in the western Mediterranean Sea, the spawning season of *Mullus barbatus* takes place in spring-summer, which coincides with data provided by Tsikliras *et al.* (2010) for other parts of the Mediterranean Sea. However, this thesis also sheds light on several essential but undocumented aspects of the reproduction of this species. For the first time, the oocyte diameter-frequency distribution and the stage-specific oocyte diameter indicated that red mullet is a batch spawner with asynchronous development of oocytes and indeterminate fecundity. We also found that there is no decrease in the lipids stored in the red mullet's muscle as the gonads develop, indicating that reproduction is fuelled by direct food intake.

With regards to *Engraulis encrasicolus*, our results indicate that, in the northwestern Mediterranean Sea, its spawning season takes place in spring-summer, which is consistent with what has been reported by Tsikliras *et al.* (2010) for other parts of the Mediterranean Sea. Although the reproductive characteristics of anchovy have been widely studied (Motos *et al.* 1996; Somarakis *et al.* 2004; Santos *et al.* 2010; Manzo *et al.* 2013; McBride *et al.* 2013; Mezedjri *et al.* 2013, among others), in this thesis we contribute to the available knowledge providing further information about the spawning strategy. We have found that, in the northwestern Mediterranean Sea, the energy allocated to the development of the gonads comes from concurrent food instead of the accumulated lipids in the muscle. Our findings also revealed that the current preponderance of small individuals is threatening the health of the stock, since it implies lower fecundity, lower lipid content and a higher intensity of certain parasites. This could have negative consequences for anchovy fisheries, because fishermen target large individuals and that affects the health and productivity of the stock, producing a negative feedback in which fish are being caught increasingly smaller and thus endangering the viability of the fishery itself.

The results of this PhD thesis indicate that, although parasitism does not always affect negatively the condition and reproduction of fishes, these three important features of the fish biology and ecology are linked. However, probably there are other environmental factors that may have influence on the effects of parasites.

For example, climate change might affect host-parasite equilibrium because parasites in aquatic systems are expected to respond directly to changes in temperature but also indirectly to changes in other abiotic parameters that are mediated though changes in the distribution of their hosts (Marcogliese 2001). Actually, Ondracková *et al.* (2010) found that some fish captured in non-native areas of distribution harbour more parasites species than those fish caught in their native areas as a result of acquiring parasites native to the new area. However, it seems that in those cases the increase of parasitism does not affect the condition because generally fish species successfully introduced in a novel area can profit from this favourable situation, attaining greater sizes and better condition.

Additionally, the synergistic effects of parasitism and pollution on host have also been identified. Pollutants may affect the immune system of fish either directly or by causing changes in water quality that in turn may reduce fish immunity to parasites (Möller 1987; Poulin 1992). For instance, Carreras-Aubets *et al.* (2011) studied the parasite fauna and histopathology of *Mullus barbatus* and *Citharus linguatula* from two sites with different degrees of pollution, and found that some parasites, as well as some histological alterations were related to pollution. The protection of marine reserves (Sasal *et al.* 2004) or fishing pressure (Marzoug *et al.* 2012) have also been recognised as factors affecting the richness, abundance and structure of some parasite communities. For instance, Sala-Bozano *et al.* (2012) analysed the hepatosomatic index of *Lithognathus mormyrus* and found that the isopod *Ceratothoa italica* caused a severe impact on the condition of those individuals subjected to higher fishing pressure.

Finally, the results of this thesis have implications for the management of Mediterranean exploited fish species. We have provided important useful data on fish health and reproductive capacity of three highly valued exploited species that could complement the standard population dynamic procedures commonly used by international scientific and management organizations.

The importance of fish health indicators to assess the status of stocks and to anticipate future consequences have been highlighted, for example, in the case of European anchovy in the Sea of Azov and the Black Sea. Shchepkina (1985) found that parasitized anchovies had lower lipid content in liver, red and white muscle, than non-parasitized ones. Shulman (2002) in turn demonstrated that the level of fat stored in anchovy is the most important endogenous factor that prepares anchovy populations for wintering migrations.

Another example of the usefulness of health indicators in the assessment and management of exploited species is the case of the well-studied Atlantic cod, *Gadus morhua*. Lambert & Dutil (1997) studied the condition and energy reserves of the Atlantic cod before and during the collapse of the northern Gulf of St. Lawrence (Canada) stock and found significant decreases in cod condition in the preceding years to the drastic reduction of stock biomass. Actually, Marshall *et al.* (1999) showed a positive association between recruitment and the liver weights of spawners cods suggesting that stock recruitment were constrained by the amount of lipid energy stored in the liver of specimens rather than to the stock spanner's biomass.

Therefore, the outcomes of this thesis can contribute to determine the status of the Mediterranean stocks of European hake, red mullet and European anchovy. The information provided on the parasitism, energy reserves and reproductive capacity of those species can be used to perform an effective and sustainable fishery management.

Recommendations for further research work

First of all, it should be noted that, disregarding discrepancies that may be due to variation in methodologies, the prevalences and intensities of parasites in the three studied species are within the average ranges reported in the previous published studies. That is to say, we did not found a significant increase of the parasitic load compared with earlier works. It is important to bear in mind this information because we did not found severe effects of parasites on host condition and reproduction but it remains unknown if a hypothetical increase of parasites, due to the biological consequences of climate and anthropogenic changes, might cause stronger effects in the near future. Therefore, I consider that a systematic monitoring of parasitism of the exploited fish species could be useful to detect any potential increase of parasitism that could then finally have a greater impact on the life history traits of fish, as well as pose an increasing risk to the health of human fish consumers.

Indeed, as anisakid nematodes can cause human disease, i.e. anisakidosis, the evaluation of parasitism in commercially exploited fish species has important implications for human health risk assessment. Actually, the Government of Catalonia via its veterinarian services (http://www.gencat.cat/salut/acsa/html/ca/Du58/index.html) has evaluated the presence of anisakid nematodes in several commercial fish species in the Catalan fishing harbours (NW Mediterranean Sea), during the period 2000-2007. Since that program has been cancelled we cannot really compare our current results with their official data but in the present study we reported substantial higher values of both prevalences and intensities of anisakids. I consider that program as very useful since it could serve as a base for an improved future project aiming to a more accurate survey on anisakid nematodes, not only in the Catalan fishing harbours but also in the main wholesale markets of the region.

With regards to human risks, I consider it is more important to inspect the samples in the markets than in the fishing harbours because a great part of the fish consumed in Catalonia comes through importation from other parts of the Mediterranean Sea or from other regions worldwide. We should bear in mind that humans can become infected with some anisakid nematodes and that infection can cause from a simple headache until a severe allergic reaction (Ishikura *et al.* 1993).

While it is true that the intensities of anisakid nematodes in the NW Mediterranean Sea are relatively low, in other zones the intensities of infection are clearly higher. For instance, whereas it was not the aim of this thesis, I had the opportunity, during my doctoral period, to search for parasites some anchovies originating from the Adriatic Sea (Fig. 7) and bought in a local market in Girona.

Fig. 7. European anchovy, *Engraulis encrasicolus*, from the Adriatic Sea with several anisakid nematodes larvae leaving the anchovies' bodies. ©Dolors Ferrer Maza.

As mentioned before, these examinations were not part of this thesis; therefore I do not have any value of the anisakid nematodes intensities. However, I could observe in real time a huge amount of nematodes leaving the anchovies' bodies. Indeed, in the Adriatic Sea, intensities of up to 70 larvae in a single anchovy have been reported (Mladineo *et al.* 2012). It is worth mentioning that the probabilities of suffering anisakidosis increases when eating raw or undercooked fish and the popular Spanish dish "anchovies marinated in vinegar" have been involved in most cases of anisakidosis in Spain (del Olmo Martínez *et al.* 2000; Repiso Ortega *et al.* 2003; González Quijada *et al.* 2005). Thus, in order to detect commercial fish heavily infected by anisakid nematodes, I suggest continuing the monitoring of the anisakid nematodes by the Catalan veterinary services.

Finally, fish health can be assessed by a variety of indicators, ranging from morphometric indices of condition, biochemical measurements (protein, lipids, etc.), evaluation of infectious diseases (parasites, virus, bacteria, etc.) or evaluation of physical damage, blood parameters, histopathological analyses, etc. (reviewed by Lloret *et al.* 2012a). Some of these indicators give accurate and detailed information on a particular aspect of fish health, however for fisheries management purposes, we should concentrate on those indicators that are better to sample in the long term, regarding time and cost, such as energy reserves and reproductive capacity.

Consequently, whereas I consider that the presence of parasites, particularly nematodes, in commercially exploited fish species must be surveyed for human health risk assessment purposes, the usefulness of studying the effects of parasites on fish condition and reproduction is rather debateable, especially taking into account that the methodology is too laborious and time-consuming to be included in long-term monitoring programs.

I consider that although in this thesis we did not found severe effects of parasites on the condition and reproduction of the studied species, the data we obtained here on the health and reproductive capacity of fish are very useful and can contribute to more extensive studies, such as those elaborated by the Scientific, Technical and Economic Committee for Fisheries (STECF). The European Commission have to take into consideration the advice from the STECF when presenting proposals on fisheries management, thus I consider that more studies on fish health are needed and the authors should, on their own initiative, provide their results to institutions like STECF.

For instance, in this thesis we suggest a precautionary increase in the minimum landing size for anchovy in the Mediterranean Sea, in order to facilitate the recovery of the stock. However, our zone of study only comprises the northwestern Mediterranean Sea and some regulatory measures are applicable throughout the Mediterranean Sea. This is why I consider very useful to report the results of the scientific studies to institutions and organisms that operate on a larger scale.

In my opinion, the major limitation of this work is that it is based on correlational study carried out with wild-caught samples. It implies that we have measured several fish variables and have found interesting relationships between them but we do not know the essential cause-and-effect relationships between these variables. Indeed, as mentioned before, there are many other uncontrolled factors that might affect the condition and reproduction of fishes and the effects of parasitism. Therefore, while correlational studies are very useful to find out relationships between variables, the next step might be to monitor the effects of parasitism on fish condition and reproduction in an experimental study.

The suitable fishes for performing that study should be species that previously had been successful raised in tanks or enclosures. Experimentally infected fish hosts could be generated by feeding them with preys previously infected by parasites. All the environmental conditions in the tanks, such as water temperature, salinity, pH, dissolved oxygen, etc. should be controlled and fixed. In this way, if a relationship between parasitism and fish health were found and the rest of variables were constant, then it might be assumed that fish health is indeed affected by parasitism.

CONCLUSIONS

This section presents the conclusions of the doctoral thesis.

Conclusions

- Parasitism, condition and reproduction of commercially exploited fish species in the western Mediterranean Sea are closely linked although their relationships do not always point in the same direction.

- Metazoan parasites negatively affect the energy reserves of *Merluccius merluccius*, and *Mullus barbatus* mainly in their spawning season, whereas the energy reserves of *Engraulis encrasicolus* are affected by parasites at any time of the year.

- The effects of metazoan parasites on reproduction are complex. No effects of parasites were found on the reproductive capacity of *Merluccius merluccius*, whereas in *Mullus barbatus*, although they were found to have a positive effect on batch fecundity, they impaired egg quality. As for the batch fecundity of anchovy, the effects of metazoan parasites can be positive or negative depending on the parasite species considered.

- *Merluccius merluccius* in the northwestern Mediterranean Sea has a protracted spawning season which continues practically all year round. Development of its gonads is detrimental to the accumulation of lipidic reserves in the liver, except in summer when the energy allocated to reproduction comes from concurrent feeding.

- *Mullus barbatus* is a batch spawner with asynchronous development of oocytes and indeterminate fecundity; its spawning season in the western Mediterranean Sea takes place in spring-summer and the energy used in gonad development comes from concurrent feeding.

- The reproduction of *Engraulis encrasicolus* in the northwestern Mediterranean Sea occurs in spring-summer and the energy allocated to gonad development comes from concurrent feeding.

- The sizes of *Merluccius merluccius*, *Mullus barbatus* and *Engraulis encrasicolus* are good indicators of their batch fecundity, showing a strong positive relationship and indicating that the removal of larger individuals can significantly affect the productivity of the populations.

Overall, the results of this thesis indicate that parasitism, condition and reproduction of Mediterranean exploited fish species are linked, although their relationships do not always point in the same direction. The relationships between energy reserves and reproduction presented in this thesis are essential for understanding the spawning strategy of the three studied species. Moreover, we have provided important information on fish health which, we believe, should be used in the assessment and management of these stocks, as well as in human health risk assessment. However, this study of the impact of parasitism on fish condition and reproduction has indicated that the effects are not always evident and straightforward, since in all probability there are others endogenous or exogenous factors that produce additive, synergistic or antagonistic effects on fish condition and reproduction.

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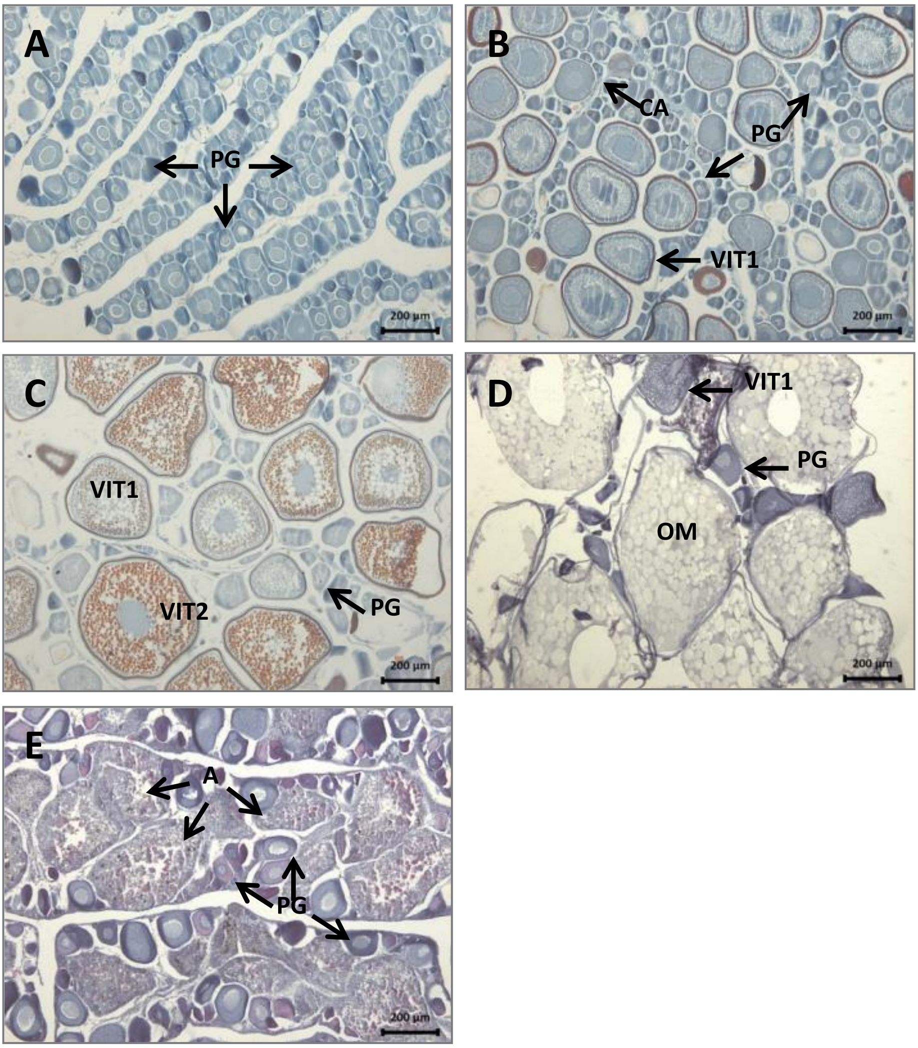
APPENDICES

In the appendices section there is supplementary material that gives useful additional content, such as microphotographs of the ovarian developmental phases of the three fish species, images of the parasites found along this study or the front pages of the three articles which compose this thesis.

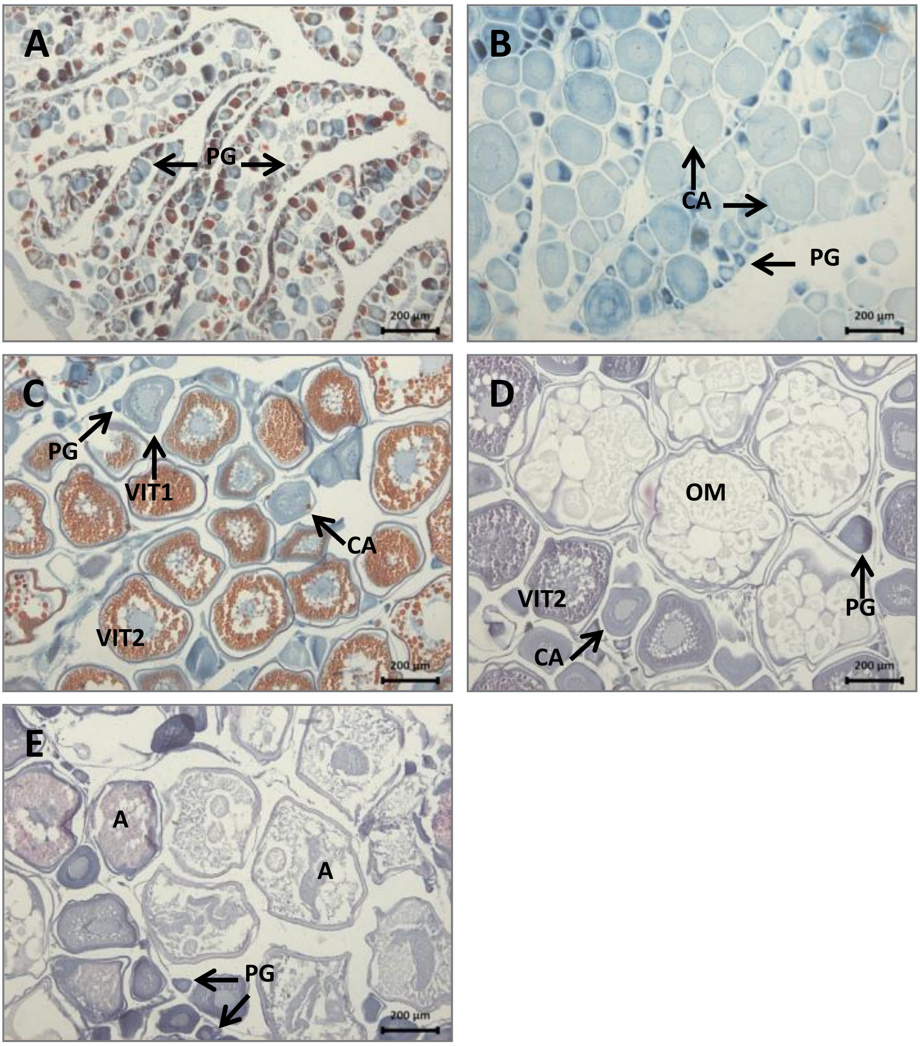
Appendix A: Gonad photomicrographs

European hake, *Merluccius merluccius*



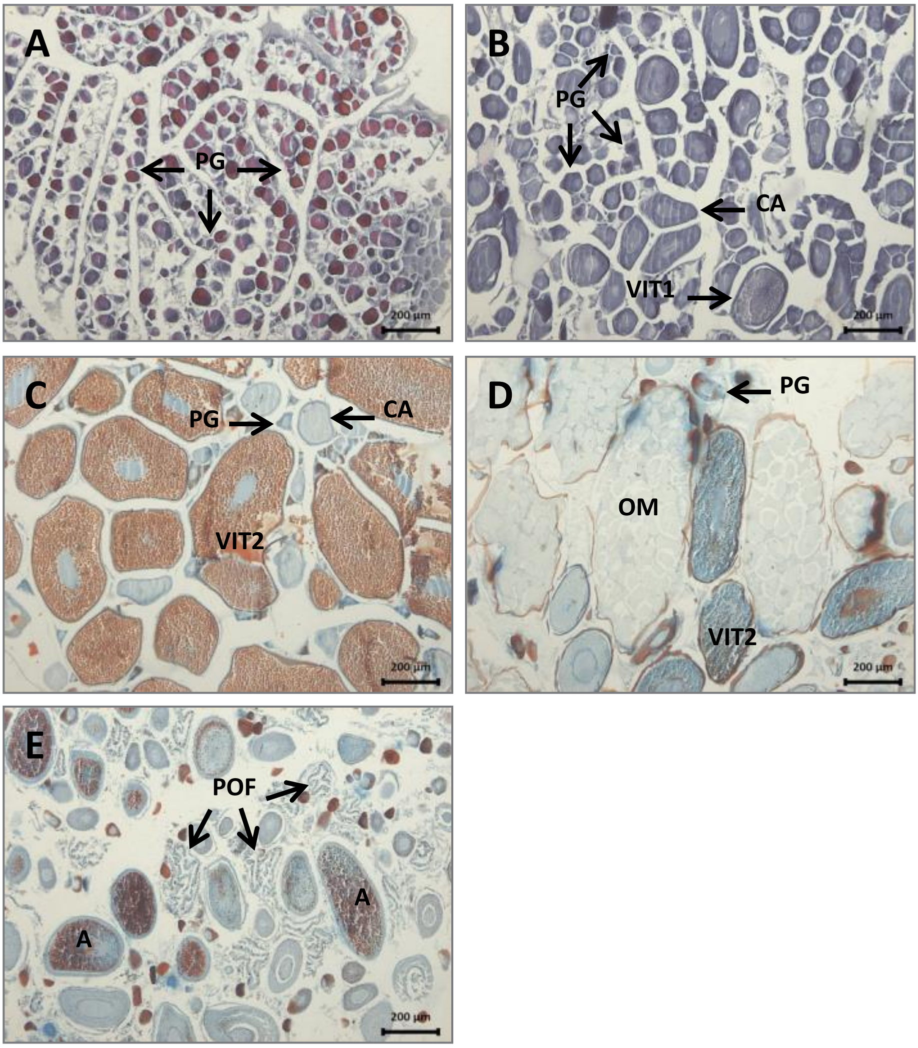
Photomicrographs of the histological slides of the European hake's ovaries in different ovarian developmental phase: A, regenerating; B, developing; C, spawning capable; D, actively spawning; and E, regressing. The oocyte developmental stages are also shown as PG, primary growth; CA, cortical alveolar; VIT1, early vitellogenesis; VIT2, late vitellogenesis; OM, maturation; and A, atresia.

Red mullet, *Mullus barbatus*



Photomicrographs of the histological slides of the red mullet's ovaries in different ovarian developmental phase: A, regenerating; B, developing; C, spawning capable; D, actively spawning; and E, regressing. The oocyte developmental stages are also shown as PG, primary growth; CA, cortical alveolar; VIT1, early vitellogenesis; VIT2, late vitellogenesis; OM, maturation; and A, atresia.

European anchovy, *Engraulis encrasicolus*

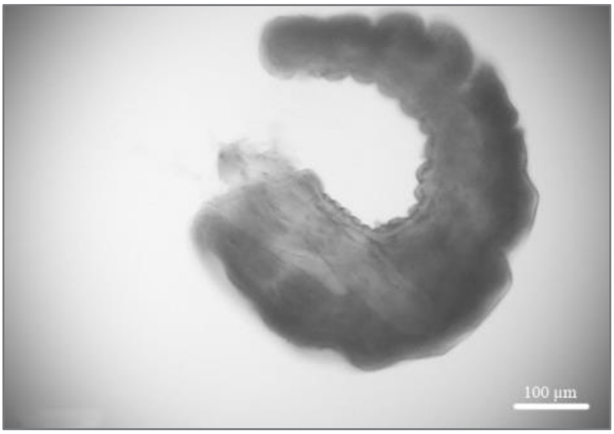


Photomicrographs of the histological slides of the European anchovy's ovaries in different ovarian developmental phase: A, regenerating; B, developing; C, spawning capable; D, actively spawning; and E, regressing. The oocyte developmental stages are also shown as PG, primary growth; CA, cortical alveolar; VIT1, early vitellogenesis; VIT2, late vitellogenesis; OM, maturation; POF, post-ovulatory follicles; and A, atresia.

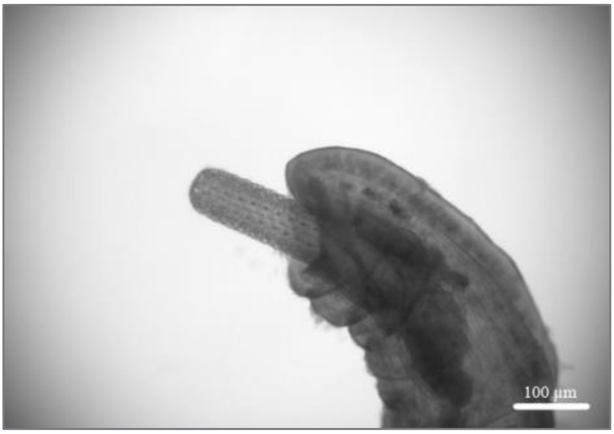
Appendix B: Parasites images

In this appendix the images of the identified metazoan parasites found in the three studied species are shown. They are grouped according to their taxa and listed alphabetically.

Acanthocephala

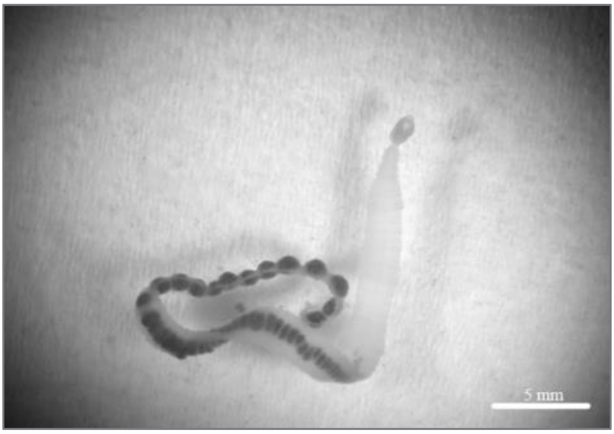


Acanthocephaloides propinquus (Dujardin, 1845)
Host: *Merluccius merluccius*

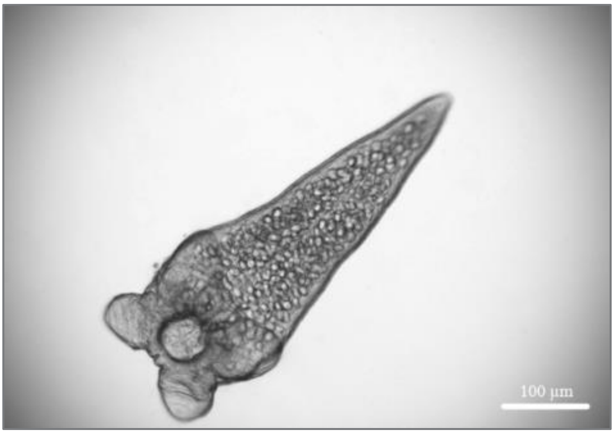


Echinorhynchus sp.
Host: *Merluccius merluccius*

Cestoda

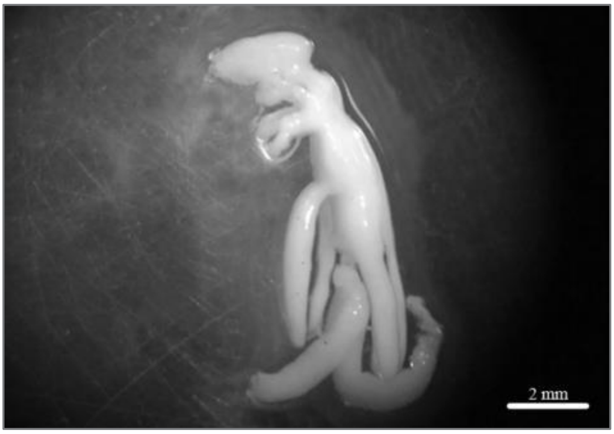


Clestobothrium crassiceps (Rudolphi, 1819)
Host: *Merluccius merluccius*

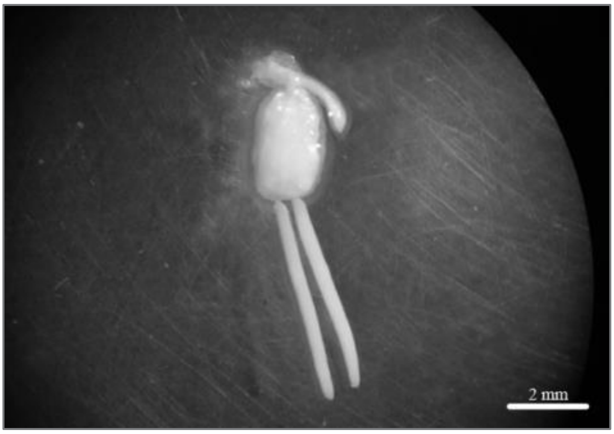


Tetraphyllidean metacestodes
Hosts: *Merluccius merluccius, Mullus barbatus, Engraulis encrasicolus*

Copepoda

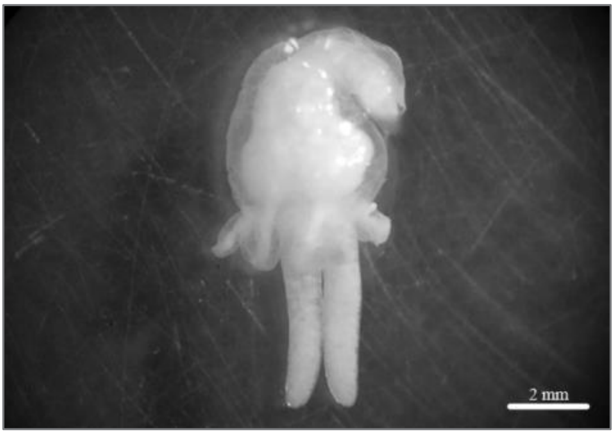


Chondracanthus merluccii (Holten, 1802)
Host: *Merluccius merluccius*



Clavella stellata (Krøyer, 1838)
Host: *Merluccius merluccius*

Parabrachiella insidiosa (Heller, 1865)
Host: *Merluccius merluccius*

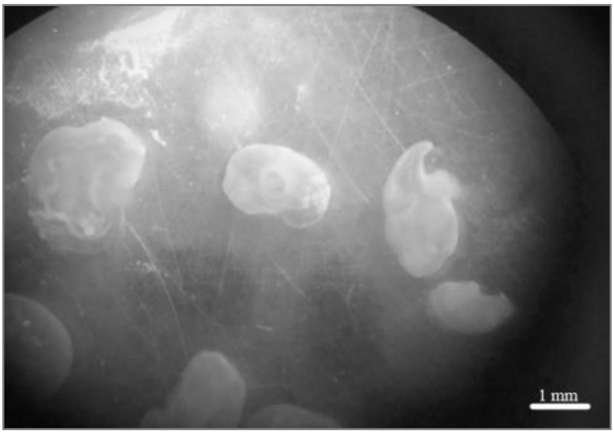


Parabrachiella merluccii (Bassett-Smith, 1896)
Host: *Merluccius merluccius*

Digenea

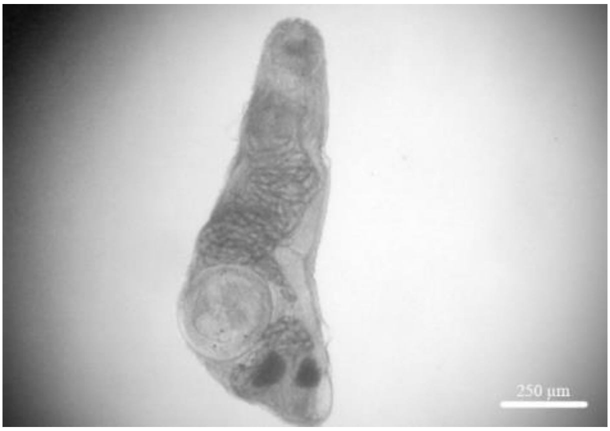
Aphanurus stossichi (Monticelli, 1891)
Host: *Engraulis encrasicolus*

Aporocotyle spinosicanalis (Williams, 1958)
Host: *Merluccius merluccius*

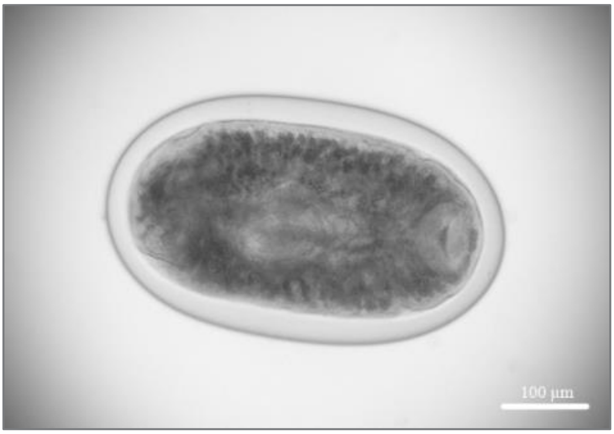


Derogenes latus (Janiszewska, 1953)
Host: *Mullus barbatus*

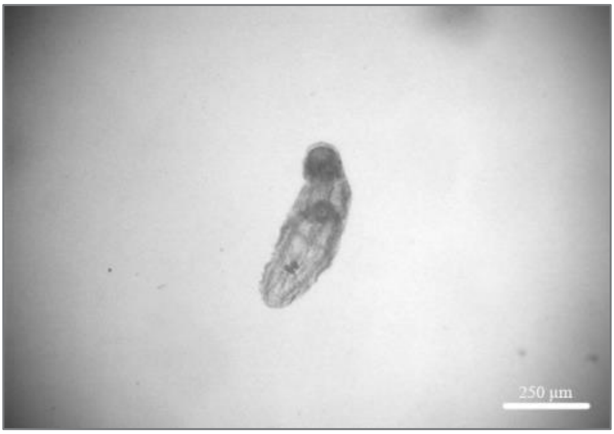
Didymozoidae metacercariae
Host: *Engraulis encrasicolus*



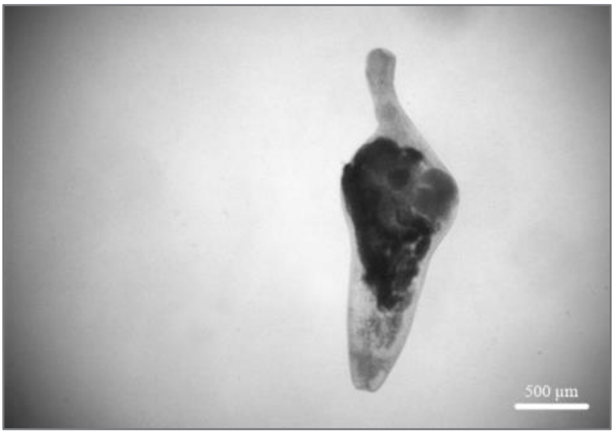
Hemipera magnaprostatica (Gaevskaya and Aleshkina, 1995)
Host: *Merluccius merluccius*



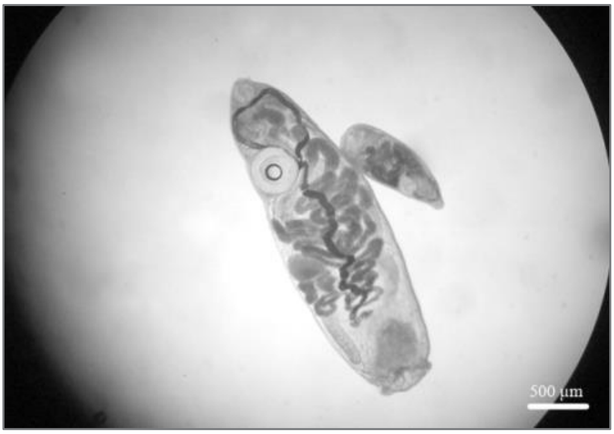
Hemiuridae metacercariae
Host: *Engraulis encrasicolus*



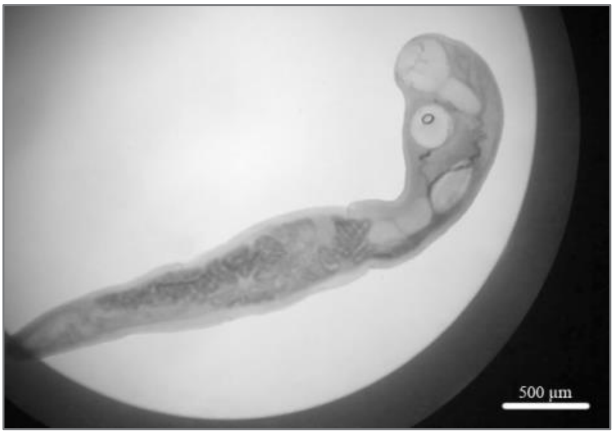
Hemiurus communis (Odhner, 1905)
Host: *Merluccius merluccius*



Lasiotocus mulli (Stossich, 1883)
Host: *Mullus barbatus*

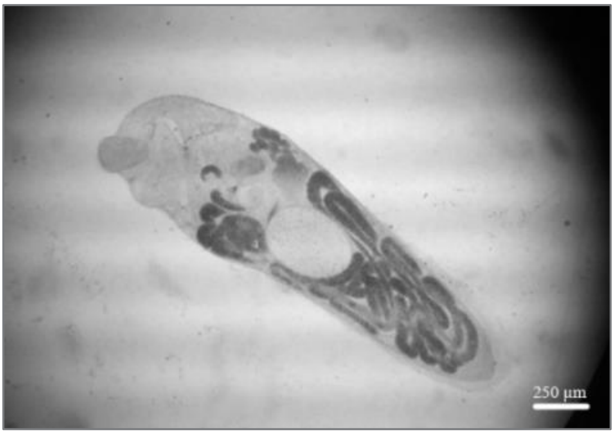


Lecithochirium musculus (Looss, 1907)
Host: *Merluccius merluccius*



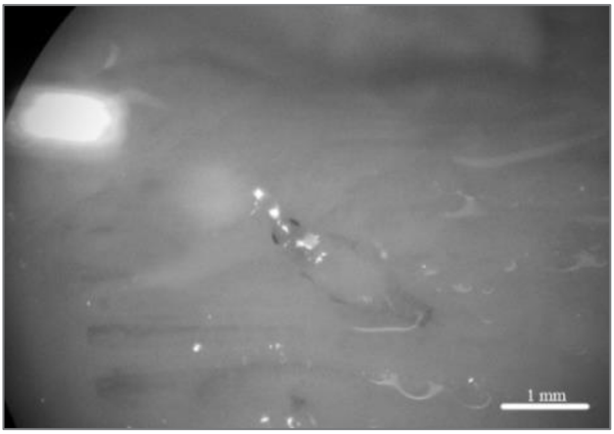
Lecithocladium excisum (Rudolphi, 1819)
Host: *Mullus barbatus*

Opecoeloides furcatus (Bremser in Rudolphi, 1819)
Host: *Mullus barbatus*



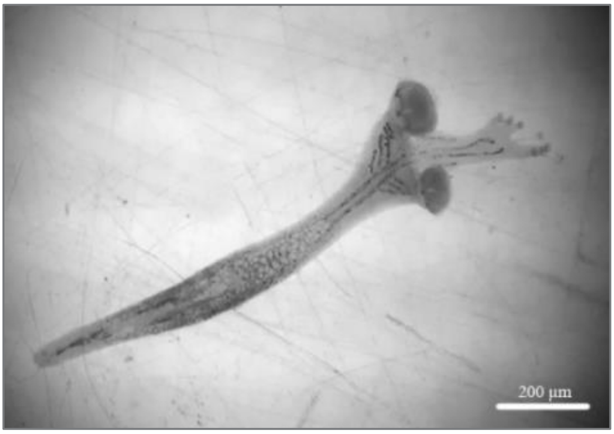
Proctotrema bacilliovatum (Odhner, 1911)
Host: *Mullus barbatus*

Isopoda



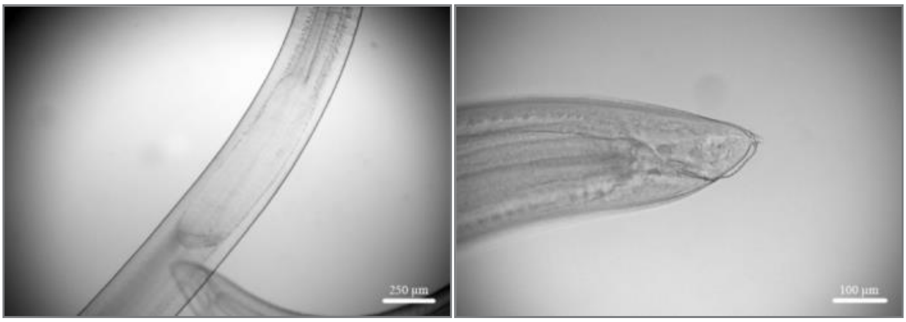
Gnathia sp.
Host: *Mullus barbatus*

Monogenea

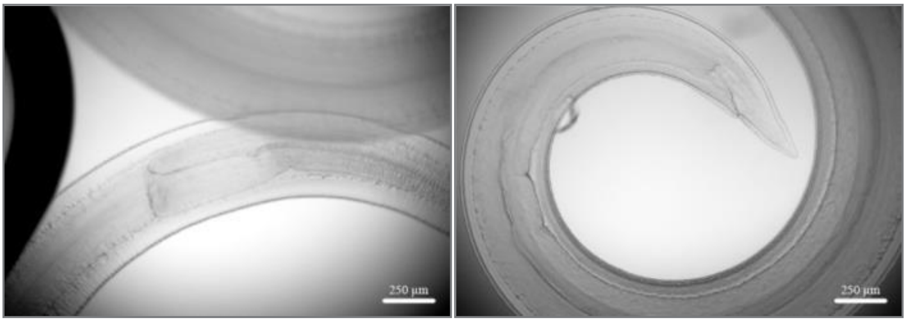


Anthocotyle merluccii (van Beneden and Hesse, 1863)
Host: *Merluccius merluccius*

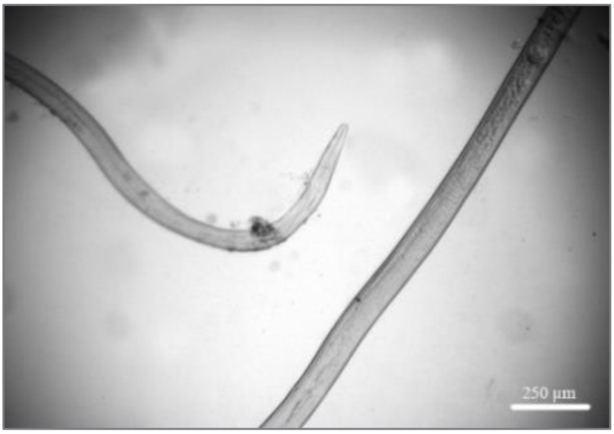
Nematoda



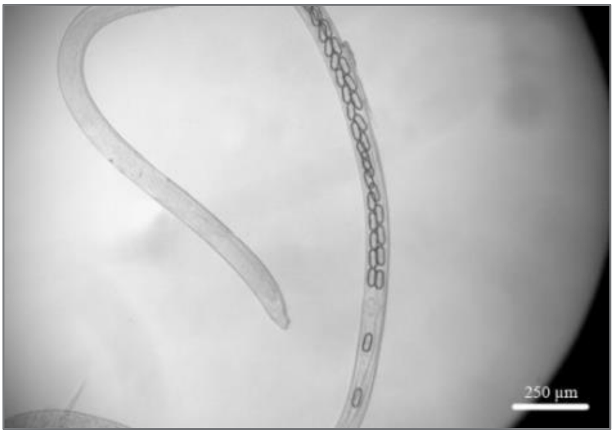
Anisakis pegreffii (Campana-Rouget and Biocca, 1955)
Hosts: *Merluccius merluccius, Engraulis encrasicolus* (*Anisakis* sp. Type I larvae)



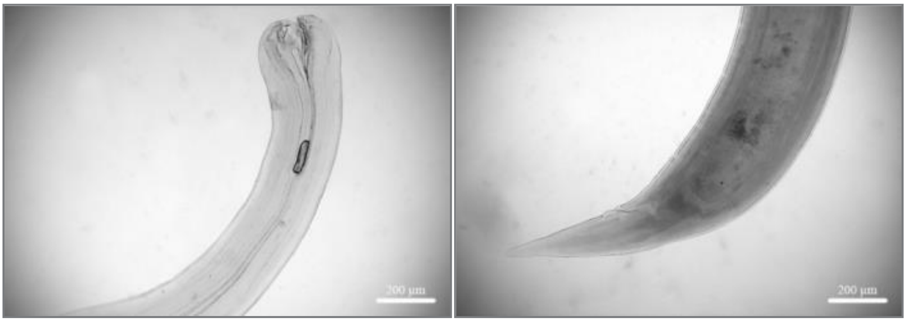
Anisakis physeteris (Baylis, 1923)
Host: *Merluccius merluccius*



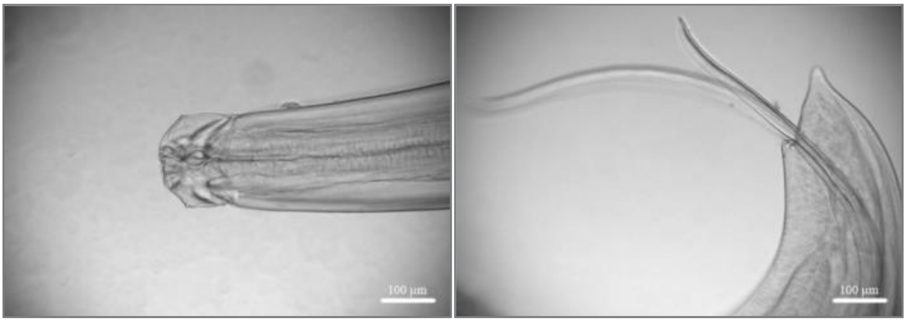
Ascarophis mullusi (Naidenova and Nikolaeva, 1968)
Host: *Mullus barbatus*



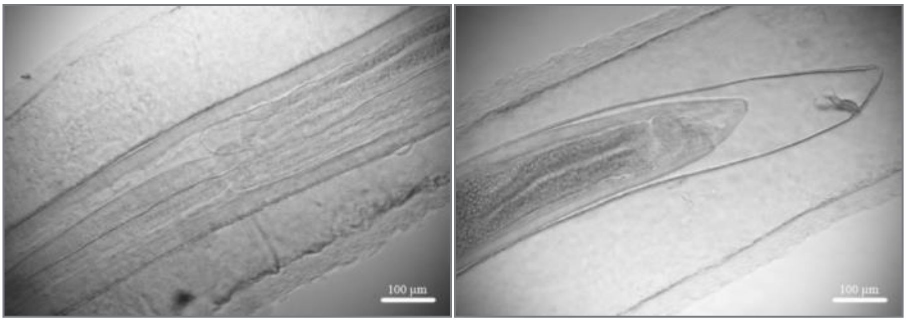
Capillaria gracili (Bellingham, 1840)
Host: *Merluccius merluccius*



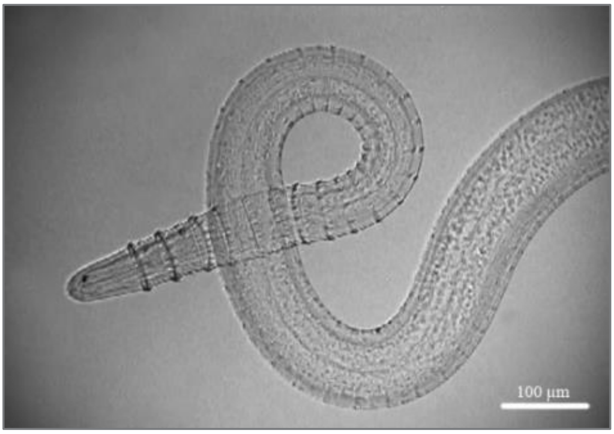
Cucullanus longicollis (Stossich, 1899)
Host: *Mullus barbatus*



Hysterothylacium aduncum (Rudolphi, 1802)
Hosts: *Merluccius merluccius, Mullus barbatus, Engraulis encrasicolus*



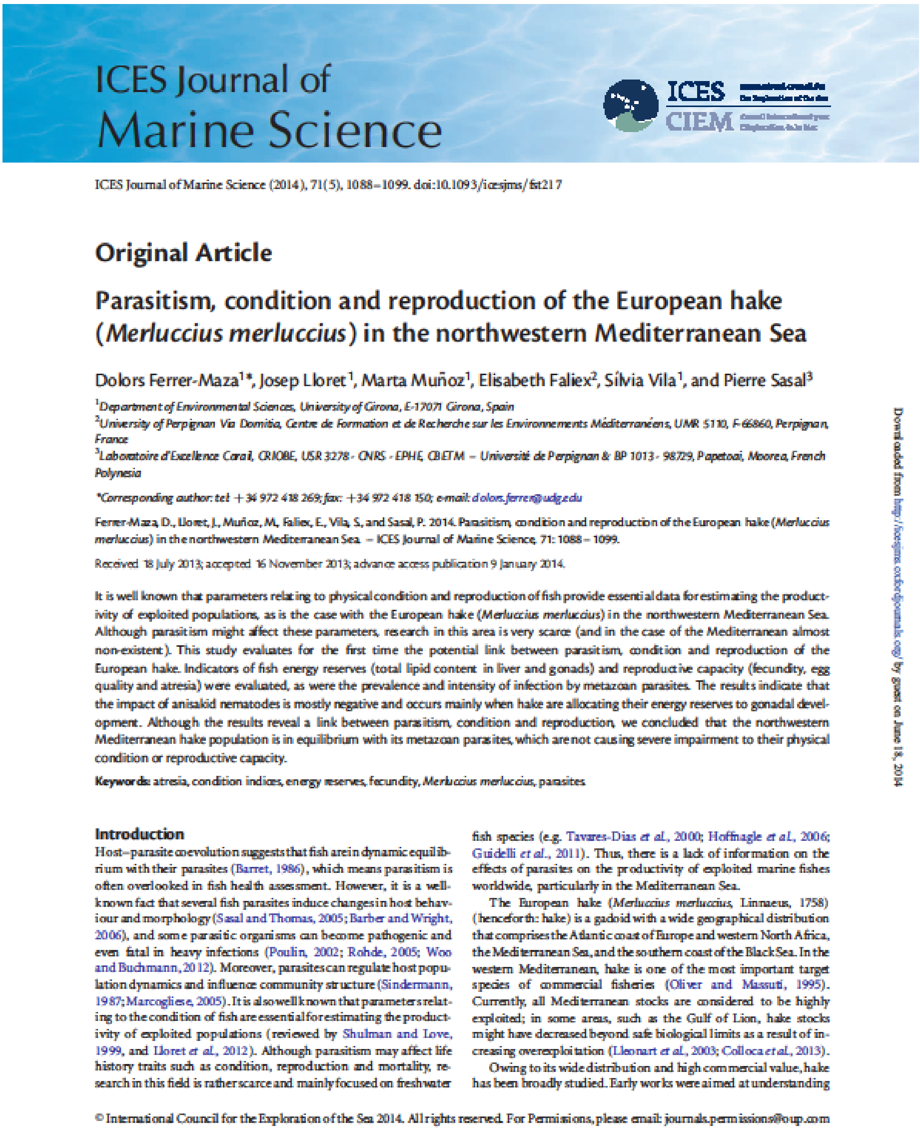
Hysterothylacium fabri (Rudolphi, 1819)
Hosts: *Merluccius merluccius, Mullus barbatus*



Spinitectus sp.
Host: *Engraulis encrasicolus*

There is no image of the two specimens of the nematode *Contracaecum* sp. found in red mullet because of a problem during preservation.

Appendix C: Front pages of the articles



ICES Journal of
Marine Science

ICES Journal of Marine Science (2014), 71(5), 1088–1099. doi:10.1093/icesjms/fst217

Original Article

Parasitism, condition and reproduction of the European hake (*Merluccius merluccius*) in the northwestern Mediterranean Sea

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Ferrer-Maza, D., Lloret, J., Muñoz, M., Faliex, E., Vila, S., and Sasal, P. 2014. Parasitism, condition and reproduction of the European hake (*Merluccius merluccius*) in the northwestern Mediterranean Sea. – ICES Journal of Marine Science, 71: 1088–1099.

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It is well known that parameters relating to physical condition and reproduction of fish provide essential data for estimating the productivity of exploited populations, as is the case with the European hake (*Merluccius merluccius*) in the northwestern Mediterranean Sea. Although parasitism might affect these parameters, research in this area is very scarce (and in the case of the Mediterranean almost non-existent). This study evaluates for the first time the potential link between parasitism, condition and reproduction of the European hake. Indicators of fish energy reserves (total lipid content in liver and gonads) and reproductive capacity (fecundity, egg quality and atresia) were evaluated, as were the prevalence and intensity of infection by metazoan parasites. The results indicate that the impact of anisakid nematodes is mostly negative and occurs mainly when hake are allocating their energy reserves to gonadal development. Although the results reveal a link between parasitism, condition and reproduction, we concluded that the northwestern Mediterranean hake population is in equilibrium with its metazoan parasites, which are not causing severe impairment to their physical condition or reproductive capacity.

Keywords: atresia, condition indices, energy reserves, fecundity, *Merluccius merluccius*, parasites.

Introduction

Host–parasite coevolution suggests that fish are in dynamic equilibrium with their parasites (Barret, 1986), which means parasitism is often overlooked in fish health assessment. However, it is a well-known fact that several fish parasites induce changes in host behaviour and morphology (Sasal and Thomas, 2005; Barber and Wright, 2006), and some parasitic organisms can become pathogenic and even fatal in heavy infections (Poulin, 2002; Rohde, 2005; Woo and Buchmann, 2012). Moreover, parasites can regulate host population dynamics and influence community structure (Sindermann, 1987; Marcogliese, 2005). It is also well known that parameters relating to the condition of fish are essential for estimating the productivity of exploited populations (reviewed by Shulman and Love, 1999, and Lloret *et al.*, 2012). Although parasitism may affect life history traits such as condition, reproduction and mortality, research in this field is rather scarce and mainly focused on freshwater fish species (e.g. Tavares-Dias *et al.*, 2000; Hoffnagle *et al.*, 2006; Guidelli *et al.*, 2011). Thus, there is a lack of information on the effects of parasites on the productivity of exploited marine fishes worldwide, particularly in the Mediterranean Sea.

The European hake (*Merluccius merluccius*, Linnaeus, 1758) (henceforth: hake) is a gadoid with a wide geographical distribution that comprises the Atlantic coast of Europe and western North Africa, the Mediterranean Sea, and the southern coast of the Black Sea. In the western Mediterranean, hake is one of the most important target species of commercial fisheries (Oliver and Massutí, 1995). Currently, all Mediterranean stocks are considered to be highly exploited; in some areas, such as the Gulf of Lion, hake stocks might have decreased beyond safe biological limits as a result of increasing overexploitation (Lleonart *et al.*, 2003; Colloca *et al.*, 2013).

Owing to its wide distribution and high commercial value, hake has been broadly studied. Early works were aimed at understanding

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PRIMARY RESEARCH PAPER

Health and reproduction of red mullet, *Mullus barbatus*, in the western Mediterranean Sea

**Dolors Ferrer-Maza · Marta Muñoz ·
Josep Lloret · Elisabeth Faliex · Sílvia Vila ·
Pierre Sasal**

Received: 20 June 2014 / Revised: 19 November 2014 / Accepted: 31 January 2015 / Published online: 7 February 2015

Abstract The reproductive and general health of exploited fish stocks is an essential element of sustainable and profitable fisheries. The main purpose of this study was to assess the relationships between reproduction and two important parameters of fish health (parasitism and energy reserves) in female specimens of red mullet, *Mullus barbatus*, from the western Mediterranean Sea. We present new data for this species on (i) the prevalence and intensity of infection by metazoan parasites; (ii) the total lipid content in muscle and gonads as a measure of condition and (iii) fecundity and egg quality as a measure of their reproductive capacity. The results show that *M. barbatus* is a batch spawner with an income breeding strategy, an asynchronous development of oocytes and indeterminate fecundity. The results also indicate that the three most abundant and prevalent parasites significantly affect the condition and reproduction of *M. barbatus*. Specifically, the digenean, *Opecoeloides furcatus*, causes a reduction in the female's energy reserves, while the nematodes, *Hysterothylacium fabri* and *H. aduncum*, produce a rise in egg production but impair egg quality. These implications of the relationships between parasitism, fish health and fish reproduction should be taken into consideration in the assessment and management of exploited species.

Keywords Egg quality · Energy reserves · Fecundity · Metazoan parasites · Reproductive strategy

Handling editor: Alison King

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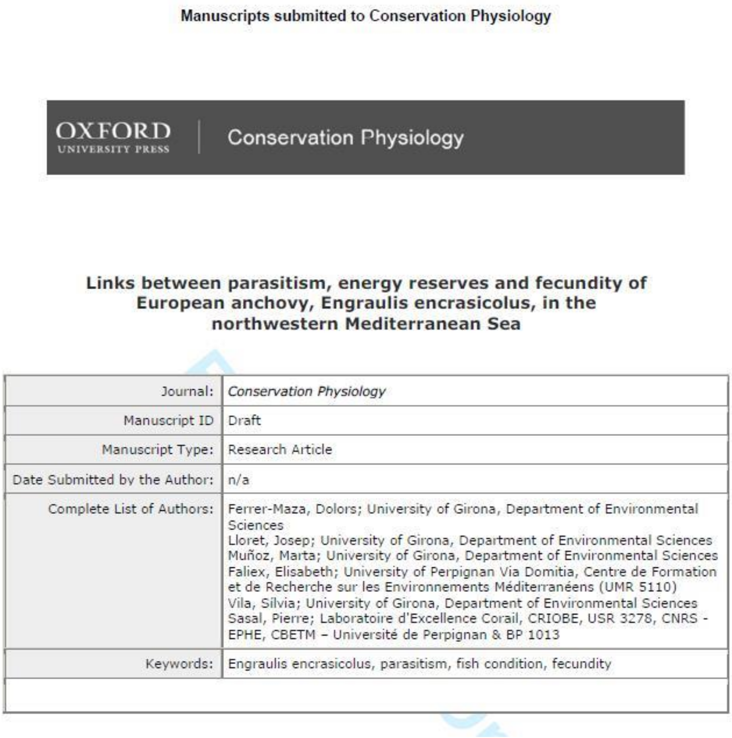
Introduction

Parasitism is usually defined as a non-mutual symbiotic relationship between species, where the parasite benefits at the expense of the host. In theory, therefore, parasitism should produce deleterious effects on fish health because the parasites drain energy from the fish that would otherwise be used to sustain the fish's condition, growth or reproduction. However, it appears that through the coevolution of host and parasite, a dynamic equilibrium has arisen between fish and their parasites (Barret, 1986). Indeed, in most cases,



Manuscripts submitted to Conservation Physiology

OXFORD UNIVERSITY PRESS | Conservation Physiology

Links between parasitism, energy reserves and fecundity of European anchovy, Engraulis encrasicolus, in the northwestern Mediterranean Sea

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Manuscript Type:	Research Article
Date Submitted by the Author:	n/a
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Keywords:	Engraulis encrasicolus, parasitism, fish condition, fecundity