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Parasite fauna of *Octopus vulgaris* (Cephalopoda: Octopodidae) and *Platichthys flesus* (Actinopterygii: Pleuronectidae): morphology, systematics, life history strategies and ecology

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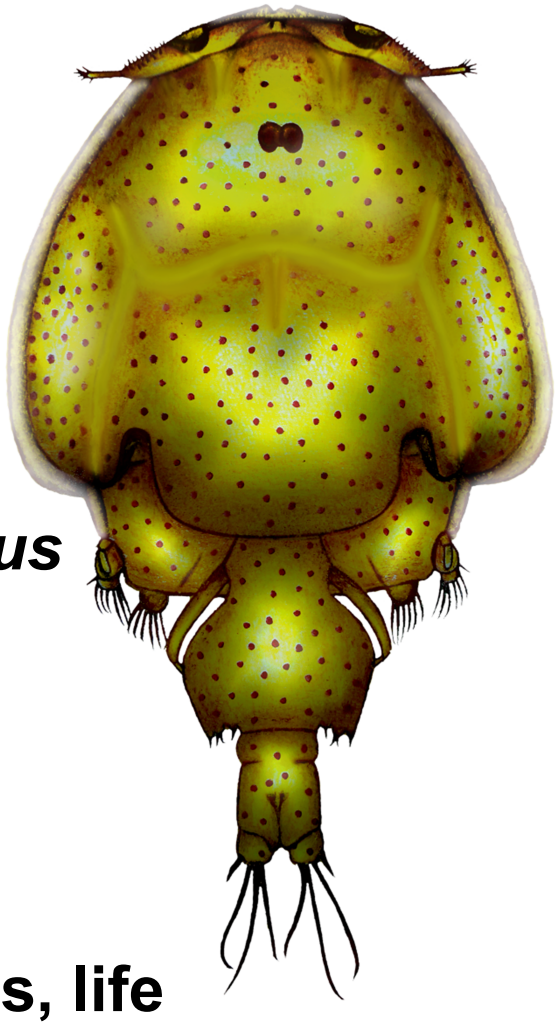

Parasite fauna of *Octopus vulgaris* (Cephalopoda: Octopodidae) and *Platichthys flesus* (Actinopterygii: Pleuronectidae): morphology, systematics, life history strategies and ecology

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This thesis is dedicated to the memory of my grandmother,

Maria Luísa Pinto Merino Nunes

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Abstract

This thesis compiles a series of papers on different aspects of the parasite fauna of an invertebrate i.e. the common octopus *Octopus vulgaris* (Cephalopoda: Octopodidae) (presently understood as a complex of species) and a vertebrate i.e. the European flounder *Platichthys flesus* (Linnaeus, 1758) (Actinopterygii: Pleuronectidae) present in Portuguese coastal waters.

Chapter 1 briefly addresses parasite diversity in morphology, systematics and life history strategies and makes a general introduction to the basic concepts and definitions in Parasite Ecology. Special emphasis is given to the proximate and ultimate causes of niche restriction in parasites, and an attempt is made to systematize the evidence on niche restriction in parasitic copepods, since the majority of the papers in this thesis respect this particular group of parasites. A few examples retrieved from studies in the literature are given. Finally, a brief introduction is made to the two hosts studied.

In chapter 2, the metazoan parasite fauna of *O. vulgaris* is characterized, for the first time, for Portuguese coastal waters. From the recorded parasitic taxa, *Octopicola superba* Humes, 1957 (Copepoda: Octopicolidae) was the only component parasite in the total sample of *O. vulgaris*. Furthermore, it was found to exhibit a marked seasonality and the recorded trend was similar to those previously reported for parasitic copepods of *P. flesus* from Portuguese waters. Also according to the evidence found, it seems likely that macroenvironmental conditions determine (at least partly) the seasonal occurrence of this and other parasitic copepods present on marine species of the Portuguese coast. The number of octopicolid copepods was significantly higher for female than for male octopuses. This, along with the fact that a significant correlation between octopus' size and parasite intensity was detected only for the female octopuses suggests a differential influence of host sex in autoinfection. The metazoan parasitic taxa so far reported for *O. vulgaris* in the studies of the literature is reviewed.

In chapter 3, the genus *Octopicola* Humes, 1957, which is exclusively found on species of octopuses, is reviewed based on the information available in the literature and morphological observations of octopicolids isolated from *O. vulgaris*. Comparative morphological analysis led to the conclusion that *Octopicola superba superba* Humes, 1957, endemic to European waters, and *O. s. antillensis* Stock, Humes & Gooding, 1963, endemic to West Indian waters, exhibit sufficient differences to be raised to

species rank. A new identification key for all the species of the genus, i.e. *O. superba* Humes, 1957, *O. antillensis* Stock, Humes & Gooding, 1963, *O. stocki* Humes, 1963 and *O. regalis* Humes, 1974, is provided.

In chapter 4, a new species of caligid copepod, *Caligus musaicus* Cavaleiro, Santos & Ho, 2010, isolated from *P. flesus*, is described. The new species is unique in that it possesses the following four character states: short abdomen; box of sternal furca carrying two parallel pointed tines; bearing a long element IV at the tip of leg 1 exopod; and a slender leg 4 exopod bearing a long outer seta at the tip of this ramus. The chosen specific name, *musaicus*, alludes to the fact that the specimens remind one of a genetic mosaic, i.e. its resemblance with several congeners.

In chapter 5, a new diplostomid metacercarial genotype isolated from the eye lenses of *P. flesus* is described. Aspects such as larval morphology, ultrastructure and morphometrics are also considered. Two distinct morphotypes, referred to as 'round' and 'long', were identified. However, these had 100% genetic homology concerning the 18S+ITS1+5.8S region of the rDNA. This was found to represent an unknown genotype, now referenced in GenBank as GQ370809. Furthermore, the molecular phylogenetic analyses, in conjunction with the principal components and cluster analyses of morphometric data indicate that the studied species of *Diplostomum* corresponds with neither *D. spathaceum* (Rudolphi, 1819) nor *D. mergi* Dubois, 1932, two species previously reported to infect *P. flesus*. The isolated marine specimens can represent a new species of *Diplostomum*, but it is more likely that they belong to a known species which has not yet been characterized in molecular terms.

In chapter 6, the trade-off between egg number and egg size is addressed for the intraspecific level of analysis, based on data recorded for adult ovigerous females of *O. superba*. The evidence found suggests that the parasite is essentially a *K*-strategist, and conforms to the general assumption that ectoparasites do not follow both an *r*- and *K*-strategy simultaneously. Furthermore, the environmental conditions seem to force them into one of the alternatives, presumably by leading to adaptive phenotypic plasticity in body dimensions and size-mediated changes in egg production. A trade-off between egg number and egg size became apparent only at high levels of fecundity, suggesting a state of physiological exhaustion.

In chapter 7, site selection is characterized in detail for *Acanthochondria cornuta* (Müller, 1776) (Copepoda: Chondracanthidae), a common parasite of *P. flesus*. A preference for the ocular side of the host's body was observed and it is speculated that this can be related with the fish's behaviour, as this fish lives partially buried in the

ocean floor. The evidence found also suggests that, as the parasite develops from one stage into another, it migrates towards different sites within the branchial chamber. This argues against the idea that the microhabitat of some parasitic copepods is determined by where infective stages settle first, i.e. that some parasitic copepods select a permanent site for living, becoming immovably fixed to it for life. The occurrence of bigamy, i.e. of bigamous females, is reported for the first time for *A. cornuta*.

In chapter 8, the occurrence of interference competition is addressed for *O. superba* and the coccidian *Aggregata* sp. (Apicomplexa: Aggregatidae), two parasites that occur at the gills of wild *O. vulgaris*. Both numerical and functional responses are analysed and both the fundamental and realized spatial niches are measured. According to the results found, the gills constitute the main and accessory site of infection of *O. superba* and *Aggregata* sp., respectively, and were simultaneously infected with the two parasites in 11 (9.2%) of the examined octopuses. While the presence of *O. superba* on gill lamellae appears to be negatively affected by the presence of *Aggregata* sp., the latter does not seem to be affected by the former.

Finally, chapter 9 presents some concluding remarks on the parasites studied. A comparative analysis of the parasite fauna recorded for the studied hosts is performed. Future lines of investigation are delineated.

Keywords: Metazoan parasites of *Octopus vulgaris*; review of *Octopicola* (Copepoda: Octopicolidae); *Caligus musaicus* sp. nov. (Copepoda: Caligidae); metacercariae of *Diplostomum* sp. from *Platichthys flesus*; trade-off between egg number and egg size; site selection of *Acanthochondria cornuta* (Copepoda: Chondracanthidae); interference competition between *Octopicola superba* (Copepoda: Octopicolidae) and *Aggregata* sp. (Apicomplexa: Aggregatidae); parasitological survey

Resumo

A presente tese compila uma série de artigos relacionados com diferentes aspetos da parasitofauna de um invertebrado i.e. o polvo comum *Octopus vulgaris* (Cephalopoda: Octopodidae) (atualmente entendido como um complexo de espécies) e de um vertebrado i.e. a solha Europeia *Platichthys flesus* (Linnaeus, 1758) (Actinopterygii: Pleuronectidae) presentes em águas costeiras Portuguesas.

O capítulo 1 considera, de forma abreviada, a diversidade morfológica dos parasitas e sua sistemática e estratégias de vida, e faz uma introdução geral aos conceitos e definições básicas em Ecologia Parasitária. É dado especial ênfase às causas próximas e últimas da restrição de nichos em parasitas, e é feito um esforço no sentido de sistematizar a evidência relativa à restrição de nichos em copépodes parasitas, dado que a maioria dos artigos apresentados nesta tese respeita este grupo particular de parasitas. São mencionados alguns exemplos encontrados nos estudos da literatura. Finalmente, é feita uma breve introdução aos dois hospedeiros estudados.

No capítulo 2, carateriza-se, pela primeira vez, a fauna de parasitas metazoários de *O. vulgaris* de águas costeiras Portuguesas. Dos taxa parasitas registados, *Octopicola superba* Humes, 1957 (Copepoda: Octopicolidae) foi o único parasita componente na amostra total de *O. vulgaris*. Adicionalmente, este parasita exibiu uma sazonalidade marcada e a tendência registada foi semelhante às anteriormente reportadas para copépodes parasitas de *P. flesus* de águas Portuguesas. De acordo ainda com a evidência encontrada, parece provável que as condições macroambientais determinem (pelo menos parcialmente) a ocorrência sazonal deste e de outros copépodes parasitas presentes em espécies marinhas da costa Portuguesa. O número de copépodes octopicolídios foi significativamente mais elevado em polvos do sexo feminino do que em polvos do sexo masculino. Isto, aliado ao fato de uma correlação significativa entre o tamanho do polvo e a intensidade parasitária ter sido detetada apenas para os polvos do sexo feminino sugere uma influência diferencial do sexo do hospedeiro na auto-infeção. É feita uma revisão dos taxa de parasitas metazoários reportados até à data para *O. vulgaris* nos estudos da literatura.

No capítulo 3, o género *Octopicola* Humes, 1957, que é exclusivamente encontrado em espécies de polvos, é revisto com base na informação disponível na

literatura e em observações morfológicas de octopicolídios isolados de *O. vulgaris*. A análise morfológica comparativa levou à conclusão de que *Octopicola superba superba* Humes, 1957, endémica de águas Europeias, e *O. s. antillensis* Stock, Humes & Gooding, 1963, endémica de águas das Índias Ocidentais, exibem diferenças suficientes para serem elevadas à categoria de espécie. É disponibilizada uma nova chave de identificação para todas as espécies do género, i.e. *O. superba* Humes, 1957, *O. antillensis* Stock, Humes & Gooding, 1963, *O. stocki* Humes, 1963 e *O. regalis* Humes, 1974.

No capítulo 4, é descrita uma nova espécie de copépode caligídio, *Caligus musaicus* Cavaleiro, Santos & Ho, 2010, isolada de *P. flesus*. Esta nova espécie distingue-se das demais por possuir as seguintes quatro caraterísticas: abdómen curto; caixa da furca esternal com duas hastes pontiagudas e paralelas; armada com um elemento IV longo na extremidade do exopodito da pata 1; e exopodito da pata 4 delgado, armado com uma cerda exterior longa na sua extremidade. O restritivo específico escolhido, *musaicus*, alude ao fato de que os espécimes fazem lembrar um mosaico genético, i.e. à semelhança da espécie relativamente a vários dos seus congéneres.

No capítulo 5, é descrito um novo genótipo de metacercárias de diplostomídio isolado da lente dos olhos de *P. flesus*. São considerados ainda aspetos como a morfologia, ultraestrutura e morfometria larvar. Foram identificados dois morfotipos distintos, referidos como ‘redondo’ e ‘longo’. Contudo, demonstrou-se que estes apresentavam 100% de homologia genética no que concerne a região 18S+ITS1+5.8S do rDNA. Descobriu-se, ainda, que esta última representava um genótipo desconhecido, agora referenciado no GenBank como GQ370809. Além disso, as análises filogenéticas moleculares, em conjugação com as análises de componentes principais e de *clusters* de dados morfométricos indicam que a espécie de *Diplostomum* estudada não corresponde nem a *D. spathaceum* (Rudolphi, 1819) nem a *D. mergi* Dubois, 1932, duas espécies que foram anteriormente reportadas para *P. flesus*. Os espécimes marinhos isolados podem representar uma nova espécie de *Diplostomum*, sendo contudo mais provável que eles pertençam a uma espécie conhecida que não foi ainda caraterizada em termos moleculares.

No capítulo 6, é considerado o *trade-off* entre o número e o tamanho dos ovos ao nível intraespecífico de análise, tendo por base dados registados para fêmeas adultas ovígeras de *O. superba*. A evidência encontrada sugere que o parasita é, essencialmente, um estrategista *K*, e está de acordo com a suposição geral de que os

ectoparasitas não seguem, simultaneamente, as estratégias *r* e *K*. Além disso, ela sugere ainda que as condições ambientais influenciam a estratégia escolhida, na medida em que são presumivelmente responsáveis por plasticidade fenotípica adaptativa ao nível das dimensões do corpo e por mudanças na produção ovígera mediadas pelas mudanças no tamanho corporal. Um *trade-off* entre o número e o tamanho dos ovos foi observado apenas a elevados níveis de fecundidade, o que sugere um estado de exaustão fisiológica.

No capítulo 7, carateriza-se, em detalhe, a seleção de sítio para *Acanthochondria cornuta* (Müller, 1776) (Copepoda: Chondracanthidae), um parasita vulgar de *P. flesus*. Foi observada uma preferência pelo lado ocular do corpo do hospedeiro, especulando-se que esta poderá estar relacionada com o comportamento do peixe, já que este vive parcialmente enterrado no fundo oceânico. A evidência encontrada sugere ainda que, à medida que o parasita se desenvolve de estádio em estádio, ele migra para diferentes sítios da cavidade branquial. Esta observação está em desacordo com a ideia de que o microhabitat de alguns copépodes parasitas corresponde ao local onde os estádios infeciosos se estabeleceram, i.e. de que alguns copépodes parasitas selecionam um sítio permanente para viver, fixando-se a ele para toda a vida. A ocorrência de bigamia, i.e. de fêmeas bígamas, é reportada pela primeira vez para *A. cornuta*.

No capítulo 8, é considerada a ocorrência de competição por interferência entre *O. superba* e o coccídio *Aggregata* sp. (Apicomplexa: Aggregatidae), dois parasitas que ocorrem nas brânquias de *O. vulgaris* de meio natural. São consideradas para análise as respostas numéricas e funcionais, e são medidos os nichos fundamental espacial e realizado espacial. De acordo com os resultados obtidos, as brânquias constituem, respetivamente, o sítio principal e acessório de infeção de *O. superba* e *Aggregata* sp., tendo sido encontradas simultaneamente infetadas pelos dois parasitas em 11 (9.2%) dos polvos examinados. Enquanto a presença de *O. superba* nas lamelas branquiais parece ser negativamente afetada pela presença de *Aggregata* sp., a última não parece ser afetada pela primeira.

Finalmente, o capítulo 9 apresenta algumas observações finais acerca dos parasitas estudados. É feita uma análise comparativa da fauna parasitária registada para os hospedeiros estudados. Linhas de investigação futura são delineadas.

Palavras-chave: Parasitas metazoários de *Octopus vulgaris*; revisão de *Octopicola* (Copepoda: Octopicolidae); *Caligus musaicus* sp. nov. (Copepoda: Caligidae); metacercárias de *Diplostomum* sp. de *Platichthys flesus*; *trade-off* entre o número e o tamanho dos ovos; seleção de sítio por *Acanthochondria cornuta* (Copepoda: Chondracanthidae); competição por interferência entre *Octopicola superba* (Copepoda: Octopicolidae) e *Aggregata* sp. (Apicomplexa: Aggregatidae); exame parasitológico

Scientific Papers

This thesis includes seven scientific papers, published, in publication or in review for publication in international journals (ISI) and concerning part of the results obtained during the experimental work.

1) **Cavaleiro, F. I.**, & Santos, M. J. (In Review for Publication). Helminth and copepod parasites of the common octopus, *Octopus vulgaris* (Cephalopoda: Octopodidae), in northwest Portuguese waters, Atlantic Ocean. *Journal of Parasitology*.

2) **Cavaleiro, F. I.**, Ho, J.-S., Iglesias, R., García-Estévez, J. M., & Santos, M. J. (2013). Revisiting the octopicolid copepods (Octopicolidae: *Octopicola* Humes, 1957): comparative morphology and an updated key to species. *Systematic Parasitology*, *86*, 77–86.

3) **Cavaleiro, F. I.**, Santos, M. J., & Ho, J.-S. (2010). *Caligus musaicus* n. sp. (Copepoda, Caligidae) parasitic on the European flounder, *Platichthys flesus* (Linnaeus) off Portugal. *Crustaceana*, *83*, 457–464.

4) **Cavaleiro, F. I.**, Pina, S., Russell-Pinto, F., Rodrigues, P., Formigo, N. E., Gibson, D. I., & Santos, M. J. (2012). Morphology, ultrastructure, genetics, and morphometrics of *Diplostomum* sp. (Digenea: Diplostomidae) metacercariae infecting the European flounder, *Platichthys flesus* (L.) (Teleostei: Pleuronectidae), off the northwest coast of Portugal. *Parasitology Research*, *110*, 81–93.

5) **Cavaleiro, F. I.**, & Santos, M. J. (In Press). Egg number-egg size: an important trade-off in parasite life history strategies. *International Journal for Parasitology*.

6) **Cavaleiro, F. I.**, & Santos, M. J. (2011). Site selection of *Acanthochondria cornuta* (Copepoda: Chondracanthidae) in *Platichthys flesus* (Teleostei: Pleuronectidae). *Parasitology*, *138*, 1061–1067.

7) **Cavaleiro, F. I.**, & Santos, M. J. (In Press). Numerical and functional responses to the presence of a competitor – the case of *Aggregata* sp. (Apicomplexa: Aggregatidae) and *Octopicola superba* (Copepoda: Octopicolidae). *Parasitology*.

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Abbreviations

A	Adult
B	*Levins' measure of niche breadth*
B_A	*Standardized Levins' measure of niche breadth*
BG	Branchial Gland
BL	Length of the Body
BLAST	Basic Local Alignment Search Tool
BS	Body Skin
BW	Width of the Body
CI	Confidence Interval
CMG	Covering Mesentery of Gonad
CO	COpepodite
CR	CRop
CT	Connective Tissue around the digestive gland
CV	Coefficient of Variation
DF	Degrees of Freedom
DFA	Discriminant Function Analysis
DR	Distal Region
DT	Digestive Tract
EG	EGgs
EY	EYes
F	Funnel
FSN	Fundamental Spatial Niche
G	Gills
GLA	Gill LAmellae
GLI	Gill LIgament
GLM	General Linear Model
HIF	Holobranch I Filaments
HIIF	Holobranch II Filaments

HIIIF Holobranch III Filaments

HIVF Holobranch IV Filaments

HL Length of the Holdfast organ

HW Width of the Holdfast organ

I Intestine

IWC Internal Wall of the Chamber

L Larva

LG Left Gill

LL Length of the Lappets

MC Mantle Cavity

MFA Multiple Factorial Analysis

MM internal surface of the Mantle Musculature

MR Middle Region

NA North America

NJ Neighbour-Joining

OE OEsophagus

OL Length of the Oral sucker

OW Width of the Oral sucker

P *Renkonen's index*

PCA Principal Component Analysis

PCR Polymerase Chain Reaction

PF Pseudobranch Filaments

PHL Length of the PHarynx

PHW Width of the PHarynx

Pol Poland

PR Proximal Region

RG Right Gill

RI Range Interval

RSN Realized Spatial Niche

S Stomach

S_b	*Bootstrap estimator of taxa richness*
SD	Standard Deviation
SE	Standard Error
SEM	Scanning Electron Microscopy
UK	United Kingdom
VD	Distance between the anterior extremity of the body and the center of the Ventral sucker
VL	Length of the Ventral sucker
VW	Width of the Ventral sucker
WaBI	Width of the body at the level of the Bifurcation of the Intestine
WaO	Width of the body at the mid-length of the Oral sucker

Chapter 1

General Introduction

"*The reality is that parasites are among the most diverse of all organisms. It could even be argued that the main purpose for preserving free-living organisms is to protect their parasites.*"

Windsor, 1995

1.1. Parasites: Diversity in Morphology, Systematics and Life History Strategies

Parasites present very different body shapes, some of which are truly bizarre! The diversity in morphology is, indeed, astounding, and reflects the wide spectrum of environments that parasites colonized during the course of evolution. Actually, parasites are present in all ecosystems on Earth, including most extreme environments, such as polar regions and abyssal depths. Furthermore, they infect all living organisms, from the simplest to the most complex, including all animal phyla. The fact that they have different life history strategies indicates that they are found on or in every different site of the body of their hosts. Ideally, different types of data (i.e., morphological, ultrastructural, genetic and morphometric) should be assembled, to characterize them fully.

1.2. Parasite Ecology: A General Overview

1.2.1. Scope, Relevance and Key Study Issues

All living organisms interact with their biotic and abiotic environment. However, their interaction varies according to species and many different factors involved. Parasites represent no exception to these general principles. However, there is a structural difference between their environment and the environment of free-living organisms, so that their ecology must be addressed from a different perspective. More specifically, the environment of parasites is unique in including two components, namely the macroenvironment, represented by the environment of the host, and the microenvironment, represented by the host (sensu Rohde, 1984). It is essentially for this reason that Parasite Ecology represents a distinct field of study. Basic concepts and definitions for this subject have been treated by Rohde (1993, 1994), Bush et al. (1997) and Poulin (2007a).

Parasite Ecology is, therefore, concerned with the interactions that parasites maintain with the biotic and abiotic components of their macro- and microenvironments. Studies are usually complex and challenging, mainly because the whole network of interactions is intricate, with factors of different nature and at different levels affecting

parasites in very different ways. Nonetheless, their number has increased exponentially in the past few years.

Several reasons justify the increasing interest in Parasite Ecology. One of them is intimately linked with the idea of parasitism as a successful lifestyle on Earth (see Windsor, 1998). Though we usually do not think of parasites as major components of biodiversity (Dobson et al., 2008), the fact is that they are cosmopolitan, as evident from the critical analysis of the literature. The variety of morphology, host associations and life strategies is staggering, reflecting the success of parasitism as a lifestyle. Furthermore, while representing the majority of species on Earth (Windsor, 1998), parasites are of great biological relevance and the study of their ecology will undoubtedly help us better understand life. Another important reason which justifies the current interest in Parasite Ecology respects the fact that a sound body of knowledge on the way in which parasites interact with their environment is necessary to define effective control and management methods in aquaculture systems, where they can cause pathological changes and a decrease in host fitness (Scholz, 1999) and lead, therefore, to significant economic losses. This aspect is particularly important nowadays since aquaculture production is increasing worldwide, representing an important source of food with high protein content.

The need for a more mechanistic understanding of some aspects of Parasite Ecology is eminent. Nonetheless, an excellent source of information has become available in the published literature. Some of the numerous key study issues are: the general laws in parasite and community ecology (Guégan et al., 2005; Poulin, 2007b); the evolution of parasite and host life history traits (e.g. Poulin, 1995a; Débarre et al., 2012); the parasite-host coevolution (e.g. May & Anderson, 1990); the nestedness in assemblages of parasites (e.g. Rohde et al., 1998); the patterns in parasite community structure and the processes operating at different spatial and temporal scales (e.g. Poulin, 1997a; Vidal-Martínez & Poulin, 2003); the competition between parasites (e.g. Dobson, 1985); the adaptations of parasites to within-host competition (e.g. Mideo, 2009); the niche restriction in parasites (Rohde, 1994); the diversity and evolution of manipulative strategies in host-parasite interactions (e.g. Lefèvre et al., 2009); the transmission of parasites and the host finding, recognition and invasion (e.g. Rea & Irwin, 1994; Haas, 2003); the biogeographic patterns and processes (e.g. Poulin et al., 2011); the occurrence of parasites in food webs (e.g. Sukhdeo, 2012); and the usefulness of parasites as bioindicators of ecosystem health i.e. environmental pollution (e.g. Vidal-Martínez et al., 2009) and climate change (e.g. Pickles et al., 2013).

Basic concepts and definitions in Parasite Ecology will be addressed in the following section. Since this thesis is focused on host-parasite systems found in the marine environment, the examples given will be confined exclusively to this environment.

1.2.2. Basic Concepts and Definitions

- The structural architecture of the parasite's environment

As stated before, the environment of a parasite presents a very unique structural architecture, including two distinct but interrelated components at different spatial scales, namely the macro- and microenvironments.

The macroenvironment is represented by a particular set of biological (i.e. species) and physicochemical (e.g. temperature, photoperiod, salinity and pH) factors. These can affect parasites directly and/or indirectly, i.e. through the host (Rohde, 1984), and in very different ways. Actually, the overall effect of macroenvironment on parasites is frequently difficult to characterize, owing to the large number of factors involved and the difficulty to measure some of them with accuracy. Furthermore, the study design is crucial when attempting to ascertain exactly how the macroenvironment is affecting parasites, and must take into account all key variables. The effect of parasites on their macroenvironment is negligible owing to their small size and the barrier represented by the host (in the case of endoparasites) (Rohde, 1984). However, the network of interactions is made more complex by the microenvironment, i.e. the host individual, which, in itself, also represents a huge source of variability (with different factors, genetically determined or not, involved). Furthermore, parasites can affect their microenvironment both mechanically and chemically, and in many different ways, depending on the species involved.

- The ecological niche: concept, types and causes of restriction

From the above considerations it is possible to conclude that parasites are simultaneously affected by a combination of macro- and microenvironmental factors. In the late 50's of the past century, Hutchinson (1957) established the concept of 'ecological niche' to refer the 'multidimensional hypervolume' determined by a set of

biotic and abiotic factors within which a species can exist. This has become a key concept in Parasite Ecology.

Depending on its origin, two types of ecological niche can be distinguished, namely the fundamental niche and the realized niche (see Severtsov, 2013). The fundamental niche is formed as a result of evolution and consists of all environmental conditions (biotic and abiotic) in which a species can live and reproduce. As for the realized niche, it consists of the subset of environmental conditions (again, biotic and abiotic) that a species actually exploits as a result of the interactions that it maintains with other species. Measures that can be used to characterize niche width include the Levin's measure of niche width (*B*), the Shannon-Wiener measure (*H'*) and the Smith's measure (*FT*) (Krebs, 1989).

There is no universal parasite, i.e. a parasite capable of infecting all tissues of all free-living species of all geographical regions of the world. In other words, niche restriction is universal among parasites. Its causes have been discussed in different works (see e.g. Rohde, 1993, 1994; Rohde & Rohde, 2005). As a rule, two general types are recognised, namely the proximate and ultimate causes. Proximate causes of niche restriction respect the causal factors that determine the species' niche, whereas ultimate causes respect all those factors that are somehow related with the biological function of the niche (Rohde & Rohde, 2005), i.e. the selection pressures leading to niche restriction. The latter are particularly difficult to address, since they cannot be demonstrated based on evidence for short ecological time-scales.

According to Rohde (1979), the number of morphological and biological aspects that can be understood as niche dimensions is almost infinite. Nonetheless, many such aspects overlap, so that it is reasonable to assume that the 'niche volume' of a parasite species can be characterized to a high degree of accuracy by considering a few dimensions only. These dimensions are regarded as the proximate causes of niche restriction. They are: host species; geographical range and macrohabitat; microhabitat(s) on or in the host; host sex and age; season of the year; food; and hyperparasites (Rohde, 1994). It has been argued that some dimensions are difficult to characterize, e.g. the exact type of food particles ingested by parasites, and that, for this reason, parasitologists decided to focus their attention on the spatial dimension of the niche (Poulin, 2007a). As for the ultimate causes of niche restriction, they include aspects such as the saturation of niches with parasite species and individuals, the avoidance of interspecific competition, the avoidance of predators, the avoidance of hyperparasites, the facilitation of mating, the reinforcement of reproductive barriers and

the adaptations to environmental complexity. It must be emphasised here that niches are dynamic, in the sense that they can be affected by a number of factors at the parasite and host levels (Rohde, 1994).

1.2.3. The Case of the Parasitic Copepods

Copepods are cosmopolitan inhabitants of the aquatic environment, being usually extremely abundant in terms of absolute numbers of individuals (Kearn, 2004). About half of the known species developed symbiotic relationships with organisms from other phyla (Huys & Boxshall, 1991; see also Boxshall & Halsey, 2004). Actually, the hosts of parasitic copepods include species from virtually all animal phyla, i.e. from sponges to vertebrates. The morphological diversity is staggering, the species in some groups being more profoundly modified than those in others (Fig. 1.1).

Fig. 1.1 – A few examples of the morphological variability found in some families of parasitic copepods. A, Octopicolidae; B, Chondracanthidae (*arrow*, male); C, Pennellidae; D, Lernaeopodidae; and E, Hatschekiidae. *Scale-bars*: A, 500 µm; B, 1.0 mm; C, 1.0 mm; D, 1.0 mm; and E, 15.0 mm.

In families such as Chondracanthidae, the males are parasites of females and incomparably smaller than them (Fig. 1.1 B), being often referred to as dwarfs in the literature.

Despite the remarkable morphological variability, all parasitic copepods present a body divided into two tagmata, i.e. an anterior prosome and a posterior urosome, with an articulation between the fourth and fifth pedigerous somites (podoplean plan) (Boxshall, 2005). Three types of parasites are recognised, namely the ectoparasites, the mesoparasites and the endoparasites. The overwhelming majority of species falls within the first type and infects external regions of the host's body (as opposed to endoparasites, which are found inside the host's body). It is a common assumption that ectoparasitic copepods may retain, or not, the freedom of their movements over the surface of their hosts (Kabata, 1981). As for the mesoparasites, they live partly embedded in the host. More specifically, in this type of parasites, the anterior end forms an anchor process which allows them to penetrate deeply into the host's tissues, while a large part of their bodies protrudes from the host and remains exposed to the external environment (Kabata, 1979, 1981; Boxshall & Halsey, 2004).

Parasitic copepods were reported to infect a wide spectrum of microhabitats (e.g. body skin, fins, nostrils, buccal and branchial cavities, eyes, mucous canals of the mandibular and preopercular areas, cephalic canal system adjacent to the nasal cavity and urinary bladder) on or in their hosts (as ecto-, meso- and endoparasites) and appear, therefore, particularly suited for addressing different aspects of Parasite Ecology. It must be emphasised that a correct identification to species is crucial, as two morphologically similar species can exhibit significant biological differences (e.g. Kabata, 1973) and, therefore, ecological differences. Proximate and ultimate causes of niche restriction in parasitic copepods are discussed in the literature. A few examples are given below, since the majority of the papers in this thesis are dealing with these parasites.

Proximate causes of niche restriction

Host species

Host specificity (sensu Rohde, 1984) varies according to parasite species and parasitic copepods represent no exception to this general principle. Physicochemical and

morphological factors seem to be particularly important in determining niche restriction in these parasites. More specifically, physicochemical factors seem to be capable of attracting larvae and ensuring the settlement on the right host. For instance, two strains of *Lepeophtheirus pectoralis* (Müller, 1777) (Copepoda: Caligidae) occurring on two closely related species of flatfish i.e. the European flounder *Platichthys flesus* (Linnaeus, 1758) (Actinopterygii: Pleuronectidae) and the plaice *Pleuronectes platessa* Linnaeus, 1758 (Actinopterygii: Pleuronectidae) (named *flesi* and *platessae*) were shown to respond to water currents, while chemical factors produced by the host are likely involved in the settlement of copepodites on the right host (see Boxshall, 1976). As for morphological factors, particular features of the attachment apparatus (among other) can be highly adapted to particular sites on or in given host species. Furthermore, the difference in activity between marine invertebrates and vertebrates may have led to the development of a more robust type of antenna, capable to secure fastening of parasites to their hosts, during switching events from invertebrate to vertebrate hosts (see e.g. Ho, 1984).

Geographical range and macrohabitat

The geographical range of a species relates to the latitudinal gradient in its occurrence and abundance. Distribution of marine parasites appears to be mainly determined by temperature (Rohde, 1994), and parasitic copepods should not represent an exception since this macroenvironmental factor has been recognised to influence development and growth of parasitic copepods in general (Kabata, 1981). However, the data currently available for parasitic copepods are still not enough to allow the establishment of a conclusion on this subject. As for the macrohabitat, i.e. the fraction of the host habitat in which the parasite is found (sensu Rohde & Rohde, 2005), the salinity appears to be a particularly relevant macroenvironmental factor affecting that of parasitic copepods. For instance, *L. pectoralis* and *Acanthochondria cornuta* (Müller, 1776) (Copepoda: Chondracanthidae) cannot develop within low salinity ranges, i.e. 7-20‰ salinity (Möller, 1978), while their host, i.e. *P. flesus*, migrates regularly between different salinity environments i.e. estuarine/brackish water environments and coastal sea areas (Nikolsky, 1961; Berg, 1962). Actually, none of those parasite species was found by Chibani & Rokicki (2004) in *P. flesus* caught in the Baltic Sea, the world's largest brackish water sea area (Leppäkoski et al., 2002).

Microhabitat(s) on or in the host

The microhabitat of a parasite corresponds to the site of infection (sensu Bush et al., 1997) on or in the host's body. Each host species exhibits a variety of microhabitats, and in some cases, a variety of very unique microhabitats. As such, hosts can be considered as an array of very different stimuli. Naturally, the challenges to survival that parasite species face should vary greatly according to microhabitat, and this probably led to specific morphological and physiological adaptations during the course of their evolution. The review of the published information reveals that the factors determining the selection of a given site by a particular parasitic copepod are mostly unknown. Despite the scarcity of information, the site of infection was suggested to have an effect on the reproductive success of the parasite (e.g. Timi et al., 2010). Furthermore, it may change during the course of the parasite's life-cycle, as reported, for instance, for *L. pectoralis* (Scott, 1901; Boxshall, 1974a). Notably, egg-producing females of this species exhibit a clear preference for the pectoral fins and are further remarkable in that they typically aggregate in close ranks (Kabata, 1979). Mated females of other groups of parasitic copepods are also remarkable for their well-defined microhabitat choice. For instance, mated female pennellids normally choose microhabitats where they have easy access to virtually unlimited blood, namely the eyes and gills (see e.g. Anstensrud & Schram, 1988; Blaylock et al., 2005) (Fig. 1.2).

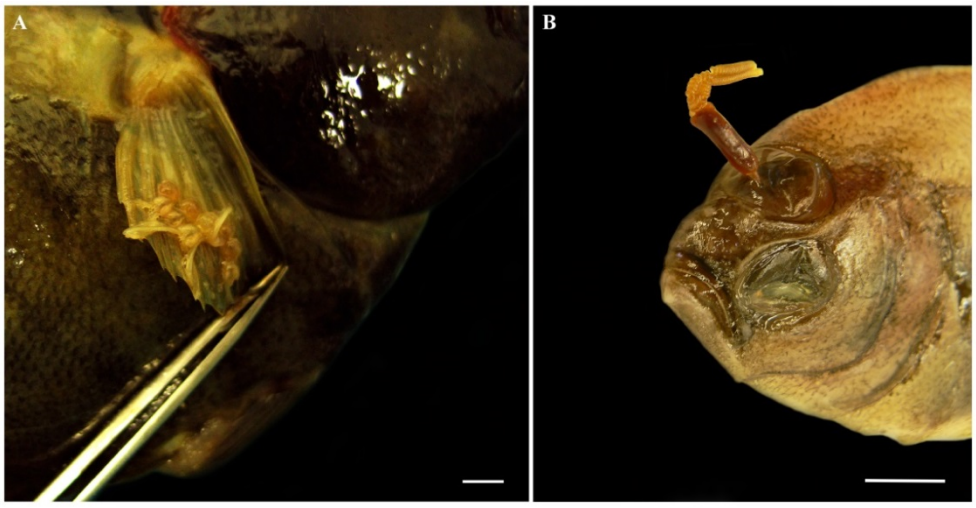


Fig. 1.2 – A, Ovigerous females of *Lepeophtheirus pectoralis* (Müller, 1777) (Copepoda: Caligidae) attached to the pectoral fin of the European flounder, *Platichthys flesus* (Linnaeus, 1758) (Actinopterygii: Pleuronectidae); and B, An ovigerous female of *Phrixocephalus cincinnatus* Wilson, 1908 (Copepoda: Pennellidae) attached to the eye of the Pacific sanddab, *Citharichthys sordidus* (Girard, 1854) (Actinopterygii: Paralichthyidae). *Scale-bars*: A, 5.0 mm; and B, 15.0 mm.

Host sex and age

It also may happen that parasites give preference to hosts of a given sex (Rohde, 1994), although the critical analysis of the literature suggests that differences of infection levels between the two sexes of a host species are not common when the infecting species is a parasitic copepod. Such differences may be due to different factors, including gender-related differences in the composition of the skin resulting in differential attraction of ectoparasites and differences in behaviour between female and male hosts. As a result of the ontogenetic changes, hosts can be thought of as a fluctuating microenvironment. Accordingly, the age of the host may represent an important proximate cause of niche restriction as well. Concerning the parasitic copepods, they may prefer to infect hosts of a certain age (Kabata, 1981), and such a preference has already been evaluated for particular species, using laboratory experiments. For instance, Anstensrud & Schram (1988) found that copepodites of *Lernaeenicus sprattae* (Sowerby, 1806) (Copepoda: Pennellidae) do not exhibit a preference for particular size groups of sprat, *Sprattus sprattus* (Linnaeus, 1758) (Actinopterygii: Clupeidae). An influence of host ontogeny on parasite's site selection was, however, suggested for fish naturally infected with parasitic copepods. For example, a displacement of *Lernanthropus cynoscicola* Timi & Etchegoin, 1996 (Copepoda: Lernanthropidae) over the gill arches of *Cynoscion guatucupa* (Cuvier, 1830) (Actinopterygii: Sciaenidae) as well as differential preferences for certain sections of the gills were observed for parasites of both sexes and suggested to be associated with the host's increasing size (understood as an indication of age) (Timi, 2003).

Season of the year

As stated before, temperature has been recognised to influence development and growth of parasitic copepods in general (Kabata, 1981). This parameter usually varies from season to season (and, more markedly, at high latitudes), being likely that the warmer seasons are more favourable for the occurrence of parasitic copepods. The season of the year should represent, therefore, an important dimension of the niche of parasitic copepods. It may further affect their occurrence by leading to changes in reproductive behaviour. For instance, Ritchie et al. (1993) reported seasonal differences in the reproductive output of winter and summer generations of females of *Lepeophtheirus salmonis* (Krøyer, 1837) (Copepoda: Caligidae). More specifically, the winter females were found to produce significantly longer egg sacs and a greater

number of smaller eggs compared with the summer females. Ritchie et al. (1993) attributed the differences in reproductive output to macroenvironmental factors such as temperature and photoperiod.

Food

Parasitic copepods can feed on host's mucus, tissues and blood (Johnson et al., 2004). More specifically, while some parasites appear to be less specific with respect to their diet – e.g. caligids can feed on those three items (Kabata, 1974; Brandal et al., 1976) – , others are definitely more restrictive – e.g. adult female pennellids usually feed on blood and lymph of their host fish (Lester & Hayward, 2006). Accordingly, in many cases, the type of food available at a particular site on or in the host's body should represent an important proximate cause of niche restriction.

Hyperparasites

The term 'hyperparasite' is used to refer any parasite of a parasite (Rohde, 2005). One of the known cases of hyperparasitism is the occurrence of udonellids on copepods parasitic on fish (Fig. 1.3). For many years, nothing was known about 'host' finding by udonellid hyperparasites. However, a recent study suggested that copepod mating represents the main route for dispersal of these hyperparasites in the 'host' population of parasitic copepods, while the contact between copepods of the same sex appears to be less important (Marin et al., 2007). It is therefore likely, that a specific chemical factor is one of the causal factors which determine the hyperparasite's niche.

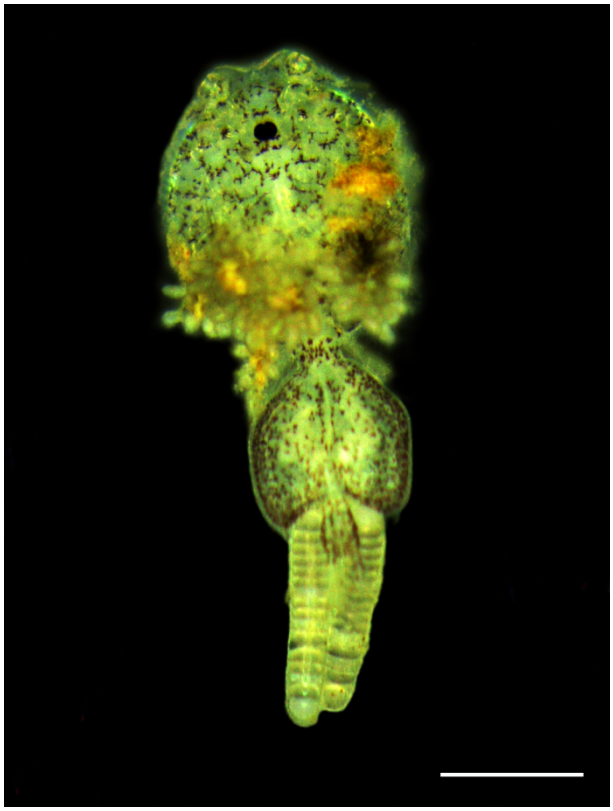


Fig. 1.3 – A case of hyperparasitism involving a parasitic copepod, with numerous eggs of *Udonella* sp. (Platyhelminthes: Monogenea) seen on the cephalothorax of an ovigerous female of *Caligus* sp. (Copepoda: Caligidae). *Scale-bar*: 1.0 mm.

Ultimate causes of niche restriction

Some of these causes are considered to be more important than others. More specifically, the common assumption is that interspecific competition is a less important ultimate cause of niche restriction. On the other hand, restriction of niches to facilitate mating and segregation of niches to avoid interspecific hybridization are, presumably, more important (e.g. Rohde, 1979, 1980).

Saturation of niches with parasite species and individuals

It is recognised that saturation of niches with parasite species and individuals results in interspecific competition. However, it is difficult to evaluate whether this sort of competition represents a relevant evolutionary/ecological agent, i.e. whether ecological niches were ‘shaped’ during the course of evolutionary time, as a result of particular interspecific competition events. Evolution of restricted niches in parasitic copepods has not been addressed frequently in the literature. One interesting case respects the

asymmetrical competition between *Lepeophtheirus thompsoni* Baird, 1850 (a specialist species) and *L. europaensis* Zeddam, Berrebi, Renaud, Raibaut & Gabrion, 1988 (a generalist species) (Copepoda: Caligidae), demonstrated experimentally by Dawson et al. (2000). These two parasites are naturally found on their sympatric hosts, i.e. the former on turbot *Scophthalmus maximus* (Linnaeus, 1758) and the latter on brill *S. rhombus* (Linnaeus, 1758) (Actinopterygii: Scophthalmidae). The experiments showed that the two parasites are able to meet, mate and hybridize on *S. maximus*. However, in natural conditions they are prevented from doing so, due to a strong host preference (when they are given a choice). Therefore, it may be that interspecific competition led to parasite species segregation between the two hosts over evolutionary time, i.e. that it represents indeed, a relevant evolutionary agent in this particular case.

Avoidance of interspecific competition

Niche restriction occurs in extant communities as a result of interspecific competition, the fundamental niche becoming a narrower realized niche (Rohde, 1994). The literature provides some evidence for the parasitic copepods. For instance, Morales-Serna & Gómez (2012) suggested that coexistence between *Acantholochus zairae* Morales-Serna & Gómez, 2010 (Copepoda: Bomolochidae) and *Pseudochondracanthus diceraus* Wilson, 1908 (Copepoda: Chondracanthidae) on the gills of *Sphoeroides annulatus* (Jenyns, 1842) (Actinopterygii: Tetraodontidae) is facilitated since intraspecific aggregation is stronger than interspecific aggregation.

Avoidance of predators

It is assumed that a given species of parasite become adapted to live in certain (i.e. protected) microhabitats to avoid being predated by animals present in the macroenvironment (Rohde, 1994). Many parasitic copepods are a potential target of cleaner fish. For instance, caligid copepods (see e.g. Treasurer, 2002) are found on different sites of the host's body, e.g. body skin, beneath the pectoral and pelvic fins and inside branchial chambers, and colonization of less exposed microhabitats by these copepods can indeed reflect an evolutionary change to avoid predators. Nonetheless, based on the information in the literature, it is not possible to make any consideration as to whether avoidance of predators has determined niche restriction in parasitic copepods over evolutionary time.

Facilitation of mating

Rohde (1976, 1977) established the 'mating hypothesis' of niche restriction, according to which, selection favoured narrow microhabitats to enhance the chances of mating, particularly in low-density infrapopulations of parasites. Timi (2003) tested this hypothesis using infection data of *L. cynoscicola* infecting *C. guatucupa*. Unlike parasites from other taxonomic groups, parasitic copepods are bisexual and, accordingly, they must find a mate to reproduce. However, Timi (2003) found no evidence supporting the mating hypothesis and concluded that the evolution of a restricted niche in *L. cynoscicola* should be more related with other reproductive benefits. More specifically, he found that individuals of the same sex were more aggregated than females and males considered together and that the intensities of females and males were negatively correlated. On the other hand, the finding that intraspecific aggregation is stronger than interspecific aggregation (see e.g. Morales-Serna & Gómez, 2012), can also be understood as an indication that niches of parasitic copepods are restricted to facilitate mating. Furthermore, the aggregated distribution of parasites among their hosts is considered a general feature of metazoan parasites (Crofton, 1971; Poulin, 2007a,b), including parasitic copepods, and can be the result of their need to reproduce (see e.g. Dippenaar et al., 2009).

Reinforcement of reproductive barriers

This aspect was commented by Rohde & Hobbs (1968). It concerns the possibility that congeneric parasites overlap less than non-congeneric ones, in spite of the fact that all of them depend on the same limited resource of space for attachment. The inevitable conclusion to draw is that the difference found cannot be explained by interspecific competition; instead, it most likely reflects a reinforcement of reproductive barriers. This aspect was addressed for parasitic copepods in the published literature. Dippenaar et al. (2009) searched for evidence of niche restriction in the spatial distribution of *Kroyeria dispar* Wilson, 1935, *K. papillipes* Wilson, 1932 (Copepoda: Kroyeriidae) and *Eudactylina pusilla* Cressey, 1967 (Copepoda: Eudactylinidae) on the gill filaments of the tiger shark *Galeocerdo cuvier* (Péron & Lesueur, 1822) (Elasmobranchii: Carcharhinidae). In spite of the fact that all those parasite species occupy the same fundamental niche, no evidence of niche restriction was found. Accordingly, the spatial distributions found do not suggest a reinforcement of reproductive barriers.

Adaptations to environmental complexity

Microhabitats are generally complex and parasites have become adapted to live in them. More specifically, they should be able to ensure their survival, by making use of a particular set of biological and physiological features. For instance, they must be able to attach to a particular substratum, gain food, resist the immune reactions of the host, react to the variations in the volume of water flushing over the gills and respond to the chemical stimuli released by mating partners present in their microhabitat (Rohde, 1994). Optimal adaptation ensures the maximum possible chances of surviving environmental changes and parasites will not occupy other microhabitats unless they are obligated to do so. With respect to parasitic copepods, the work of Timi (2003) is particularly relevant. In that work, it is suggested that adaptations to environmental complexity, rather than increasing intraspecific contact, are more likely ultimate causes of niche restriction.

1.3. The Studied Hosts

1.3.1. The Common Octopus, *Octopus vulgaris* (Cephalopoda: Octopodidae)

The common octopus, *Octopus vulgaris* (Cephalopoda: Octopodidae) (Fig. 1.4), is a neritic, nektobenthic species, commonly found in moderately warm, shallow coastal waters (< 200 m deep) (Hastie et al., 2009). Its geographic distribution is wide, comprising the tropical, subtropical and temperate regions of the Atlantic, Indian and Pacific Oceans, and also, the adjacent seas (Mangold, 1983). Actually, it has been argued that it represents a complex of species rather than a single cosmopolitan species (see e.g. Leite et al., 2008). Each species in the complex should be adapted to the local environmental conditions (e.g. Guerra, 1982) and, accordingly, there can be differences in the parasite fauna of different species.

The species in the *O. vulgaris* complex are marketed fresh, frozen, dried salted and canned, representing an important food item and source of income to many people throughout the world. In Portugal, *O. vulgaris* usually occurs in commercial landings in fisheries off mainland Portugal, Madeira and the Azores Islands. The total world catch has decreased in recent years (Food and Agriculture Organization, 2013), and the cephalopod is presently considered a candidate species for marine aquaculture

(Estefanell et al., 2013), owing to its high food conversion rate (Wells, 1978), fast growth rate (Mangold & Boletzky, 1973) and high protein content (Lee, 1994). This justifies the importance of characterizing its parasite fauna in detail.

It should be noted here that since some parasites, i.e. octopicolid copepods, are exclusively found on octopuses (Boxshall & Halsey, 2004), their study can be particularly relevant in systematic terms. Besides, it should also be noted that several features might impair the host-to-host transmission of parasites in populations of these marine invertebrates, especially, the transmission of parasites with monoxenous life-cycles. More specifically, octopuses typically have short lives (for details see e.g. Hastie et al., 2009), engaging in solitary and sedentary lifestyles, and, at least, some species appear to be semelparous (Mangold, 1987) i.e. reproduce once in its life. These issues should be considered for analysis in parasitological studies of *O. vulgaris*, which have not yet been conducted for the Portuguese coast.

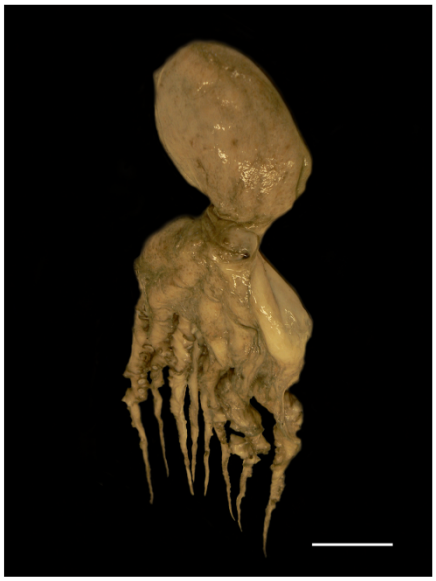


Fig. 1.4 – The common octopus, *Octopus vulgaris* (Cephalopoda: Octopodidae). *Scale-bar*: A, 10.0 cm.

1.3.2. The European Flounder, *Platichthys flesus* (Actinopterygii: Pleuronectidae)

The European flounder, *Platichthys flesus* (Linnaeus, 1758) (Actinopterygii: Pleuronectidae) (Fig. 1.5) is a demersal flatfish that spends most of its life in estuarine and brackish water environments. It swims close to the sea bed, and is usually found in shallow waters (< 100 m deep) and environments where the pH is 7.5 to 8.2 (Froese &

Pauly, 2008). In early spring, it migrates to coastal sea areas to spawn. Its geographic distribution is wide, extending along the Atlantic coast from the White Sea to the northern Africa, including the Mediterranean and the Black seas (Lucas & Baras, 2001). It is one of the most important fish species landed in Portugal, being found along the entire coast (Sobral & Gomes, 1997).

The occurrence of parasites in this flatfish, namely of metazoan ectoparasites, is well characterized in the published literature (see the review of Cavaleiro & Santos, 2007). Caligids and chondracanthids are among the most common ectoparasites. The body of the former can be conveniently divided into four tagmata i.e. cephalothorax, fourth pediger, genital complex and abdomen (Ho & Lin, 2004). As for chondracanthids, their body consists of three tagmata i.e. cephalosome (or true cephalothorax), trunk and genito-abdomen in females; in males, the body plan varies more or less markedly from the original structural plan of the free-swimming podoplean (Kabata, 1979). It should be noted that the occurrence of parasites in this flatfish can be influenced by its movements between different salinity environments, namely because some parasites are stenohaline. This issue is relevant and must be taken into account in parasitological studies of *P. flesus*. Particular aspects of the life history strategy of some parasites have already been addressed in the literature, e.g. the life-cycle and spatial distribution of *L. pectoralis* on the host's body (Scott, 1901; Boxshall, 1974b) and the life-cycle of *A. cornuta* (Heegaard, 1947), but many other remained to be elucidated.

Fig. 1.5 – The European flounder, *Platichthys flesus* (Linnaeus, 1758) (Actinopterygii: Pleuronectidae). *Scale-bar*: 5.0 cm.

1.4. Study Aims

This thesis enlarges the body of knowledge about the parasite fauna of *O. vulgaris* and *P. flesus*, by providing new information on the parasites occurring on or in these two species of hosts. Specific aims were as follows:

1) To characterize, for the first time, the metazoan parasite infections occurring in *O. vulgaris* from Portuguese coastal waters, including aspects such as seasonality trends and ecology of established host-parasite relationships.

2) To review the current knowledge on a poorly studied group of parasitic copepods, i.e. the octopicolid copepods. This is exclusively found on species of octopuses and was isolated during the parasitological survey of *O. vulgaris* caught in Portuguese coastal waters.

3) To describe a new and rare species of caligid copepod, *Caligus musaicus* sp. nov. (Copepoda: Caligidae), isolated from *P. flesus* of Portuguese coastal waters.

4) To describe, in detail, the morphology, ultrastructure, genetics and morphometrics of a diplostomid metacercaria isolated from the eye lenses of *P. flesus*.

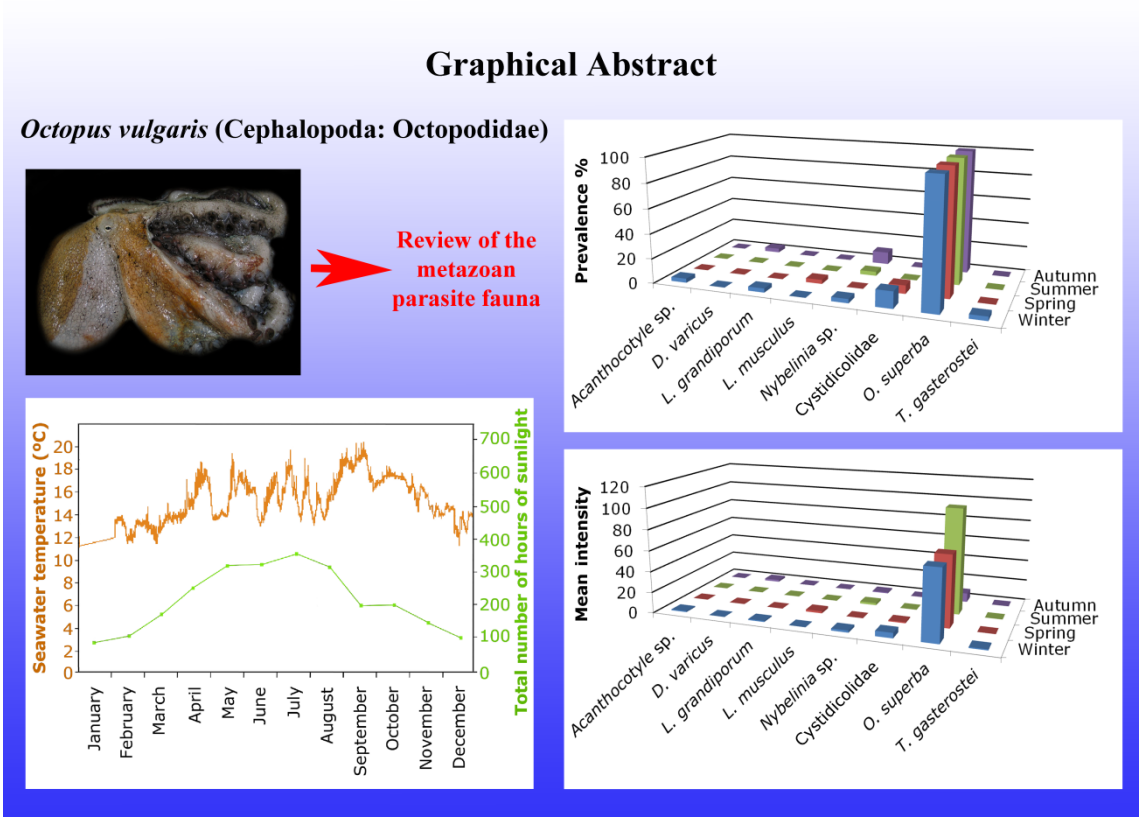
5) To study the trade-off between egg number and egg size at the intraspecific level, based on data recorded for adult ovigerous females of *Octopicola superba* Humes, 1957 (Copepoda: Octopicolidae).

6) To evaluate whether copepod parasites other than *L. pectoralis*, i.e. *A. cornuta*, also exhibit a particular spatial distribution on the body of *P. flesus*.

7) To evaluate the evidence supporting the occurrence of interference competition between an endoparasitic microparasite, i.e. *Aggregata* sp. (Apicomplexa: Aggregatidae), and an ectoparasitic macroparasite, i.e. *O. superba*, that co-occur at the gills of *O. vulgaris*.

Chapter 2

Helminth and copepod parasites of the common octopus, *Octopus vulgaris* (Cephalopoda: Octopodidae), in northwest Portuguese waters, Atlantic Ocean



This chapter has been adapted from:

Cavaleiro, F. I., & Santos, M. J. (In Review for Publication). Helminth and copepod parasites of the common octopus, *Octopus vulgaris* (Cephalopoda: Octopodidae), in northwest Portuguese waters, Atlantic Ocean. *Journal of Parasitology*.

2.1. Abstract

Octopus vulgaris (Cephalopoda: Octopodidae) from northwest Portuguese waters was collected seasonally for one year and examined for metazoan parasites. Eight parasitic taxa were found, including six taxa of helminths and two taxa of copepods. They are: *Acanthocotyle* sp. (Monogenea: Acanthocotylidae); *Derogenes varicus* (Digenea: Derogenidae); *Lecithochirium grandiporum*, and *L. musculus* (Digenea: Hemiuridae); *Nybelinia* sp. (Cestoda: Tentaculariidae); Cystidicolidae (Nematoda: Spiruroidea); *Octopicola superba* (Copepoda: Octopicolidae); and *Thersitina gasterostei* (Copepoda: Ergasilidae). *O. superba* was the only component parasite in the total sample of *O. vulgaris*. It exhibited a marked seasonality, with the lowest and highest mean intensity levels recorded for autumn and summer, respectively. According to the evidence found in this and other studies, the seawater temperature and the total number of hours of sunlight influence the infection levels of parasitic copepods. Furthermore, significantly higher numbers of octopicolid copepods were recorded for the female octopuses. This, along with the fact that a significant correlation between octopus' size and parasite intensity was detected only for the female octopuses suggests a differential influence of host sex in autoinfection. The infection levels recorded for *Octopicola* spp. infecting *O. vulgaris* in contiguous estuarine waters off Galicia were lower, which suggests that octopicolids are stenohaline.

2.2. Introduction

The common octopus, *Octopus vulgaris* (Cephalopoda: Octopodidae), is exploited by commercial fisheries, commanding high prices in the market place (Food and Agriculture Organization of the United Nations, 2007). Despite the fact that it is presently considered a candidate for aquaculture (Vaz-Pires et al., 2004; Estefanell et al., 2013), there is still little information about the parasite fauna of wild and reared specimens. Phylogenetic analyses of mitochondrial genes indicate that it represents a complex of species rather than a single cosmopolitan species (e.g. Söller et al., 2000; Warnke et al., 2004; Guerra et al., 2010). This study aimed: (i) qualitative and quantitative characterization of the metazoan parasite fauna of *O. vulgaris* from waters off the coast of northern Portugal; (ii) comparison of the recorded parasite fauna with that reported in the literature; and (iii) characterization of host-parasite relationships (component parasites).

2.3. Materials and Methods

O. vulgaris was caught seasonally (N = 30 per season; on 2 March, 24 and 31 May, 7 September, and 22 November) for one year (2010) off Matosinhos (41º10'N, 8º42'W), northeast Atlantic Ocean. The specimens were caught by a boat that fishes for *O. vulgaris* exclusively, and the landed catch was collected and kept in a box for a few hours, separated from the species fished by other boats. Octopuses were frozen, defrosted days later and examined for metazoan parasites and gross pathology. The total length and sex were recorded for all of them. External body surfaces were washed with saline solution (3.5%) to isolate the ectoparasites and all organs were examined for endoparasites. Mesozoans are not included in the survey because they could not be detected in the frozen material. Parasites were fixed and preserved in 70% ethanol. Later, they were identified to the lowest possible taxonomic level, according to Monticelli (1899), Overstreet & Hochberg (1975), Kabata (1979), Gibson & Bray (1986), Gestal et al. (1999), Palm (2004), Chabaud (2009) and Cavaleiro et al. (2013). Digeneans and the single monogenean specimen were stained with iron acetocarmine (Georgiev et al., 1986); nematodes (Hoffman, 1999) and copepods (Humes & Gooding, 1964) were cleared in lactophenol and lactic acid, respectively. Infection parameters (number of infected octopuses/prevalence [95% confidence interval] % and mean intensity ± SD [range]) were assessed for each parasitic taxon and considering the seasonal and total samples of octopuses. Bootstrap estimator values of taxa richness (S_b) (Poulin, 2007a) were also assessed for the seasonal and total samples. Seasonal,

sex and size differences in infection were evaluated for the component taxa (sensu Bush et al., 1990; considering the total sample of octopuses) exclusively. Intensity data were compared using the Kruskal-Wallis' test (comparison between the four samples) and the Mann-Whitney's *U* test (pairwise sample comparisons). A significant correlation between octopus' size and parasite intensity was evaluated using the Spearman's test (females and males were considered separately for analysis). Significance was set at $P < 0.05$ for all statistical tests (performed using SPSS, version 19.0) except the pairwise sample comparisons (Bonferroni-adjusted level: 0.008(3)). Lastly, the effect of temperature (data assessed from: Portuguese Hydrographic Institute, 2012) and sunlight (data derived from: Portuguese Meteorology Institute, 2012) in infection levels was evaluated. Terminology (locality, site, prevalence, intensity, and mean intensity) follows Bush et al. (1997).

2.4. Results

O. vulgaris was infected with eight taxa of metazoan parasites: *Acanthocotyle* sp. (Monogenea: Acanthocotylidae); *Derogenes varicus* (Müller, 1784) (Digenea: Derogenidae); *Lecithochirium grandiporum* (Rudolphi, 1819) Lühe, 1901, and *L. musculus* (Looss, 1907) (Digenea: Hemiuridae); *Nybelinia* sp. (Cestoda: Tentaculariidae); Cystidicolidae (Nematoda: Spiruroidea); *Octopicola superba* Humes, 1957 (Copepoda: Octopicolidae); and *Thersitina gasterostei* (Pagenstecher, 1861) (Copepoda: Ergasilidae) (Table 2.1). Gross pathology was not observed in any of the examined octopuses.

Table 2.1 – Metazoan parasitic taxa isolated from the seasonal and total samples of *Octopus vulgaris* (Cephalopoda: Octopodidae), observed life-cycle stages, sites, infection parameters (number of infected octopuses/prevalence [95% confidence interval] %, mean intensity ± SD [range]), and values for the bootstrap estimator of taxa richness (abbreviations: A, Adult; BS, Body Skin; CO, COpepodite; CMG, Covering Mesentery of Gonad; EY, EYes; F, Funnel; G, Gills; I, Intestine; L, Larva; MM, internal surface of the Mantle Musculature; OE, OEsophagus; and S, Stomach).

Group: Family **Taxon**	**Life-cycle stage**	**Site**	**Seasonal sample** ***N*** $_{Octopus\ vulgaris}$ **– Total;♀♀;♂♂** **Mean total length ± SD (range) (cm)**				**Total sample**
			Winter 30;13;17 69.8±8.2 (56.6–86.0)	Spring 30;15;15 68.3±10.9 (53.4–88.7)	Summer 30;17;13 65.8±10.8 (50.2–90.1)	Autumn 30;11;19 66.9±7.9 (53.4–89.1)	120;56;64 67.7±9.5 (50.2–90.1)
Monogenea: Acanthocotylidae							
Acanthocotyle sp.	A	BS	1/3.3 (0.6–16.7) 1 (1)	–	–	–	1/0.8 (0.2–4.6) 1 (1)
Digenea: Derogenidae							
Derogenes varicus	A	S	–	–	–	1/3.3 (0.6–16.7) 2 (2)	1/0.8 (0.2–4.6) 2 (2)
Digenea: Hemiuridae							
Lecithochirium grandiporum	A	S	1/3.3 (0.6–16.7) 1 (1)	–	–	–	1/0.8 (0.2–4.6) 1 (1)
Lecithochirium musculus	A	S	–	1/3.3 (0.6–16.7) 2 (2)	–	–	1/0.8 (0.2–4.6) 2 (2)
Cestoda: Tentaculariidae							
Nybelinia sp.	L	S;I	1/3.3 (0.6–16.7) 2 (2)	–	1/3.3 (0.6–16.7) 2 (2)	3/10.0 (3.5–25.6) 1 (1)	5/4.2 (1.8–9.4) 1.4±0.5 (1–2)

Table 2.1 (continuation) – Metazoan parasitic taxa isolated from the seasonal and total samples of *Octopus vulgaris* (Cephalopoda: Octopodidae), observed life-cycle stages, sites, infection parameters (number of infected octopuses/prevalence [95% confidence interval] %, mean intensity ± SD [range]), and values for the bootstrap estimator of taxa richness (abbreviations: A, Adult; BS, Body Skin; CO, COpepodite; CMG, Covering Mesentery of Gonad; EY, EYes; F, Funnel; G, Gills; I, Intestine; L, Larva; MM, internal surface of the Mantle Musculature; OE, OEsophagus; and S, Stomach).

Group: Family	**Life-cycle stage**	**Site**	**Seasonal sample**				**Total sample**
Taxon			$N_{Octopus\ vulgaris}$ **– Total;♀♀;♂♂** **Mean total length ± SD (range) (cm)**				
			Winter 30;13;17 69.8±8.2 (56.6–86.0)	Spring 30;15;15 68.3±10.9 (53.4–88.7)	Summer 30;17;13 65.8±10.8 (50.2–90.1)	Autumn 30;11;19 66.9±7.9 (53.4–89.1)	120;56;64 67.7±9.5 (50.2–90.1)
Nematoda: Cystidicolidae							
	L	OE;S;I	4/13.3 (5.3–29.7) 5±7.3 (1–16)	2/6.7 (1.9–21.3) 1 (1)	–	–	6/5.0 (2.3–10.5) 3.7±6.1 (1–16)
Copepoda: Octopicolidae							
Octopicola superba	CO;A	BS;G;CMG;MM;EY;F	30/100 (88.7–100) 67.0±26.9 (18–119)	30/100 (88.7–100) 67.9±88.4 (1–230)	30/100 (88.7–100) 100.8±72.8 (7–235)	30/100 (88.7–100) 8.5±9.7 (1–38)	120/100 (88.7–100) 61.1±67.2 (1–235)
Copepoda: Ergasilidae							
Thersitina gasterostei	A	G	1/3.3 (0.6–16.7) 1 (1)	–	–	–	1/0.8 (0.2–4.6) 1 (1)
		S_b	7.5	3.5	2.4	3.4	9.8

Parasite taxa richness varied from season to season, with the minimum (two) and maximum (six) numbers of taxa recorded for summer and winter, respectively. *O. superba* was the only component parasite (overall prevalence = 100%) in the total sample of *O. vulgaris*. Mean intensity of this parasite varied according to season, with the minimum and maximum levels recorded for autumn and summer, respectively (Kruskal-Wallis' test [*P*-value]: < 0.0001; Mann-Whitney's *U* test [*P*-value]: 0.036 [winter *vs* spring]; 0.395 [winter *vs* summer]; < 0.0001 [winter *vs* autumn; summer *vs* autumn]; 0.003 [spring *vs* summer]; and 0.221 [spring *vs* autumn]). A difference in intensity levels recorded for female and male octopuses was also statistically confirmed (♀♀ octopuses [mean ± SD] = 89.4±78.5 parasites; ♂♂ octopuses: 36.3±42.4 parasites; Mann-Whitney's *U* test [*P*-value]: < 0.0001). A positive correlation between octopus' size and parasite intensity was detected for females but not for males (Spearman's test: r_s = 0.551, *P* < 0.0001, *N* = 56 [♀♀ octopuses]; r_s = 0.045, *P* = 0.726, *N* = 64 [♂♂ octopuses]). Temporal variations in seawater temperature and total number of hours of sunlight (from January to December 2010) at the sampled area are depicted in Fig. 2.1.

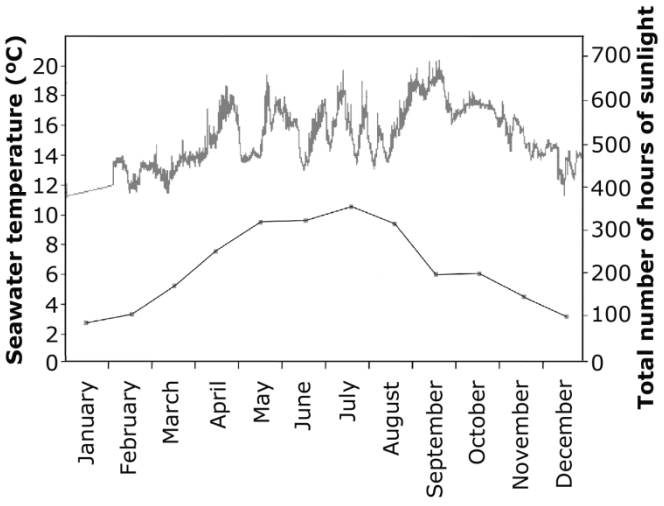


Fig. 2.1 – Temporal trends in seawater temperature (at 83 m depth) and total number of hours of sunlight recorded for the sampled area (off Matosinhos, northwest Portuguese coast) (upper trend line, temperature levels; and lower trend line, total number of hours of sunlight).

The lowest and highest levels were recorded during winter and summer seasons, respectively, both for temperature and sunshine total duration.

2.5. Discussion

From the metazoan parasites recorded in this study, *Acanthocotyle* sp., *D. varicus*, *L. grandiporum*, *L. musculus* and *T. gasterostei* are new records for the *O. vulgaris* complex (see the review presented in Table 2.2). The highest number of parasitic taxa was recorded in winter, which suggests a reduced resistance to infections in this season of the year. The recorded bootstrap values indicate that more parasitic taxa should have been found in the winter and total samples. The unfound species likely represent rare parasites of *O. vulgaris*. *Acanthocotyle* spp. are typically found on the skin of elasmobranchs (Yamaguti, 1963); accordingly, *Acanthocotyle* sp. is probably an accidental parasite of *O. vulgaris* (only one specimen found through the examination of 120 octopuses), i.e. its occurrence on *O. vulgaris* most likely reflects the absence of suitable hosts. The larvae of *Nybelinia* were rare and exhibited a great morphological similarity to *Nybelinia lingualis* Cuvier, 1817 (according to Palm, 2004). Nonetheless, the assignment of larvae to this species must be confirmed by molecular analyses. The infection levels of Cystidicolidae were also low, which indicates that this taxon is also uncommon in *O. vulgaris* in the sampled area. Similar evidence was found by Pascual et al. (1996) for *Cystidicola* sp. (prevalence = 11.4%) and Gestal et al. (1999) for Cystidicolidae larvae (prevalence = 16%; mean intensity = 1.46 worms/host) infecting *O. vulgaris* from the Ría de Vigo, a large estuary in northwestern Spain. The infection levels of *O. superba* suggest that this is a common parasite of *O. vulgaris* in waters off the coast of northern Portugal. Moreover, the seasonal trend of *O. superba* is similar to the trends found for other parasitic copepods present at the sampled area, i.e. *Lepeophtheirus pectoralis* (Müller, 1777) (Copepoda: Caligidae) and *Acanthochondria cornuta* (Müller, 1776) (Copepoda: Chondracanthidae) (see Cavaleiro & Santos, 2009). The temporal trends of the considered environmental variables were consistent enough between the two years studied to underpin the hypothesis that high water temperature and large photoperiod have a positive effect on the infection with parasitic copepods. *T. gasterostei* is a common parasite, usually found on species of sticklebacks and others (Kabata, 1979). Furthermore, the copepod is cosmopolitan at higher latitudes of the northern hemisphere, which helps to justify its accidental occurrence on *O. vulgaris* (only one specimen found through the examination of 120 octopuses). *Octopicola* spp. occurred less frequently on *O. vulgaris* from estuarine waters of Ría de Vigo (34.3%) and Ribadeo (38.5%) (Pascual et al., 1996) compared with *O. superba* on *O. vulgaris* from waters off Matosinhos (100%). This suggests that octopicolids are stenohaline, which conforms to what has been said for the parasitic copepods (Kabata, 1979; Knudsen & Sundnes, 1998). The spatial distribution of *O. superba* on the body of *O.*

vulgaris was addressed in a previous study (Cavaleiro & Santos, In Press). The results of the present study shed further light on that host-parasite system, since they suggest an influence of host sex on parasite life strategy. More specifically, not only the intensity was significantly higher in female octopuses, as a significant positive correlation between octopus' size and parasite intensity was recorded only for the subsample of females. This evidence suggests that significant autoinfection takes place in female octopuses and that these have a key role in host-to-host transmission of *O. superba*. The infection with *O. superba* did not cause gross pathology; accordingly, it should not cause economic losses to fisheries. It can however become problematic in intensive rearing systems, and prophylactic measures can be crucial to preventing economic losses.

Table 2.2 – Metazoan parasites recorded for the *Octopus vulgaris* (Cephalopoda: Octopodidae) complex in the literature and respective localities and sites (abbreviations: BS, Body Skin; CR, CRop; CT, Connective Tissue around the digestive gland; DT, Digestive Tract; EG, EGgs; G, Gills; I, Intestine; MC, Mantle Cavity; and S, Stomach).

Group: Family **Taxon**	**Locality**	**Site**	**Reference**
Aspidogastrea: Aspidogastridae			
Lobatostoma sp.	Off Durban, Natal, South Africa, Indian Ocean	CR,S	Bray (1984)
Digenea: Fellodistomidae			
Proctoeces maculatus (Looss, 1901)	Off Natal, South Africa, Indian Ocean	CR,S	Bray (1983)
Proctoeces sp.	Off Peru, South Pacific Ocean	MC	Reategui et al. (1989)
Digenea: Opecoelidae			
Podocotyle scorpaenae (Rudolphi, 1919)	Northern coast of the western Mediterranean	–	Bartoli & Gibson (2007)
Digenea (*incertae sedis*)			
Distoma octopodis	Off Naples, Italy, Mediterranean Sea	–	Delle Chiaje (1822, 1829, 1841); Blanchard (1847); Carus (1885); Dollfus (1958)
Cestoda: Phyllobothriidae			
Phyllobothrium sp.	Ría de Vigo, Spain, Ibero-Atlantic waters	DT	Pascual et al. (1996)
Cestoda: Tentaculariidae			
Nybelinia lingualis Cuvier, 1817[a]	North Atlantic Ocean	–	Diesing (1850); Mingazzini (1904); Redi (1684); Vaullegeard (1899)
Nematoda: Anisakidae			
Anisakis simplex (Rudolphi, 1809)	Off the coast of Galicia, Spain, Ibero-Atlantic waters	–	Abollo et al. (1998)
Nematoda: Cystidicolidae			
	Ría de Vigo, Spain, Ibero-Atlantic waters	CR,CT,I	Gestal et al. (1999)
Cystidicola sp.	Ría de Vigo, Spain, Ibero-Atlantic waters	S	Pascual et al. (1996)
Copepoda: Octopicolidae			
Octopicola antillensis Stock, Humes and Gooding, 1963	Off Curaçao and Barbados, Caribbean Sea, North Atlantic Ocean	–	Stock et al. (1963)
Octopicola superba Humes, 1957	Off Roscoff, France, North Atlantic Ocean	BS,MC,G	Bocquet & Stock (1960)
	Off Banyuls-sur-Mer, France, Mediterranean Sea	BS,MC	Debouttéville et al. (1957)
	Off Banyuls-sur-Mer, France, Mediterranean Sea	BS,EG	Humes (1957)
Octopicola spp.	Ría de Vigo, Spain, Ibero-Atlantic waters	G	Pascual et al. (1996)
Copepoda: Pennellidae			
Pennella spp.	Ría de Vigo, Spain, Ibero-Atlantic waters	G,MC	Pascual et al. (1996)
	Off Ribadeo, Spain, Cantabric Sea	G,MC	Pascual et al. (1996)

[a]Referred as *Tetrabothriorhynchus octopodiae* Diesing, 1850 and *Tetrarhynchus megabothrium* Rudolphi, 1810.

2.6. Acknowledgements

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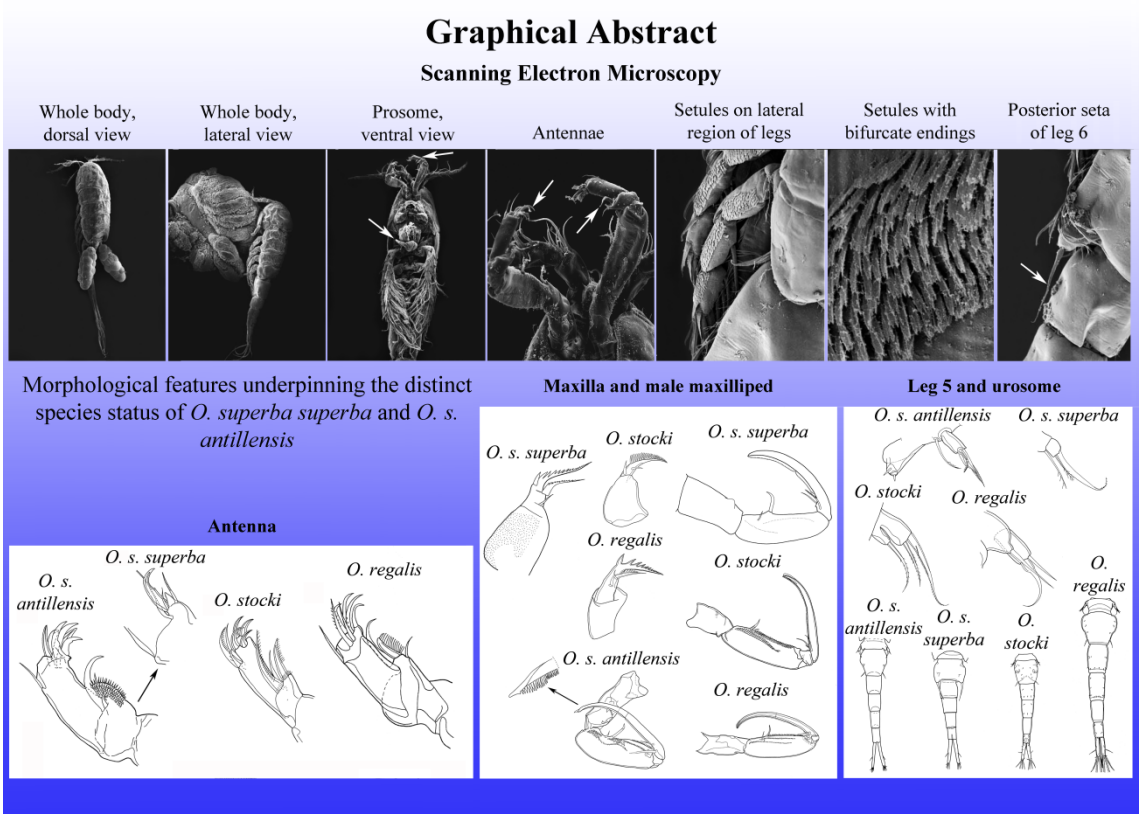
Chapter 3

Revisiting the octopicolid copepods (Octopicolidae: *Octopicola* Humes, 1957): comparative morphology and an updated key to species



This chapter has been adapted from:

Cavaleiro, F. I., Ho, J.-S., Iglesias, R., García-Estévez, J. M., & Santos, M. J. (2013). Revisiting the octopicolid copepods (Octopicolidae: *Octopicola* Humes, 1957): comparative morphology and an updated key to species. *Systematic Parasitology*, *86*, 77–86.

3.1. Abstract

A review of the present state of knowledge on the octopicolid copepods (Octopicolidae: *Octopicola* Humes, 1957) is presented. Characteristic morphological features are illustrated with scanning electron micrographs of *Octopicola superba superba* Humes, 1957. Comparative morphology analysis led to the conclusion that there is sufficient evidence to justify raising the two subspecies of *O. superba* to full species rank. A new identification key for the four species of *Octopicola* Humes, 1957, i.e. *O. superba* Humes, 1957, *O. antillensis* Stock, Humes & Gooding, 1963, *O. stocki* Humes, 1963 and *O. regalis* Humes, 1974, is proposed after evaluation of the morphological characters which vary more markedly between them. Among other characters, these species differ in the ornamentation of the third antennal segment, maxilla and male maxilliped. They are further distinguished by a combination of several character states concerning the fifth pedigerous somite.

3.2. Introduction

The octopicolids (Octopicolidae: *Octopicola* Humes, 1957) are tiny, mobile, poecilostomatoid copepods. As suggested by their name, they live in exclusive association with octopuses (Cephalopoda: Octopodidae) (see Humes, 1974; Hochberg, 1983; Boxshall & Halsey, 2004). Their bodies are cyclopiform, meaning that the general body shape closely resembles that of *Cyclops* spp. Although octopicolids seem to prefer the mantle cavity of their hosts (Hochberg, 1983), different sites on the body surface may be found infected (Cavaleiro & Santos, In Press; Humes & Stock, 1973); they can be also found amongst the eggs (Humes, 1957, 1974; Humes & Stock, 1973; Hochberg, 1983). While in the mantle cavity, octopicolids either move about freely over the gills or attach to the arterial stems beneath the branchial leaflets (Hochberg, 1983). At least one species of octopicolid copepod has been observed to exhibit a circadian rhythm in site occupation, inhabiting the mantle cavity of the host during daytime and moving out on the surface of the body after dark (Debouttéville et al., 1957).

The genus *Octopicola* Humes, 1957 was erected by Humes (1957), but it was only 39 years later, in 1996, that a new family, named Octopicolidae Humes & Boxshall, 1996 has been established to accommodate it (Humes & Boxshall, 1996). These authors argued that the octopicolids are the only copepods in the lichomolgoidean complex of families (following Humes & Boxshall, 1996) to retain the primitive six-segmented condition of the female urosome and that they should therefore be included in a separate family. Currently, three species of octopicolid copepods are recognised: *Octopicola superba* Humes, 1957, *O. stocki* Humes, 1963, and *O. regalis* Humes, 1974; the former comprised of two subspecies, *O. s. superba*, endemic to European waters and corresponding to the species described by Humes (1957); and *O. s. antillensis* Stock, Humes & Gooding, 1963, endemic to West Indian waters (Humes, 1957, 1963, 1974; Stock et al., 1963; Humes & Stock, 1973).

The key to the species and subspecies of the genus *Octopicola* of Humes & Stock (1973) is based on the morphological variability exhibited by the females and males of *O. s. superba*, *O. s. antillensis* and *O. stocki* but does not include *O. regalis*, described one year after its publication. Therefore, it needs a revision to include all species described to date. Furthermore, a close examination of the morphology of *O. s. superba* and *O. s. antillensis* suggested that these subspecies exhibit sufficient differences to be raised to full species rank.

The present study aimed: (i) illustration of the characteristic morphological features of the octopicolid copepods using scanning electron microscopy examination of *O. s. superba*, (ii) discussion of the morphological evidence which justifies raising the two subspecies of *O. superba* to full species rank; and (iii) elaboration of a key for identification of the species of *Octopicola*.

3.3. Materials and Methods

Collection and identification of the octopicolid copepods

Unlike other parasitic copepods, whose presence on host tissues is readily detected by naked eye, octopicolids will most certainly go unnoticed by the casual observer. Moreover, their relatively small size, associated with their sometimes transparent appearance, render it unlikely that an observer would be able to recognize them with ease, without using appropriate instrumentation. Indeed, the detection of these parasites can be problematic even under a stereomicroscope. Not infrequently, infected host tissues are covered with a dark black ink expelled by the octopus. Additionally, the large size of many species of octopuses renders them difficult to handle, making it almost impossible to observe the parasites in situ, under a stereomicroscope. Due to all these constraints, a particular method is to be followed while examining octopuses for octopicolid copepods. In this study, *O. s. superba* was used to illustrate characteristic morphological features of the octopicolid copepods. The parasite was isolated from the body of naturally infected specimens of the common octopus, *Octopus vulgaris* Cuvier, after washing the body, internal surface of the mantle musculature and external surface of the organs with saline solution (3.5%); *O. s. superba* was isolated from the sediment under a stereomicroscope. The specimens were cleaned of mucus and other debris in saline solution (3.5%) and fixed in 70% ethanol. Later, they were cleared in a drop of 90% lactic acid (Humes & Gooding, 1964) and identified to the subspecies level (Humes & Stock, 1973) under a compound microscope (Carl Zeiss Axiophot Photomicroscope) at magnifications of up to 1000×.

Scanning electron microscopy of O. s. superba

A few specimens of *O. s. superba* were selected for study by scanning electron microscopy (SEM). Their preparation for SEM examination included fixation in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 for about 2–3 h and in 1%

osmium tetroxide for 30 min. Afterwards, the specimens were dehydrated through a graded ethanol series, i.e., 50, 70, 80, 90% and absolute (2×), remaining immersed for about 20–30 min in each of these ethanol solutions. The copepods were then transferred to 25, 50, 75% (in ethanol) and pure isoamyl-acetate (2×15 min in each solution), critical point-dried in CO_2, mounted on stubs, and coated with a 15 nm layer of gold using an automated sputter coater (Emitech K550X). They were observed under a scanning electron microscope (Philips XL30) at an accelerating voltage of 5–10 kV.

Family Octopicolidae Humes & Boxshall, 1996

Genus *Octopicola* Humes, 1957

Diagnosis

Body elongate and slender in both sexes; prosome formed by cephalosome and 4 free, subequal pedigers; urosome 6-segmented in both sexes, with genital and first abdominal somites separate; first pediger not fused with cephalosome. Caudal ramus long, narrow, with 6 setae (4 terminal, 1 subterminal, 1 on external margin) and several minute setules. Antennule 7-segmented (armature formula 4, 13, 6, 3, 4 + 1 aesthetasc, 2 + 1 aesthetasc, 7 + 1 aesthetasc), long, shorter than prosome, with sclerotisation between second and third segments (especially ventrally) suggesting an intercalary piece (incomplete segment). Rostrum triangular, slightly pointed, bearing setules of variable length. Antenna uniramous, 4-segmented, with coxa and basis fused to form coxobasis, armed with recurved spines on third and fourth segments. Labrum with 2 elongate posteroventral lobes, delimited medially by deep incision of posterior margin. Mandible strongly sclerotised with pointed tooth and wide, pointed lobe, bearing row of spinules along inner margin. Paragnath a small unornamented lobe at region of labrum. Maxillule a small lobe, bearing 3 setae, one much smaller. Maxilla 2-segmented; first segment largest and tooth-like; second segment slender, culminating in tapered process with graduated teeth along one side. Maxilliped in females 3-segmented: first segment elongate, unornamented, second segment elongate, slightly sinuous, bearing 2 small, naked setae and distal patch of small spinules, third segment small, bearing terminally 3 claw-like processes; in males 4-segmented (assuming that proximal part of claw represents fourth segment): first segment unornamented, second segment armed with 2 unequal inner setae and numerous spinules arranged in rows, third segment very small and unornamented, last

segment a very long, slender claw, bearing fused setal element. Legs 1-4 with 3-segmented rami, except leg 4 endopod which is 1-segmented; first and second segments of legs 1-3 exopods and all segments of leg 4 exopod armed with numerous small setules on lateral region, some with bifurcate endings. Leg 5 on urosome, with protopodite and exopodite incorporated into somite, bearing 1 and 2 very unequal setae, respectively. Leg 6 represented by genital opercula, bears up to 2 setae; genital somite conspicuous, with paired genital apertures, dorsolateral in females and ventral in males. Ovigerous females with paired, multiseriate egg-sacs. *Type-species*: *Octopicola superba* Humes, 1957.

Characteristic morphological features of octopicolid copepods

Characteristic morphological features of octopicolid copepods are illustrated with scanning electron micrographs of *Octopicola superba superba* Humes, 1957 in Figs. 3.1 and 3.2, and the typical pattern of ornamentation of legs 1-4 is shown below (spines indicated by Roman numerals; setae indicated by Arabic numerals).

	Coxa	Basis	Exopod	Endopod
Leg 1	0-0	1-0	I-0; I-1; III,I,4	0-1; 0-1; I,5
Leg 2	0-0	1-0	I-0; I-1; III,I,5	0-1; 0-2; I,I + 1, 3
Leg 3	0-0	1-0	I-0; I-1; III,I,5	0-1; 0-2; I,I + 1, 2[a]
Leg 4	0-0	1-0	I-0; I-1; II,I,5	II, 1

[a]The drawing of the leg 3 presented in the original description of *O. stocki* (see Humes, 1963) was made from an aberrant specimen, as later confirmed by the author (Humes, 1974).

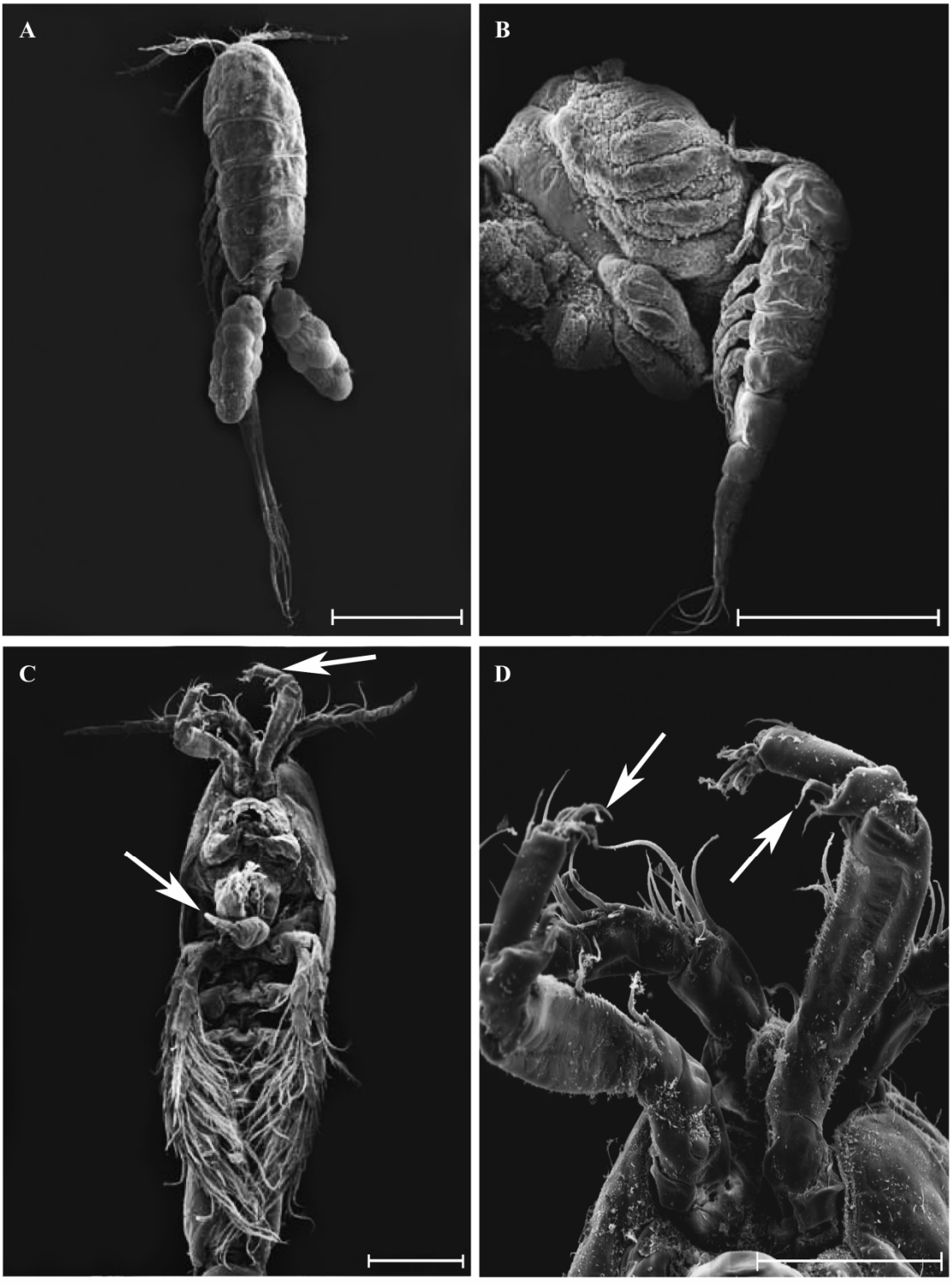


Fig. 3.1 – Scanning electron microscopy of characteristic morphological features of *Octopicola superba superba*, isolated from the common octopus *Octopus vulgaris*. A, Adult ovigerous female, dorsal view; B, Specimen attached to host gill, lateral view; C, Prosome of male, ventral view (*upper arrow*, antenna; and *lower arrow*, claw of maxilliped); and D, Detail of the claws (*arrows*) on the antenna. *Scale-bars*: A, B, 500 µm; C, 200 µm; and D, 100 µm.

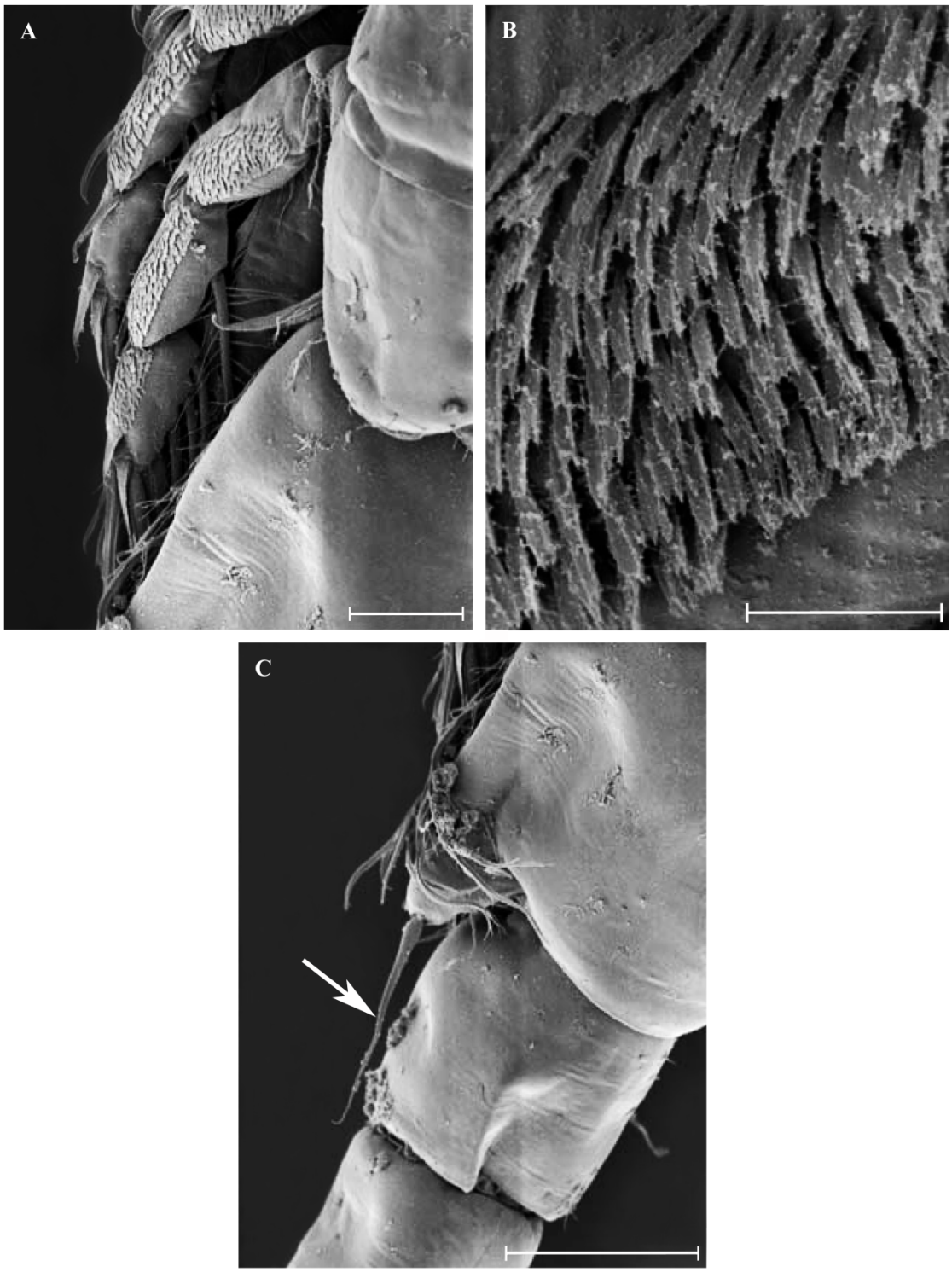


Fig. 3.2 – Scanning electron microscopy of characteristic morphological features of *Octopicola superba superba*, isolated from the common octopus, *Octopus vulgaris*. A, Detail of the ornamentation seen on the lateral region of legs 3 (upper leg) and 4 (lower leg); B, Detail of the setules with bifurcate endings on the lateral region of the legs; and C, Detail of the longer of the two setae of leg 6 (*arrow*) on the posterior lateral corner of the genital somite. *Scale-bars*: A, 50 µm; B, 10 µm; and C, 100 µm.

Species and subspecies distinction

Tables 3.1 and 3.2 summarise the relevant information on species biology, ecology and morphometry.

Table 3.1 – Host and distribution data for the known taxa of octopicolid copepods.

Species	***O. s. superba*** **Humes, 1957**	***O. s. antillensis*** **Stock, Humes & Gooding, 1963**	***O. stocki*** **Humes, 1963**	***O. regalis*** **Humes, 1974**
Host	*Octopus vulgaris* Cuvier	*Octopus briareus* Robson; *O. vulgaris* Cuvier	*Octopus cyaneus* Gray[a]	*Octopus cyaneus* Gray
Colour (live, under light microscope)	White	–	Transparent to opaque	Opaque
Geographical distribution (source)	Off Mediterranean coast of France, Atlantic Ocean (Humes, 1957); off Channel coast of France, Atlantic Ocean (Bocquet & Stock, 1960); off Portuguese coast, Atlantic Ocean (present study)	Off Florida, Atlantic Ocean (Humes & Stock, 1973; *O. briareus*); off West Indies, Atlantic Ocean (Stock et al., 1963; *O. vulgaris*); off Florida, Atlantic Ocean (Humes & Stock, 1973; *O. vulgaris*)	Off Madagascar Island, Indian Ocean (Humes, 1963; unidentified species of octopus); off Madagascar Island, Indian Ocean (Humes, 1963)	Off New Caledonia Islands and Eniwetok Atoll, Marshall Islands, Pacific Ocean (Humes, 1974)

[a]And not on *Octopus* (*Tritaxeopus*) *cornutus* Owen, 1881 as reported in the species description (see Boxshall & Halsey, 2004).

Table 3.2 – Summary of metrical data for the known species and subspecies of *Octopicola*.

Species /character	***O. s. superba***		***O. s. antillensis***		***O. stocki***		***O. regalis***	
Source	**Humes (1957, 1963, 1974)**		**Stock et al. (1963)**		**Humes (1963, 1974)**		**Humes (1974)**	
	♀	♂	♀	♂	♀	♂	♀	♂
Body length × width (mm)	1.8×0.4	1.9×0.3	1.5–2.2[a]	1.2–1.8[a]	1.7×0.3	1.3×0.3	2.2×0.4	1.6×0.3
Length to width ratio of caudal ramus	≈ 9:1		–		≈ 7.6:1		≈ 4.7:1	
Last segment of antenna (µm)	94×22		40×22[b]		44×18		65×24	
Endopod of leg 4 (µm)	143×39		–		85×36		125×44	
Egg-sac length × width (µm)	648×229		≈ 582×221		650×210		858×286	

[a]Only range for length available.

[b]Length taken along the inner margin; width taken at the middle of segment.

The morphological features that vary among species and subspecies are summarised below. The antennule, rostrum, labrum and oral area, postoral protuberance, mandible, maxillule, maxilla, female maxilliped and legs 1-4 of *O. s. antillensis* do not exhibit significant departures from the structural plan described for *O. s. superba* (see Stock et al., 1963).

Ornamentation of the antenna (Fig. 3.3 A-D)

The third segment of the antenna of *O. s. superba* exhibits a finely denticulate, triangular process, whereas that of *O. s. antillensis* has a very prominent projection (about half the length of the accompanying claw), massively covered with long spinules. The segment bears one spine and two setae in *O. s. superba* and *O. s. antillensis*; two spines and one seta in *O. stocki*; and one claw-like jointed spine, one blunt spine with rows of long hairs along the inner margin and one small smooth seta in *O. regalis*. Differences are also observed in the ornamentation of the fourth antennal segment i.e. all three setae are subterminal in *O. stocki*, whereas in *O. s. superba*, *O. s. antillensis* and *O. regalis* two of the setae are terminal and the third is clearly subterminal.

Ornamentation of the maxilla (Fig. 3.3 E-G)

The first segment of the maxilla of *O. s. superba* has numerous minute setules distributed over almost its entire surface and one distal, small, smooth seta, whereas that of *O. stocki* and *O. regalis* is unornamented. The second segment of the maxilla possesses a spine-like seta with spinules along one side in *O. s. superba* and *O. regalis* but not in *O. stocki*. The first of the graduated teeth at the tapered process of the second segment is tooth-like in *O. s. superba* and *O. regalis* but not in *O. stocki*.

Ornamentation of the male maxilliped (Fig. 3.3 H-L)

The second segment of the maxilliped in the males of *O. s. superba*, *O. s. antillensis* and *O. stocki* bears two rows of spinules along the inner surface whereas three rows of spinules are present on the corresponding region of the maxilliped in the male of *O. regalis*. Groups of spinules connecting the rows of spinules are present in *O. s. antillensis* but were not reported for *O. s. superba*, *O. stocki* and *O. regalis* (see Humes, 1957, 1963, 1974). A conspicuous hyaline process is seen at the base of the claw of the maxilliped of the male of *O. stocki*, whereas a very small prominence is seen at the corresponding region of the maxilliped in the male of *O. s. superba*. The hyaline membrane near the tip of the claw (convex surface) is bluntly pointed and smooth in *O. s. superba* and prolonged into a small element in *O. s. antillensis*, *O. stocki* and *O. regalis*. This element is armed with a group of small spinules on its base in *O. s. antillensis*.

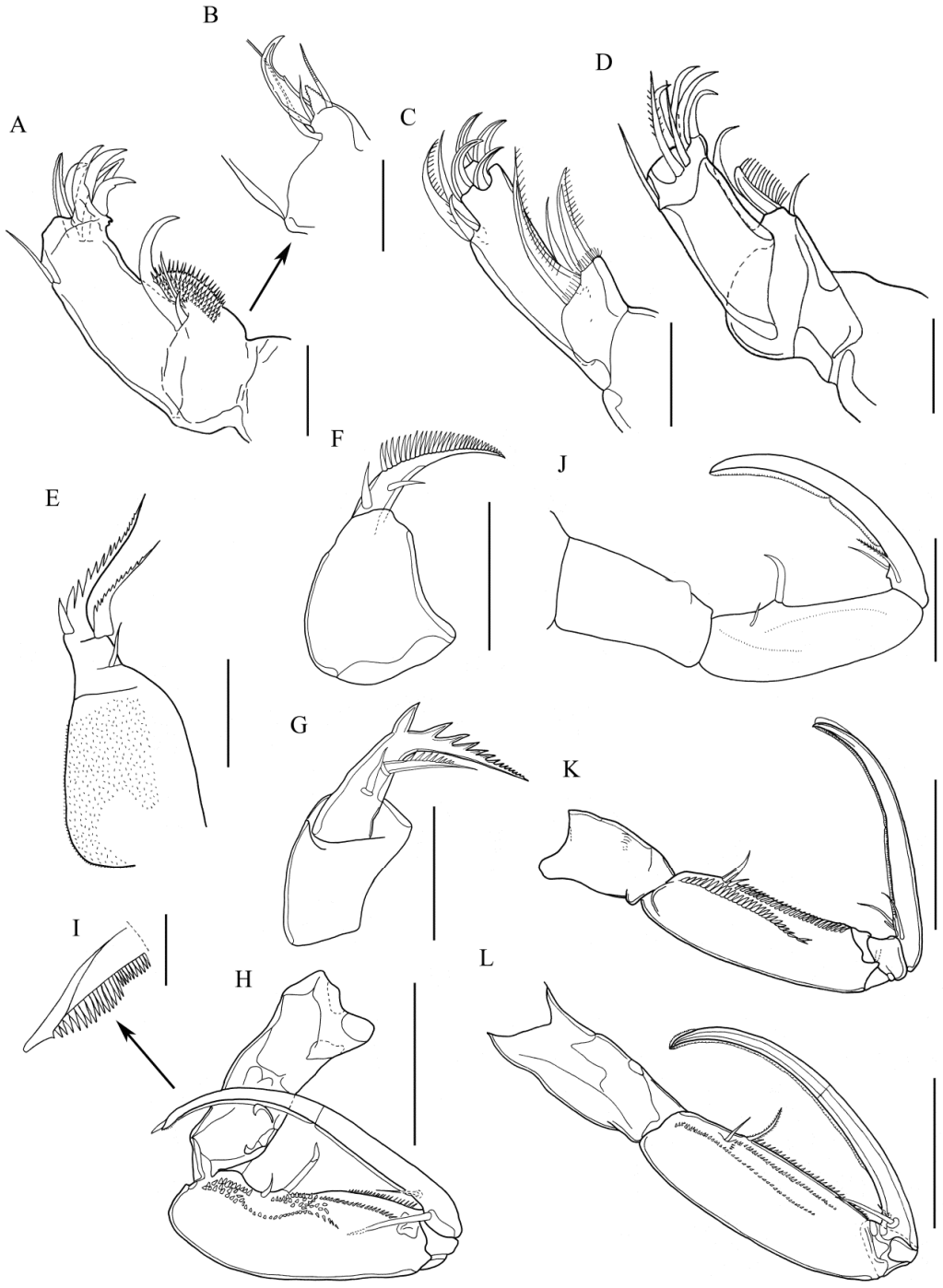


Fig. 3.3 – Morphological variations in octopicolid copepods. A, Third and fourth antennal segments of *Octopicola superba antillensis*; B, Third antennal segment of *Octopicola superba superba*; C, Third and fourth antennal segments of *Octopicola stocki*; D, Third and fourth antennal segments of *Octopicola regalis*; E, Maxilla of *O. s. superba*; F, Maxilla of *O. stocki*; G, Maxilla of *O. regalis*; H, Maxilliped of the male of *O. s. antillensis*; I, Detail of the claw of the maxilliped of the male of *O. s. antillensis* showing the small spinules at the base of the element at the dactylus; J, Maxilliped of the male of *O. s. superba*; K, Maxilliped of the male of *O. stocki*; and L, Maxilliped of the male of *O. regalis*. *Scale-bars*: A-D, 30 µm; E-G, 50 µm; I, 10 µm; and H, J, K, L, 100 µm. Redrawn after Humes (1957) (E, J); Bocquet & Stock (1960) (B); Humes (1963) (C, F, K); Stock et al. (1963) (A, H, I); and Humes (1974) (D, G, L).

Leg 5 and surrounding area of fifth pedigerous somite (Fig. 3.4 A-D)

The shape of the free segment of leg 5 varies from subquadrate in *O. s. superba* to subrectangular in *O. stocki* (length to width ratio 1.8:1) and *O. regalis* (length to width ratio 2.4:1). The larger of the two setae on this segment exhibits a swollen base in *O. regalis*. The seta dorsal to the free segment is inserted into a lobe in *O. stocki* and *O. regalis*, whereas in *O. s. superba* and *O. s. antillensis* it arises directly from the body wall. Tergal plates were reported for the fifth pedigerous somite of *O. s. antillensis* exclusively (Stock et al., 1963).

Relative length of setae of leg 6 (Fig. 3.4 E-H)

The two setae of leg 6 are short in *O. s. antillensis*, *O. stocki* and *O. regalis*, the most posterior seta (the one on the posterolateral area of the genital somite) extends only slightly beyond the posterior margin of its own somite. In *O. s. superba* the most posterior seta of leg 6 is long, reaching to the posterior margin of the next urosomal somite or beyond.

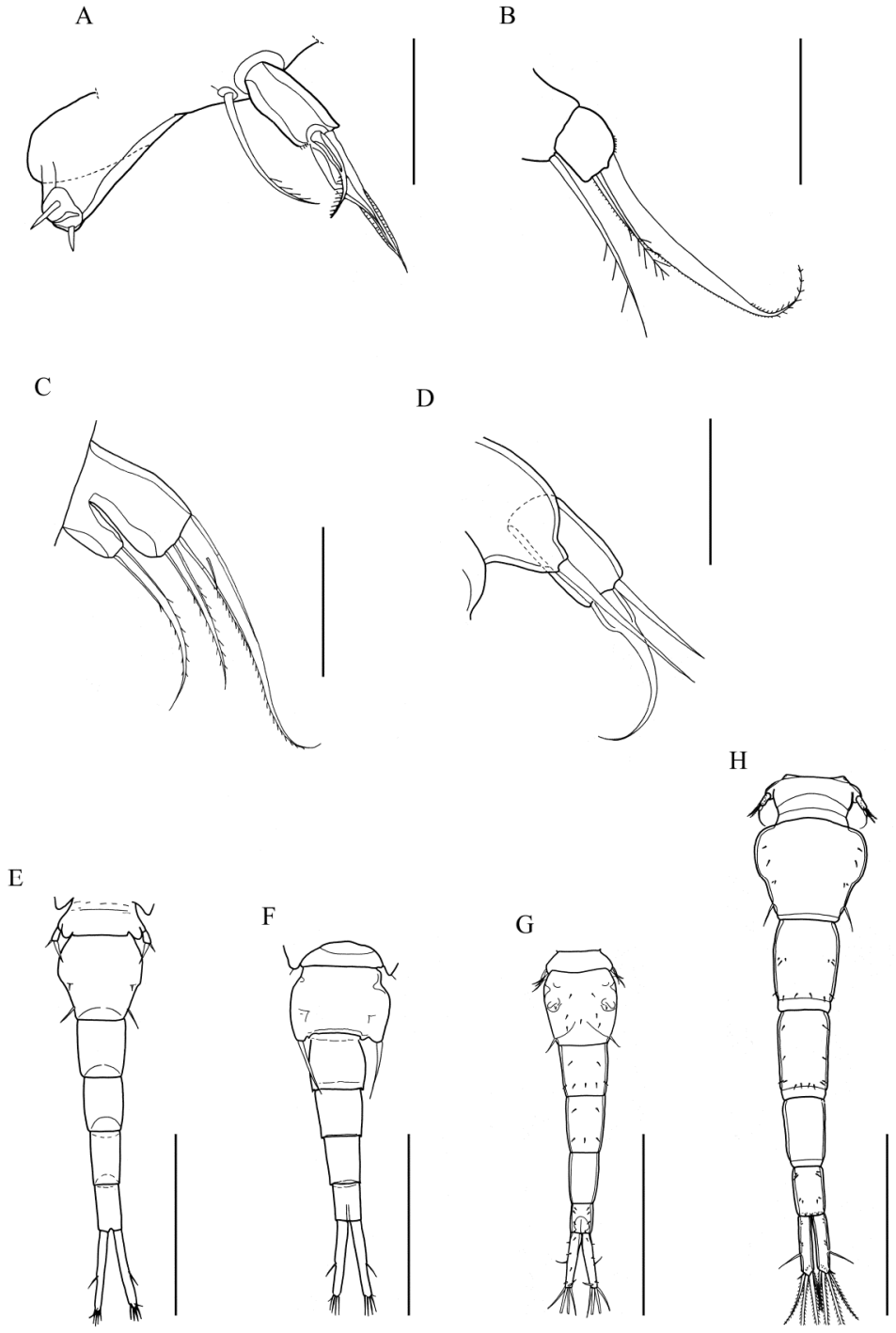


Fig. 3.4 – Morphological variations in octopicolid copepods. A, Detail of the fifth pedigerous somite of *Octopicola superba antillensis* showing leg 5, adjacent seta and tergal plate; B, Leg 5 of *Octopicola superba superba* and adjacent seta; C, Leg 5 of *Octopicola stocki* and adjacent seta; D, Leg 5 of *Octopicola regalis* and adjacent seta; E, Urosome of *O. s. antillensis*; F, Urosome of *O. s. superba*; G, Urosome of *O. stocki*; and H, Urosome of *O. regalis*. *Scale-bars*: A, B, D, 50 µm; C, 30 µm; and E-H, 500 µm. Redrawn after Humes (1957) (B); Humes (1963) (C, G); Stock et al. (1963) (A, E, F); and Humes (1974) (D, H).

3.4. Discussion

The studies on the octopicolid copepods are scarce and date back to the past century. This has motivated the present work, in which scanning electron microscopy was used for the first time to illustrate relevant aspects of their morphology.

While comparing between the descriptions in the literature (Humes, 1957; Bocquet & Stock, 1960; Humes, 1963; Stock et al., 1963; Humes, 1974), we found convincing morphological evidence to justify raising the two subspecies of *O. superba* to full species status. At the time of description of *O. s. antillensis*, only one other octopicolid copepod, i.e. *O. superba*, had been described. The latter had also been isolated from *O. vulgaris* and the specimens isolated from the West Indian octopuses exhibited only small differences from the description of *O. superba*; this has led Stock et al. (1963) to consider that their specimens represent a new subspecies and not a new species. A critical analysis of the descriptions in the literature indicates that *O. stocki* and *O. regalis* also exhibit small differences from *O. s. superba* and *O. s. antillensis*, concerning the ornamentation of certain segments of given appendages; nonetheless, they are regarded as different species.

The best features underpinning the distinct species status of *O. s. superba* and *O. s. antillensis* are the characteristics of the antenna. This appendage exhibits the greatest morphological variability in octopicolid copepods, which conforms with what has been said before, that the antenna of the copepods in the lichomolgoidean complex of families (following Humes & Boxshall, 1996) is particularly vulnerable to morphological adaptations to the parasitic mode of life, i.e. changes that ensure an effective attachment to the host (see e.g. Ho, 1984). Furthermore, all species can be distinguished from one another by the specific features of the third antennal segment, as illustrated in this work (see Fig. 3.3 A-D). In recognising the existence of four different species of octopicolid copepods (i.e., *O. superba*, *O. antillensis*, *O. stocki* and *O. regalis*) it must be said that two other appendages, i.e. the maxilla and the male maxilliped, are also useful for identifying octopicolid copepods to the species level.

However, the differences between species in relation to the morphology of these appendages are not as conspicuous as those associated with the morphology of the antenna. Furthermore, the species differ from one another in the presence of setules on the first segment of the maxilla (ornamented with numerous small setules in *O. superba* and *O. antillensis vs* unornamented in *O. stocki* and *O. regalis*) and the ornamentation of the second segment, i.e. the number and the type of setae: one

spine-like seta in *O. superba*, one smooth seta plus one spine-like seta in *O. antillensis* and *O. regalis*, and two smooth setae in *O. stocki*. In the specific case of the male maxilliped, it was found that certain features are exclusively observed in particular species: *O. antillensis* is unique in bearing groups of spinules connecting the two rows of spinules on the second segment and small spinules at the base of the element at the dactylus; *O. regalis* is unique in bearing three rows of spinules on the second segment; and *O. stocki* is unique in bearing a conspicuous hyaline process near the base of the seta on the basal concave margin of the claw. However, the males of both *O. superba* and *O. stocki* exhibit two rows of spinules on the second segment of the maxilliped, the difference being in the degree of development of the spinules. Moreover, the latter appear to be larger in *O. stocki* (compare Fig. 3.3 J, K). *Octopicola superba* possesses a very small prominence at the base of the claw, which perhaps represents a hyaline process, as in *O. stocki*. The latter species is also unique in that the outermost seta on the endopod segment of leg 4 exhibits sexual dimorphism. This seta is spiniform and armed with short lateral spinules in females, and distinctly spiniform, sinuous, and armed with prominent lateral spinules in males.

As for the remaining species, *O. antillensis* is unique in bearing tergal plates on the fifth pedigerous somite, *O. superba* in having a subquadrate leg 5 and a very long seta representing leg 6 on the posterolateral area of the genital somite, and *O. regalis* in possessing larger body dimensions. Further species differences are seen in the combination of the following character states concerning the fifth pedigerous somite: (i) free segment of leg 5 subquadrate/subrectangular; (ii) larger of the two setae on the free segment of leg 5 with swollen base; (iii) seta dorsal and adjacent to the free segment of leg 5 arising directly from the body wall/arising from a distinct lobe; and (iv) presence of tergal plates.

It is worth noting that despite the large number of species of octopuses known to date, little attention has so far been devoted to their parasites. Therefore, it is highly likely that more octopicolid copepods remain to be discovered.

Key to the species of *Octopicola*

A critical analysis of the literature (Humes, 1957; Bocquet & Stock, 1960; Humes, 1963, 1974; Stock et al., 1963) resulted in identification of the morphological features that vary among species and subspecies. The existence of sufficient morphological

evidence to justify raising the two subspecies of *O. superba* to full species rank was evaluated; this resulted in the elaboration of the identification key provided below.

Females

1 Seta dorsal and adjacent to free segment of leg 5 arises directly from body wall ……..…….. 2

– Seta dorsal and adjacent to free segment of leg 5 arises from distinct lobe ……...……..…… 3

2 Third antennal segment with finely denticulated triangular process in addition to a spine and two setae; fifth pedigerous somite without tergal plates; most posterior seta on leg 6 long, reaches to posterior margin of next urosomal somite or beyond……...……..……. *O. superba*

– Third antennal segment with very prominent projection, half the length of accompanying claw, densely covered with long spinules in addition to a spine and two setae; fifth pedigerous somite with tergal plates; most posterior seta on leg 6 short, extends only slightly beyond the posterior margin of its own somite ……. *O. antillensis*

3 Third antennal segment bears two spines and one seta; second segment of maxilla armed with two smooth setae; first of graduated teeth on tapered process of second segment of maxilla not tooth-like.……...…...…….…… *O. stocki*

– Third antennal segment bears a claw-like jointed spine, a blunt spine with rows of long hairs along inner margin and a small naked seta; second segment of maxilla armed with one smooth seta plus one spine-like seta with spinules along one side; first of graduated teeth on tapered process of second segment of maxilla tooth-like……... *O. regalis*

Males

1 Seta dorsal and adjacent to free segment of leg 5 arises directly from body wall……. 2

– Seta dorsal and adjacent to free segment of leg 5 arises from distinct lobe ……..……..……. 3

2 Third antennal segment with finely denticulated triangular process in addition to a spine and two setae; inner surface of second segment of maxilliped without groups of spinules connecting rows of spinules; hyaline membrane near tip of claw (convex surface) bluntly pointed and smooth……. *O. superba*

– Third antennal segment with very prominent projection, half as long as accompanying claw, densely covered with long spinules in addition to a spine and two setae; inner surface of second segment of maxilliped with groups of spinules connecting rows of spinules; hyaline membrane near tip of claw (convex surface) prolonged into a small element with small spinules at its base……..…….. *O. antillensis*

3 Third antennal segment bears two spines and one seta; inner surface of second segment of maxilliped bears two rows of spinules; conspicuous hyaline process present at base of claw of maxilliped; outermost seta on endopod of leg 4 distinctly spiniform, sinuous and armed with prominent lateral spinules…..……..… *O. stocki*

– Third antennal segment bears a claw-like jointed spine, a blunt spine with rows of long hairs along inner margin, and a small naked seta; inner surface of second segment of maxilliped bears three rows of spinules; hyaline process at base of claw of maxilliped absent; outermost seta on endopod of leg 4 not spiniform, not sinuous and unarmed…….…..… *O. regalis*

3.5. Acknowledgements

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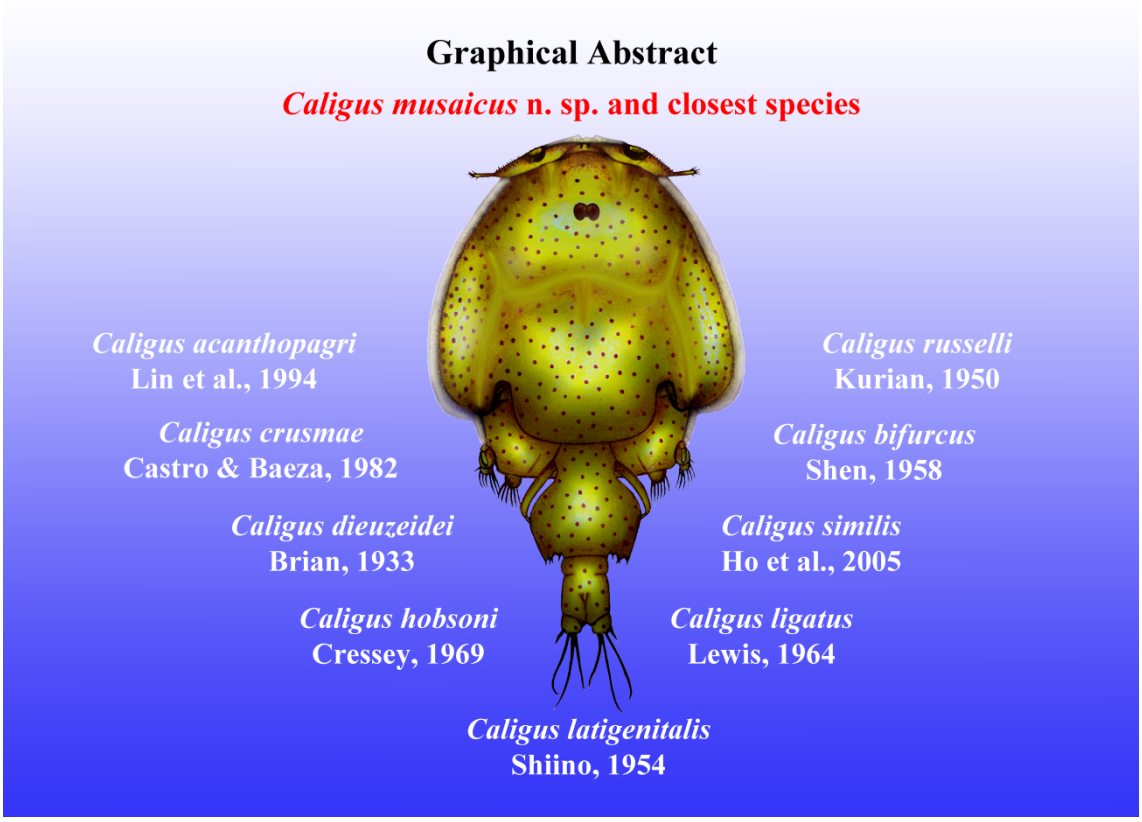
Chapter 4

Caligus musaicus n. sp. (Copepoda, Caligidae) parasitic on the European flounder, *Platichthys flesus* (Linnaeus) off Portugal



This chapter has been adapted from:

Cavaleiro, F. I., Santos, M. J., & Ho, J.-S. (2010). *Caligus musaicus* n. sp. (Copepoda, Caligidae) parasitic on the European flounder, *Platichthys flesus* (Linnaeus) off Portugal. *Crustaceana*, *83*, 457–464.

4.1. Abstract

A new species of caligid copepod, *Caligus musaicus* n. sp., is described from the European flounder, *Platichthys flesus* (Linnaeus, 1758), caught off the northern coast of Portugal. The new species is distinguished from its congeners by the combination of the following character states: (1) equipped with a short abdomen (about 1/3 the length of the thoracic zone of the cephalothoracic shield); (2) armed with a pair of parallel pointed tines on the box of the sternal furca; (3) bearing a long element IV (about 3 times as long as the next longest element) at the tip of leg 1 exopod; and (4) with a slender leg 4 exopod bearing a long outer seta (about 3 times as long as the next longest seta) at the tip of this ramus.

4.2. Introduction

The European flounder, *Platichthys flesus* (Linnaeus, 1758), is a demersal and catadromous fish with high commercial value. It is widely distributed in coastal and brackish waters of western Europe, extending from the White Sea to the Mediterranean and the Black Sea (Froese & Pauly, 2008). So far as we are aware, five species of parasitic copepods have been reported from this species of flounder. They are: *Acanthochondria cornuta* (Müller, 1776) reported by Ho (1970); *Caligus elongatus* von Nordmann, 1832 reported by Boxshall (1974a); *Lepeophtheirus europaensis* Zeddam, Berrebi, Renaud, Raibaut & Gabrion, 1988 reported by Zeddam et al. (1988); *L. pectoralis* (Müller, 1777) reported by Boxshall (1974a); and *Lernaeocera branchialis* (Linnaeus, 1767) reported by Polyanski (1955). While the first four species of parasites were found as adults on the flounder, the last one utilizes the flounder as an intermediate host; in other words, only the chalimus stages were seen.

While one of us (F.I.C.) was studying the Crustacea infections on the European flounder occurring off the northern coast of Portugal (Cavaleiro, 2007; Cavaleiro & Santos, 2007), a species of *Caligus* was occasionally encountered. It is a rare species of sea louse, with only 11 specimens being found through the examination of 210 host fish collected between September 2005 and May 2006. Close studies of this parasite revealed that it represents a new species. Inasmuch as both sexes are represented in this rare collection, a full description of the species is given in the following.

4.3. Materials and Methods

Flounders collected at Matosinhos fish harbour (in northern Portugal) were brought back to the laboratory on the campus of the Universidade do Porto for examination. The copepod parasites were removed from the fish host and were preserved in 70% ethanol. Later, the preserved parasites were cleared in 90% lactic acid for about 1 hour before making dissection in a drop of lactic acid. The dissected body parts and appendages were examined using a Zeiss Axiophot Photomicroscope at magnifications of up to 1000×. All drawings were made with the aid of a camera lucida. Measurements given are the mean followed by the range in parentheses. The description of the female is given in full but that of the male is confined only to those parts showing sexual dimorphism.

4.4. Results

CALIGIDAE Burmeister, 1835

Caligus Müller, 1785

Caligus musaicus n. sp. (Figs. 4.1-4.3)

Material examined. – Eleven specimens (4 ♀♀; 7 ♂♂) parasitic on the body skin and the pectoral and ventral fins of the European flounder, *Platichthys flesus* (Linnaeus, 1758) (Teleostei: Pleuronectidae), landed at Matosinhos fishing port, Portugal (41º10'N 8º42'W), as follows: 1 ♀ from body skin (blind side) of 1 flounder collected on 2 September 2005; 1 ♂ from body skin (ocular side) of 1 flounder collected 2 September 2005; 1 ♂ from body skin (blind side) of 1 flounder collected 2 September 2005; 1 ♂ from pectoral fin (ocular side) of 1 flounder collected 2 September 2005; 1 ♂ from ventral fin (ocular side) of 1 flounder collected 2 September 2005; 1 ♂ from body skin (ocular side) of 1 flounder collected 23 May 2006; 2 ♂♂ from body skin (blind side) of 2 flounders collected 23 May 2006; and 3 ♀♀ from body skin (ocular side) of 3 flounders collected on 23 May 2006.

All isolated parasite specimens were adults, the females being non-ovigerous. One holotype (USNM 1136866) and an allotype (USNM 1136867) are deposited in the Smithsonian Institution, Washington, D.C., and two paratypes have been deposited in the Natural History Museum, London, (Catalogue numbers: NHM 2010.248 and NHM 2010.249). The remaining specimens have been retained in the personal collections of the authors.

Female. – Body (Fig. 4.1 A) 4.41 (3.75–5.07) mm long, excluding setae on caudal rami. Cephalothoracic shield roughly triangular in shape, 2.53 (2.08–3.00) × 2.24 (1.94–2.41) mm, excluding lateral hyaline membrane; frontal plates well developed and carrying moderately large lunules (width slightly less than 1/3 that of the plates); free margin of thoracic zone projecting slightly beyond tips of lateral zones; sinuses deep. Fourth pediger wider than long, 0.26 (0.20–0.32) × 0.64 (0.52–0.85) mm, not separated from genital complex. Genital complex subcircular, 1.07 (0.75–1.22) × 1.24 (0.85–1.43) mm, about equally long or slightly longer than thoracic zone of cephalothoracic shield. Abdomen (Fig. 4.1 B) short, 1-segmented, measuring 0.47 (0.44–0.50) × 0.43 (0.40–

0.47) mm; bearing 8 papillae on dorsal surface, 6 with single setule and 2 with multiple setules. Caudal ramus about as long as wide, 0.16 (0.13–0.18) × 0.13 (0.10–0.16) mm; armed with 2 short, 1 medium, and 3 long plumose setae in addition to a setule-bearing papilla on dorsal surface and a row of setules on medial margin.

Antennule (Fig. 4.1 C) 2-segmented; proximal segment carrying 25 setae on anterodorsal surface, 2 of them naked, plus 2 small setae on ventral surface; distal segment with 1 subterminal seta on posterior margin and tipped with 11 setae plus 2 aesthetascs. Antenna (Fig. 4.1 D) 3-segmented; proximal segment smallest, with short, pointed posteromedial process; middle segment subrectangular and armed with 1 corrugated and well developed adhesion pad near medial region of medial border; distal segment long, curved claw bearing 2 setae, 1 proximal and broad, the other comparatively thinner and close to medial region. Postantennal process a large hook with 2 basal setule-bearing papillae; another similar papilla on sternum. Maxillule comprising short but pointed dentiform process and basal papilla tipped with 3 setae. Mandible (Fig. 4.1 E) with 4 sections, bearing 12 teeth on medial margin of distal blade. Maxilla (Fig. 4.1 F) 2-segmented and brachiform; proximal segment (lacertus) unarmed; distal segment (brachium) carrying small, subterminal hyaline membrane (flabellum) on outer edge and 2 unequal elements at terminal end, a short canna, and a long calamus. Maxilliped (Fig. 4.1 G) 3-segmented; proximal segment (corpus) largest but unarmed; middle segment (shaft) carrying small, digitiform process at mediodistal corner; distal segment (claw) with long medial barbel. Box of sternal furca (Fig. 4.1 H) quadrangular and carrying 2 parallel pointed tines, fringed with membrane along their entire length and shorter than box.

Formula of armature of rami on legs 1-4 as follows (Roman numerals indicating spines and Arabic numerals indicating setae):

	Exopod	**Endopod**
Leg 1	1-0; III, I, 3	(vestigial)
Leg 2	I-1; I-1; II, I, 5	0-1; 0-2; 6
Leg 3	I-0; I-1; 7	0-1; 6
Leg 4	I-0; I, III	(absent)

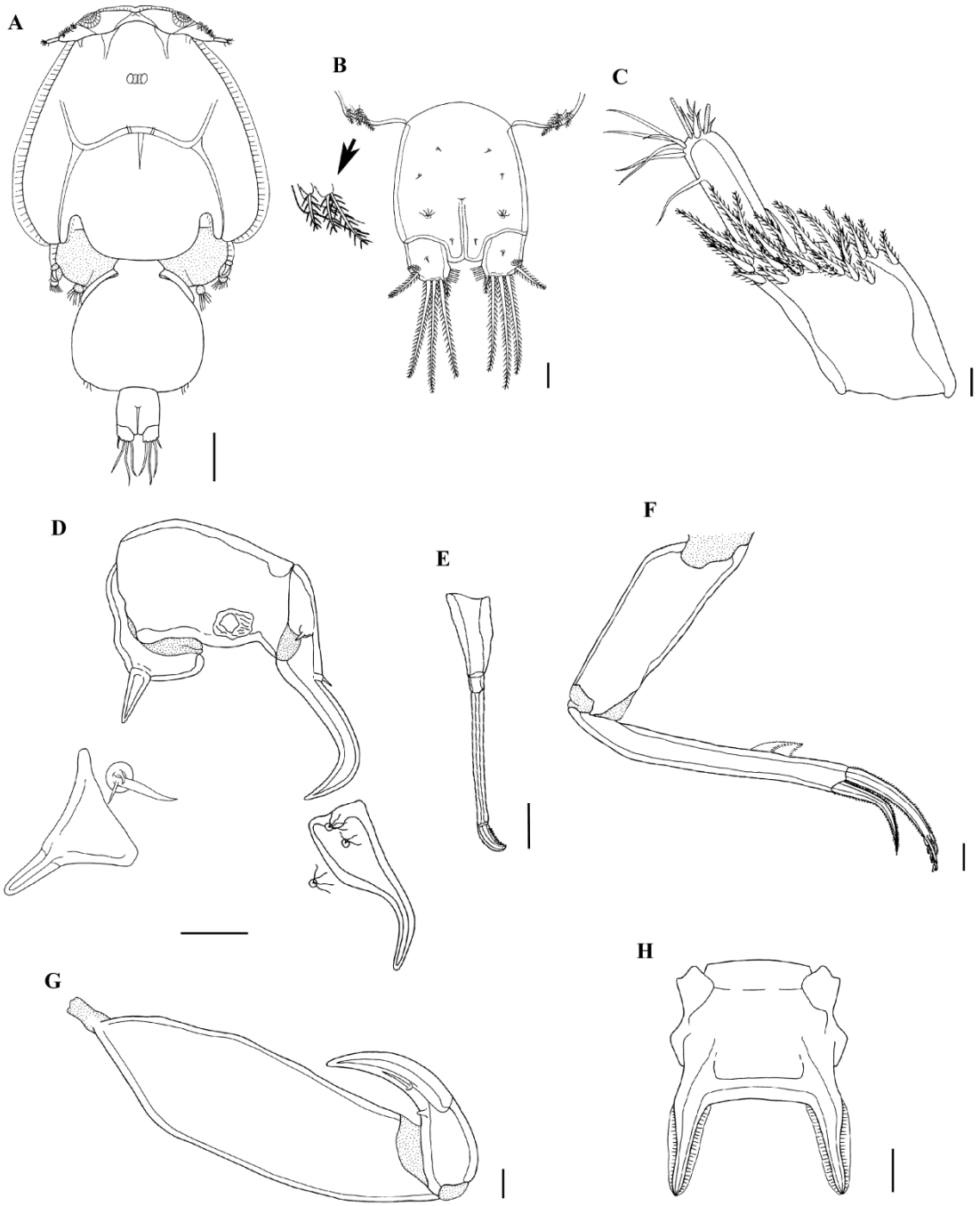


Fig. 4.1 – *Caligus musaicus* n. sp., female. A, Habitus, dorsal; B, Abdomen and caudal rami; C, Antennule; D, Antenna, postantennal process and maxillule; E, Mandible; F, Maxilla; G, Maxilliped; and H, Sternal furca. *Scale-bars*: A, 0.5 mm; B, D, 100 µm; C, 50 µm; and E-H, 50 µm.

Protopod of leg 1 (Fig. 4.2 A) with long plumose outer seta and another similar inner seta, in addition to a papilla bearing 2 setules on outer margin of coxa. Endopod a small inconspicuous process. First segment of exopod with a row of setules on posterior edge and small spiniform seta on outer distal corner; middle two of 4 terminal

elements on last segment of exopod with accessory process; element 4 about 3 times as long as element 2 and bearing setules only on outer margin. Leg 2 (Fig. 4.2 B) coxa small, with large plumose inner seta on posterior edge and long setule-bearing papilla on ventral surface. Basis carrying long seta on outer edge in addition to long setule-bearing papilla on ventral surface, close to base of posterior marginal membrane. Anterodistal surface of basis and first segment of exopod with large marginal membrane. Outer margin of 3 endopodal segments with a tuft or row of small setules. Leg 3 (Fig. 4.2 C) protopod (apron) with small outer and large inner plumose setae, in addition to an outer and a posterior marginal membrane; ventral surface of protopod with small setule-bearing papilla at both ends of that membrane; velum well developed and fringed with marginal setules. Leg 4 (Fig. 4.2 D) protopod large, with plumose seta at outer distal corner; exopod 2-segmented, due to fusion of distal two segments; pecten at base of each seta on exopod; outer terminal seta about 3 times as long as middle one. Leg 5 (Fig. 4.1 B) represented by 2 small papillae on posterolateral corner of genital complex, one tipped with a single and the other with 2 small, plumose setae.

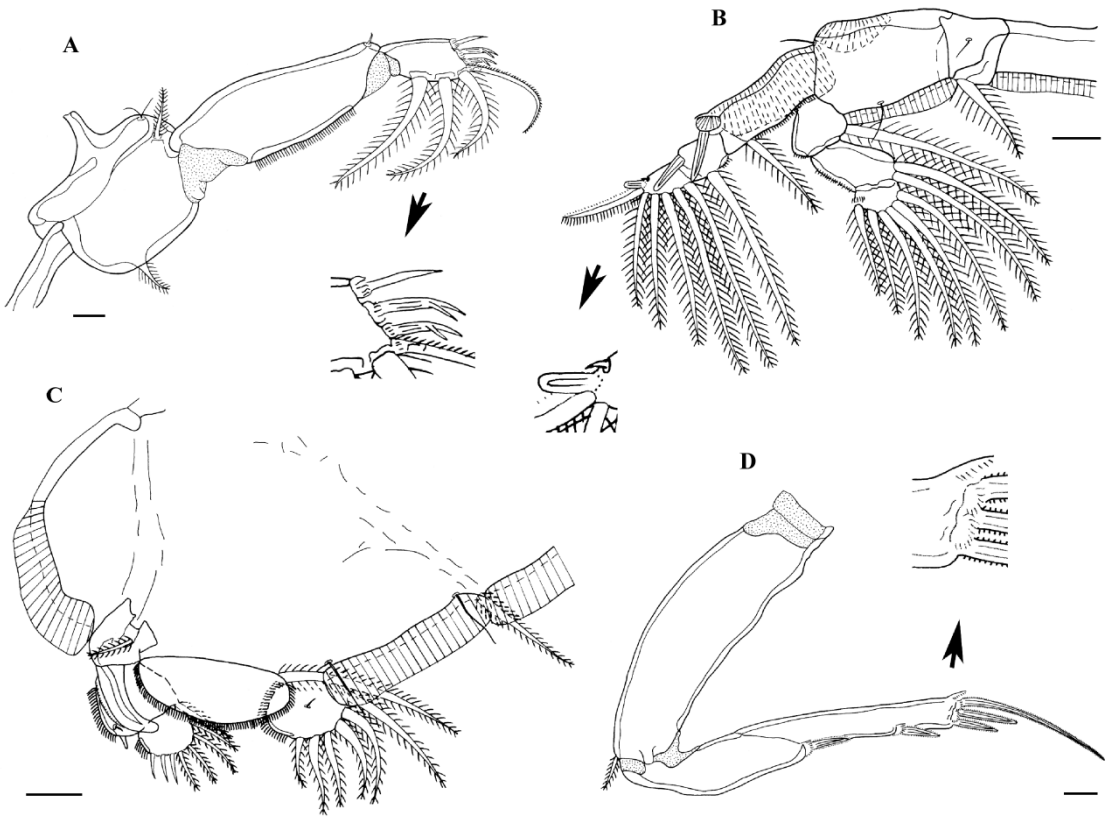


Fig. 4.2 – *Caligus musaicus* n. sp., female. A, Leg 1; B, Leg 2; C, Leg 3; and D, Leg 4. *Scale-bars*: A, D, 50 µm; and B, C, 100 µm.

Male. – Body (Fig. 4.3 A) 3.42 (3.25–3.64) mm long, excluding setae on caudal rami. Cephalothoracic shield roughly triangular in shape, 2.09 (1.91–2.26) × 1.93 (1.81–2.03) mm; frontal plates well developed and carrying moderately large lunules (width slightly less than 1/3 that of the plates); free margin of thoracic zone projecting slightly beyond tips of lateral zones; sinuses deep. Fourth pediger not separated from genital complex, roughly hexagonal in shape and about 2 times as wider as long, 0.20 (0.16–0.28) × 0.46 (0.41–0.50) mm. Genital complex subrectangular, 0.54 (0.50–0.59) × 0.76 (0.73–0.80) mm, smaller than thoracic zone of cephalothoracic shield, and with 2 small protuberances on posterolateral corners. Abdomen (Fig. 4.3 B) partially 2-segmented; proximal somite smallest and distinctly wider than long, 0.46 (0.45–0.49) × 0.39 (0.35–0.42) mm; anal somite subsquare, 0.36 (0.31–0.41) × 0.38 (0.34–0.41) mm. Caudal ramus about equally long as wide, 0.16 (0.14–0.18) × 0.15 (0.13–0.17) mm, armed as in female. Antenna (Fig. 4.3 C) 3-segmented; proximal segment slender, armed with long corrugated pad on outer surface; middle segment largest, armed with 3 pads in addition to a corrugated band; terminal segment smallest, armed with 2 basal setae and 2 overlapping cuticular flaps bearing pointed tips. Maxilliped (Fig. 4.3 D) generally as in female except for corpus being more robust and bearing in myxal region a small dentiform protuberance and another bipartite protuberance. Leg 5 (Fig. 4.3 B) located on outer protuberance on posterolateral corner of genital complex comprising 2 papillae, one tipped with 1 and the other with 2 plumose setae. Leg 6 represented by a posterolateral ridge on genital complex carrying a protuberance tipped with 1 plumose seta.

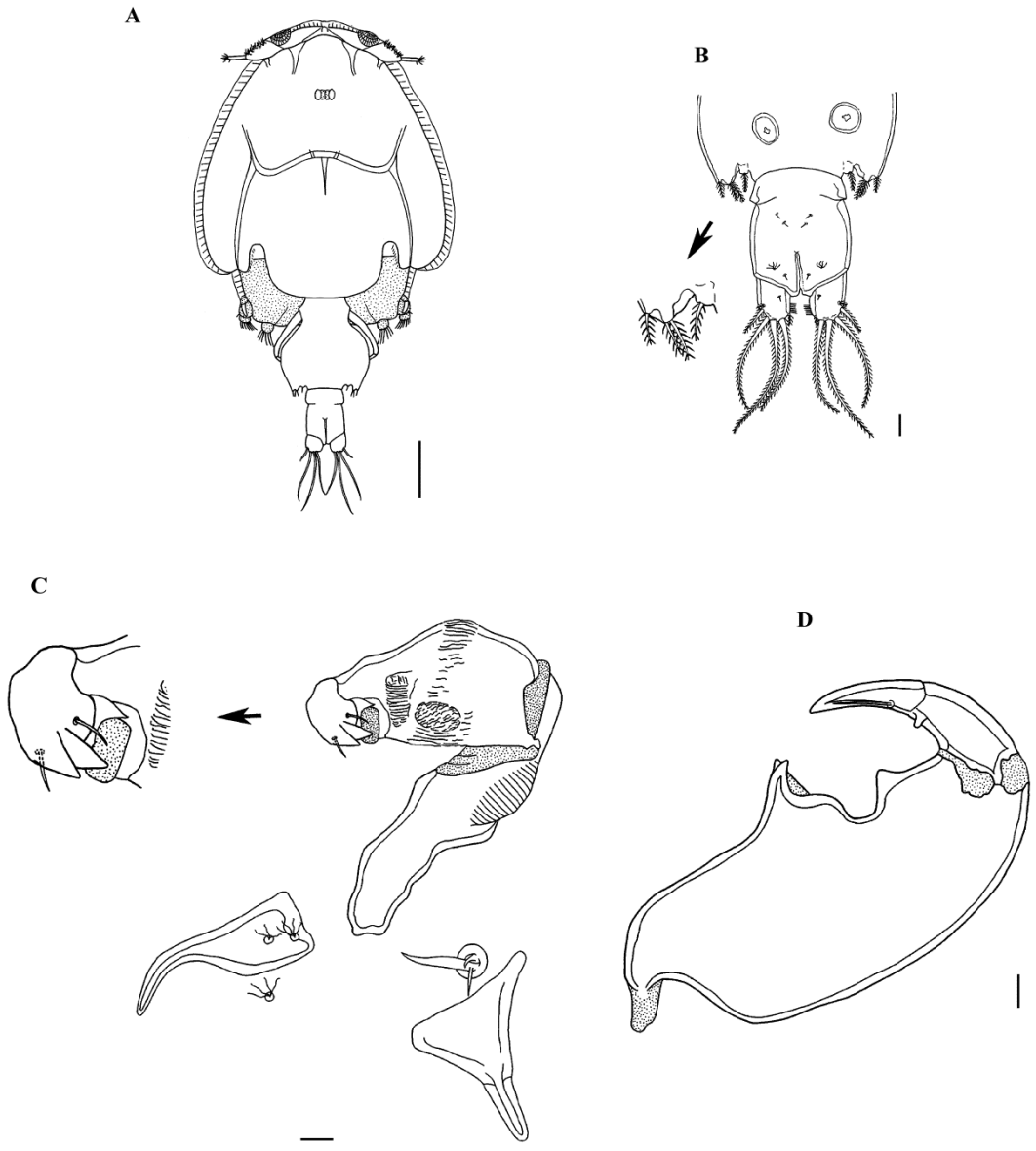


Fig. 4.3 – *Caligus musaicus* n. sp., male. A, Habitus, dorsal; B, Abdomen and caudal rami; C, Antenna, postantennal process and maxillule; and D, Maxilliped. *Scale-bars*: A, 0.5 mm; B, 100 µm; and C, D, 50 µm.

Etymology. – The species name *musaicus* is the Latin word for mosaic. It alludes to the species' resemblance with several of its congeners, in such a way that it reminds of a genetic mosaic, i.e., an organism whose body consists of a mixture of cells of two or more different genotypes.

4.5. Discussion

Caligus Müller, 1785 is the largest genus of parasitic copepods, containing over 250 species (Ho & Lin, 2004). Since the male remains unknown for many of them, comparison of our specimens obtained from the flounder with its congeners is accordingly restricted to the female.

As far as we can find, there are 9 species of *Caligus* showing closeness to *C. musaicus* n. sp. in sharing the following 3 character states with the new species: (1) a short abdomen (about 1/3 the length of the thoracic zone of the cephalothoracic shield), (2) bearing a long seta IV (about 3 times as long as the next longest element) at the tip of leg 1 exopod, and (3) with a slender, 2-segmented leg 4 exopod bearing a long outer seta (about 3 times as long as the next longest seta) at the tip of this ramus. Those 9 species of *Caligus* are: *C. acanthopagri* Lin et al., 1994; *C. crusmae* Castro & Baeza, 1982; *C. dieuzeidei* Brian, 1933; *C. hobsoni* Cressey, 1969; *C. latigenitalis* Shiino, 1954; *C. ligatus* Lewis, 1964; *C. similis* Ho et al., 2005; *C. bifurcus* Shen, 1958; and *C. russelli* Kurian, 1950. Nevertheless, the new species can be distinguished from the first 7 species mentioned above in the possession of a pair of parallel pointed tines on the sternal furca (see Fig. 4.1 H). Of the remaining two species, *C. bifurcus* can be distinguished from the new species by the structure of the sternal furca (being narrower), and *C. russelli*, in the structure of the postantennal process and the corpus of the maxilliped. Besides, seta IV (the longest element) at the tip of the exopod of leg 1 in the new species is unusual in bearing setules only on one side (outer margin) of the element.

4.6. Acknowledgements

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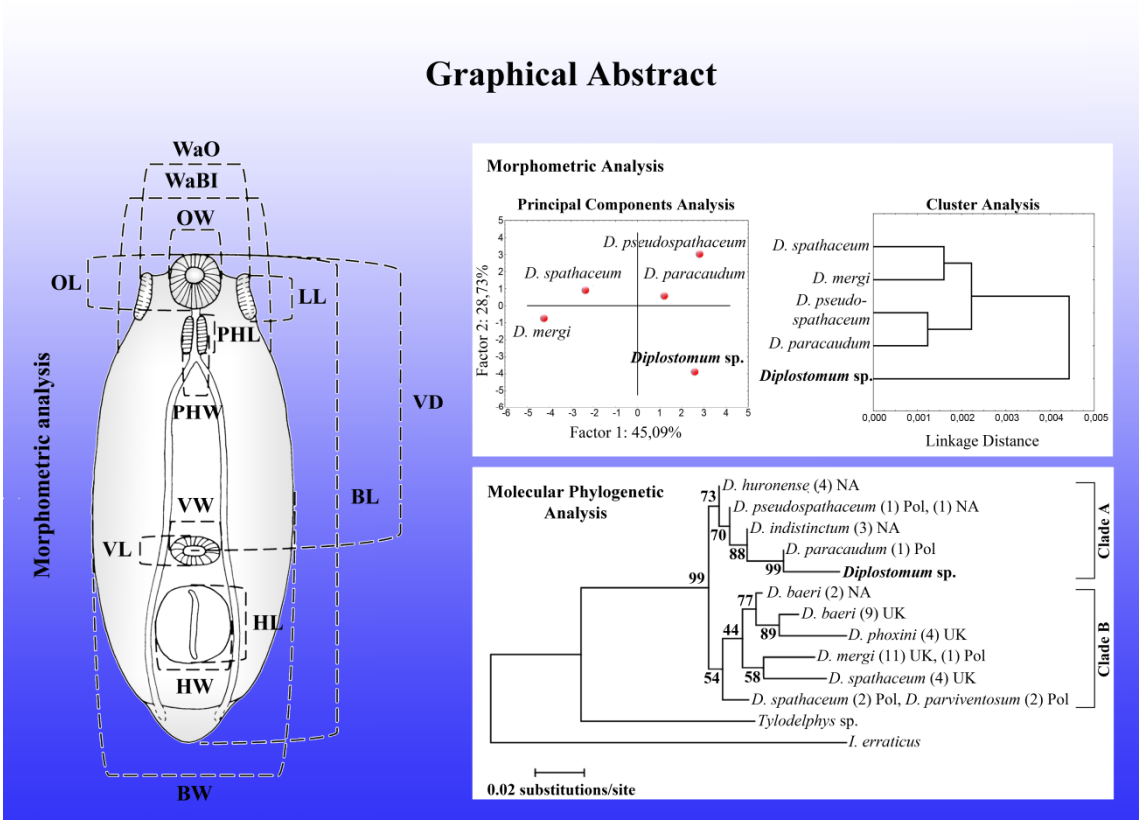
Chapter 5

Morphology, ultrastructure, genetics, and morphometrics of *Diplostomum* sp. (Digenea: Diplostomidae) metacercariae infecting the European flounder, *Platichthys flesus* (L.) (Teleostei: Pleuronectidae), off the northwest coast of Portugal



This chapter has been adapted from:

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5.1. Abstract

The morphology, ultrastructure, genetics, and morphometrics of a species of *Diplostomum* von Nordmann 1832 (Digenea: Diplostomidae), isolated from the European flounder (*Platichthys flesus* (L.)) caught off the northwest coast of Portugal, are characterized. The metacercarial stage was found unencysted in the lens capsule of the eye. Light microscopical observations revealed the existence of some variability in specimen shape and size, with two morphotypes, referred to as 'round' and 'long', being apparent. Scanning electron microscopy revealed a smooth, unarmed tegument, with the lappet region being the most irregular and porose. Both the oral and ventral suckers were provided with a series of papillae, which presented very distinctive ultrastructural features and were particularly conspicuous in the case of the ventral sucker. The two morphotypes detected were found to have 100% genetic correspondence in the 18S+ITS1+5.8S region of the rDNA. Since the genetic data for this metacercaria differed from those of the species of *Diplostomum* available in GenBank, a description of a new genotype (accession number GQ370809) is provided. The molecular phylogenetic analyses, in conjunction with principal components and cluster analyses based on morphometric data, revealed the existence of consistent differences between the *Diplostomum* sp. metacercariae from flounder compared with *Diplostomum spathaceum*, *Diplostomum mergi*, *Diplostomum pseudospathaceum*, and *Diplostomum paracaudum*. The latter of these species was found to be the most similar to the present material. Our results do not support an evolutionary separation of the European and North American species of *Diplostomum*.

5.2. Introduction

The lens of the eyes in freshwater fishes has frequently been highlighted in the literature as the site of infection of metacercariae of *Diplostomum spathaceum* (Rudolphi, 1819) (Digenea: Diplostomidae) (see e.g., Kennedy & Burrough, 1978; Conneely & McCarthy, 1984; Dwyer & Smith, 1989; Inchausty et al., 1997; Moravec, 2003). Indeed, for many years, it was a common procedure to assign all metacercarial specimens isolated from the lens to that particular species, whereas those isolated from the vitreous body and retina were generally assumed as representatives of *Diplostomum gasterostei* Williams, 1966 or simply *Diplostomum* sp. (Valtonen & Gibson, 1997). Over the years, despite a huge amount of work on metacercariae of species of *Diplostomum* von Nordmann 1832 and even a book (Shigin, 1986) and a key (Shigin, 1976), identification has remained problematical. This dilemma was commented on by Chappell (1995), Niewiadomska & Niewiadomska-Bugaj (1995), and Gibson (1996). Despite the fact that numerous techniques have been used, e.g., chaetotaxy and multivariate analysis, morphometric studies have invariably led to misidentifications or at least questionable identifications. Attempts at growing metacercariae in birds, invariably in unnatural species (likely definitive hosts are often protected), and even eggs have been disappointing. Nevertheless, some studies have attempted to discriminate between different species, e.g., *D. spathaceum* from *Diplostomum baeri* Dubois, 1937 (see Höglund & Thulin, 1992), *D. spathaceum* from *Diplostomum pseudobaeri* Razmaskin & Andrejak, 1978 (see Field & Irwin, 1995), *Diplostomum paracaudum* (Iles, 1959) from *Diplostomum pseudospathaceum* Niewiadomska, 1984 (see Niewiadomska & Niewiadomska-Bugaj, 1995), and *D. spathaceum* from *Diplostomum mergi* Dubois, 1932 (see Niewiadomska & Niewiadomska-Bugaj, 1998), by comparing their morphometrics. In the past, great efforts have been made to complete the life-cycles of *Diplostomum* species in order to achieve an accurate identification (Field et al., 1994; Field & Irwin, 1995; McKeown & Irwin, 1995). Presently, it is expected that a reliable identification of metacercariae to the species level may only be assumed if different kinds of data, e.g., morphological, ultrastructural, genetic, and morphometric, are linked in one and the same study, especially when experimental infection data are used to help confirm the species identity.

During the course of a recent investigation, metacercarial forms of *Diplostomum* were isolated with some regularity from the eye lens of the European flounder, *Platichthys flesus* (Linnaeus, 1758) (Teleostei: Pleuronectidae), caught off the northwest coast of Portugal. The marine situation for larval *Diplostomum* is unusual,

except in regions of low salinity, such as the Baltic Sea. However, flounders are euryhaline, spending part of the year in estuaries and even moving deep into freshwater (Lucas & Baras, 2001). The present study is intended to provide a full characterization of these metacercariae from flounders, in an attempt to determine their identity. Aspects of the morphology, ultrastructure, genetics, and morphometrics are characterized, and the resulting data are compared with those available in the literature and in the GenBank in an attempt to identify the specimens to the specific level.

5.3. Materials and Methods

Collection and identification of the metacercariae

Flounder specimens that were captured by beam trawling in northwest Portuguese offshore waters were brought to the laboratory at Porto University campus for parasitological examination. After dissection and removal from the fish, the eyes were opened to reveal the lens, vitreous body, and subretinal regions, which were examined for metacercariae. The worms recovered were washed in 0.9% saline solution and roughly identified using the descriptions of and keys to the metacercarial diplostomoids in Hughes (1929) and Gibson et al. (2002), and then following the identification key to the metacercariae in fishes available in Gibson (1996). The further processing of specimens depended on the analysis to be performed, i.e., morphology, ultrastructure, genetics, or morphometrics and is described below.

Morphological analysis

In order to characterize the general body morphology, isolated metacercariae were first examined alive under a stereomicroscope. Next, they were mounted in a drop of 0.9% saline solution and observed using light microscopy (Carl Zeiss Axiophot Photomicroscope) at magnifications of up to 1000×. Images of the entire worms and the relevant structural details were recorded at different magnifications.

Ultrastructural analysis

Specimens fixed in 70% ethanol were cleaned and prepared for scanning electron microscopy (SEM). The technique, slightly modified from that described in Felgenhaeur (1987), was as follows:

(1) Specimens were transferred to vials containing a 16% glycerol solution prepared with distilled water, and the vials were placed in a shaker table overnight with the aim of removing the mucus from the body surface; (2) the glycerol solution was completely emptied from the vials; these were then filled with 20% ethanol and placed in the shaker table for 10 h to remove all traces of glycerol; (3) the metacercariae were dehydrated through a graded ethanol series, i.e., 30%, 50%, 80%, and 100% ethanol, remaining immersed for about 15 min in each of these solutions; (4) the vials were carefully sonicated for about 10 s; (5) the metacercariae were critical point-dried in CO_2, then mounted on stubs using slow cure Araldite, i.e., epoxy glue, allowed to dry overnight and coated with 20 nm of gold-palladium; finally, they were examined in a scanning electron microscope (Philips XL30 FEG) at an accelerating voltage of 5 kV.

Genetic analysis

DNA extraction, PCR amplification, and sequencing

DNA from 20 ‘round’ and 18 ‘long’ metacercariae recovered from naturally infected *P. flesus* was extracted using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma, St. Louis, MO) according to the manufacturer’s instructions.

The 18S+ITS1+5.8S region of the rDNA was amplified using a primer located about 141 bp from the 3′ end of the conserved region of the ssrDNA (18S-ITS1: 5′-CCG TCG CTA CTA CCG ATT GAA-3′) and a primer located about 95 bp from the 5′ end of the 5.8S region (5.8S-ITS1: 5′-CGCAATGTGCGTTCAAGATGTC-3′).

A polymerase chain reaction (PCR) was carried out in a total volume of 50 µl consisting of 10× PCR reaction volume, 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, 0.4 µM of each primer, 1 U platinum Taq polymerase, and 2 µl genomic DNA. The cycling conditions were as follows: one cycle of initial denaturation at 94°C for 5 min; 40 cycles at 94°C for 30 s, 54°C for 30 s, and 72°C for 2 min; plus a final extension at 72°C for 10 min. Samples without DNA were included in each amplification run to exclude contamination.

Amplified PCR products were analysed by electrophoresis in a 1.0% agarose gel stained with ethidium bromide, purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and sequenced. The obtained sequence that included the partial 18S, ITS1 (complete), and partial 5.8S was submitted to GenBank under accession number GQ370809.

Molecular phylogenetic analysis

Nucleotide sequence data were compared for similarity by searching the GenBank-NCBI database using the Basic Local Alignment Search Tool (BLAST, www.ncbi.nlm.nih.gov/blast), and multiple sequence alignments were performed using Multalin (available at http://bioinfo.genotoul.fr/multalin/multalin.html) and the ClustalW version 2 software (Larkin et al., 2007).

Partial and complete ITS1 sequences of identified species of *Diplostomum* as well as of two out-group species have been retrieved from GenBank for molecular and phylogenetic studies. *Tylodelphys* sp. (Diplostomidae), which has been indicated as a genus closely ancestral to *Diplostomum* by Galazzo et al. (2002), together with *Ichthyocotylurus erraticus* (Rudolphi, 1809) (Strigeidae) were selected as out-groups. Taxonomic names, developmental stage, hosts, ITS1 length, collecting sites, and GenBank accession numbers are provided in Table 5.1.

Table 5.1 – Digenean species used in this study, their hosts, ITS1 length, geographical origin, and GenBank accession numbers for the corresponding sequences.

Digenean taxa	Stage	Host species	ITS1 length (bp)	Geographical origin	GenBank no.
Family Diplostomidae					
Diplostomum sp.	Metacercaria	*Platichthys flesus*	607	Portugal, Matosinhos	GQ370809
D. baeri	Cercaria	*Lymnaea peregra*	650[a]	UK, Scotland	AY386162
	Metacercaria	*Gasterosteus aculeatus*	650[a]	UK, Scotland	AY386149
		Oncorhynchus mykiss	650[a]	UK, Scotland	AY386145-48
		O. mykiss	650[a]	UK, Scotland	AY386152
		Perca flavescens	604	Canada, Montreal	AY123042
		Pimephales notatus	585[a]	Canada, Quebec	GQ292505
		Rutilus rutilus	649[a]	UK, England	AY386150
		Scardinius erythrophthalmus	650[a]	UK, England	AY386151
D. huronense	Metacercaria	*Catostomus commersoni*	603	Canada, Montreal	AY123044
		C. commersoni	591[a]	Canada, Quebec	GQ292507
		C. commersoni	600[a]	Canada, Quebec	GQ292509
		C. commersoni	592[a]	Canada, Quebec	GQ292513
D. indistinctum	Metacercaria	*C. commersoni*	607	Canada, Montreal	AY123043
		C. commersoni	594[a]	Canada, Quebec	GQ292508
		Neogobius melanostomus	589[a]	Canada, Quebec	GQ292506
D. mergi	Cercaria	*Radix ovata*	580[a]	Poland, Warsaw	AF419279
	Metacercaria	*Abramis bramae*	650[a]	UK, England	AY386140
		Cyprinus carpio	650[a]	UK, England	AY386137
		O. mykiss	648[a]	UK, Scotland	AY386134
		O. mykiss	650[a]	UK, Scotland	AY386135-36
		O. mykiss	650[a]	UK, Scotland	AY386138-39
		O. mykiss	650[a]	UK, Scotland	AY386141
		Salmo trutta	651[a]	UK, Scotland	AY386142-43
		S. salar	650[a]	UK, Scotland	AY386144

Table 5.1 (continuation) – Digenean species used in this study, their hosts, ITS1 length, geographical origin, and GenBank accession numbers for the corresponding sequences.

Digenean taxa	Stage	Host species	ITS1 length (bp)	Geographical origin	GenBank no.
D. pseudospathaceum	Cercaria	*Lymnaea stagnalis*	578[a]	Poland, Warsaw	AF419273
	Metacercaria	*Micropterus salmoides*	595[a]	Canada, Quebec	GQ292511
D. spathaceum	Cercaria	*R. ovata*	579[a]	Poland, Warsaw	AF419275-76
	Metacercaria	*G. aculeatus*	650[a]	UK, Scotland	AY386153
		O. mykiss	650[a]	UK, Scotland	AY386155-56
		S. salar	650[a]	UK, Scotland	AY386154
D. parviventosum	Cercaria	*R. ovata*	586[a]	Poland, Warsaw	AF419277-78
D. phoxini	Metacercaria	*Phoxinuns phoxinus*	648[a]	UK, Scotland	AY386157-60
D. paracaudum	Cercaria	*R. ovata*	579[a]	Poland, Warsaw	AF419272
Tylodelphys sp.	Metacercaria	*R. rutilus*	652[a]	UK, Scotland	AY386164
Family Strigeidae					
Ichthyocotylurus erraticus	Metacercaria	*Coregonus lavaretus*	781	Finland	AJ301887
		C. albula	781	Finland	AJ301887

[a]ITS1 rDNA partial sequence.

Phylogenetic and molecular evolutionary analyses were conducted on the aligned partial nucleotide sequences of ITS1 using MEGA software version 4 (Tamura et al., 2007). The neighbour-joining (NJ) method (Saitou & Nei, 1987) was performed using the program's default settings. The reliability of internal branches in the NJ trees was assessed using bootstrap analysis with 10,000 replicates. The resulting networks were rooted with the out-group taxa.

Morphometric analysis

The morphometric data were assessed using a Carl Zeiss Axiophot Photomicroscope equipped with an Axiocam ICc3 camera and connected to a computer with version 4.6.3 of the Axiovision digital image processing software (Carl Zeiss Microimaging Inc., Thornwood, NY, USA). A series of 14 metric dimensions were assessed from the

metacercariae (*N* = 30), i.e., the length (BL) and width (BW) of the body; the length (OL) and width (OW) of the oral sucker; the length (PHL) and width (PHW) of the pharynx; the length (VL) and width (VW) of the ventral sucker; the length (HL) and width (HW) of the holdfast organ; the distance between the anterior extremity of the body and the center of the ventral sucker (VD); the length of the lappets (LL); and the width of the body at the level of the bifurcation of the intestine (WaBI) and at the mid-length of the oral sucker (WaO) (see Fig. 5.1), plus eight indices, i.e., BW/BL (in percent); BL×BW/HL×HW; BL×BW/VL×VW; OL×OW/VL×VW; HL×HW/VL×VW; OL×OW/PHL×PHW; VD/BL (in percent); and WaO/WaBI, including the corresponding means, ranges, coefficients of variation, and limits of the 95% confidence interval for the population means (Niewiadomska & Niewiadomska-Bugaj, 1995, 1998). Those metric dimensions and indices contributing most to the variability found among the isolated specimens were evaluated by running a multiple factorial analysis on version 8.0 of the statistical program package STATISTICA for Windows (StatSoft Inc., Tulsa, USA). The morphometric segregation of the species of *Diplostomum* isolated in this study from *D. paracaudum*, *D. pseudospathaceum*, *D. spathaceum*, and *D. mergi* was evaluated by running principal component and cluster (similarity measure, 1−Pearson's correlation coefficient) analyses using the same software. Morphometric comparisons were limited to those made possible by the data available in the literature. Moreover, the data used in such comparisons were retrieved from Niewiadomska & Niewiadomska-Bugaj (1995) for *D. paracaudum* and *D. pseudospathaceum* and from Niewiadomska & Niewiadomska-Bugaj (1998) for *D. spathaceum* and *D. mergi*.

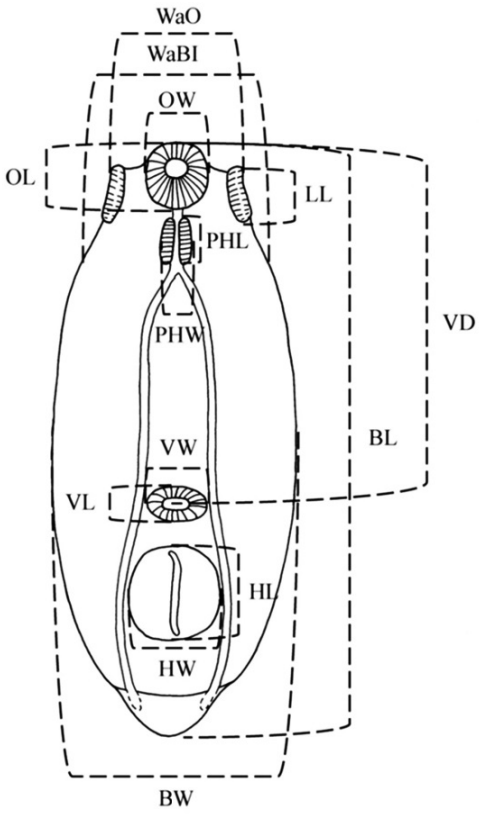

Fig. 5.1 – Measurements taken from the metacercariae of *Diplostomum* sp. isolated from the lens of the eye of the European flounder, *Platichthys flesus*, caught off the northwest coast of Portugal (abbreviations: BL, Length of the Body; BW, Width of the Body; OL, Length of the Oral sucker; OW, Width of the Oral sucker; PHL, Length of the PHarynx; PHW, Width of the PHarynx; VL, Length of the Ventral sucker; VW, Width of the Ventral sucker; HL, Length of the Holdfast organ; HW, Width of the Holdfast organ; VD, Distance between the anterior extremity of the body and the center of the Ventral sucker; LL, Length of the Lappets; WaBI, Width of the body at the level of the Bifurcation of the Intestine; and WaO, Width of the body at the mid-length of the Oral sucker).

5.4. Results

Identification of the metacercariae

The isolated metacercariae were unencysted. They were site-specific, i.e., exclusively found in the lens capsule, presenting accelerated and rhythmical lengthening and shortening movements when alive. All were identified as specimens of *Diplostomum*.

Morphological analysis

Body thin, varying considerably in shape and size, with two distinct morphotypes, herein referred to as ‘round’ and ‘long’, being recognised among the isolated specimens (Fig. 5.2 A, B). ‘Round’ and ‘long’ morphotypes coexist in the same lens.

The anterior end of the body trilobate, with small lateral protuberances (lappets) on either side of the oral sucker; the ventral sucker, oval to round, located post-equatorially and similar in size or slightly larger than the oral sucker. Just posterior to and comparatively larger than the ventral sucker is the holdfast organ (Fig. 5.2 A, B). The digestive tract comprises a prepharynx, wide pharynx, short oesophagus, and two blind intestinal caeca which terminate close to the posterior end of the body and often contain granules of irregular shape. Primordial gonads are generally visible as long, irregular, pale brown structures located around or posterior to the holdfast organ. The paranephridial part of the excretory system is readily visible and consists of the excretory bladder, which appears distinctly as two large outgrowths at the posterior end of the body, and one median and two lateral longitudinal canals provided with ramifications that terminate in spherical pockets filled with excretory concretions (calcareous corpuscles). The protonephridial part of the excretory system, i.e., the flame-cell system, is usually difficult to discern, even in fresh worms (Fig. 5.2 C).

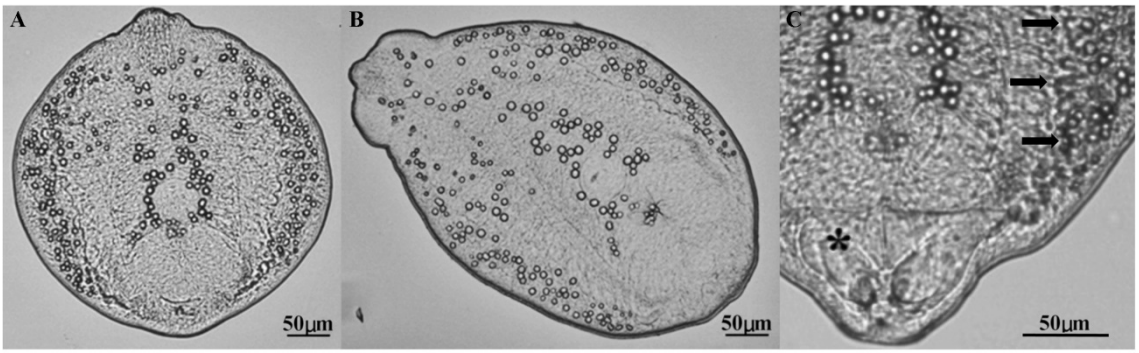

Fig. 5.2 – The *Diplostomum* sp. metacercariae, isolated from the lens of the eye of the European flounder, *Platichthys flesus*, caught off the northwest coast of Portugal. Two morphotypes A 'round', B 'long', and C a detail of the posterior region of the body and excretory system (*asterisk*, excretory bladder; and *arrows*, excretory canal).

Ultrastructure

Additional features were visible using the SEM (Fig. 5.3 A-F).

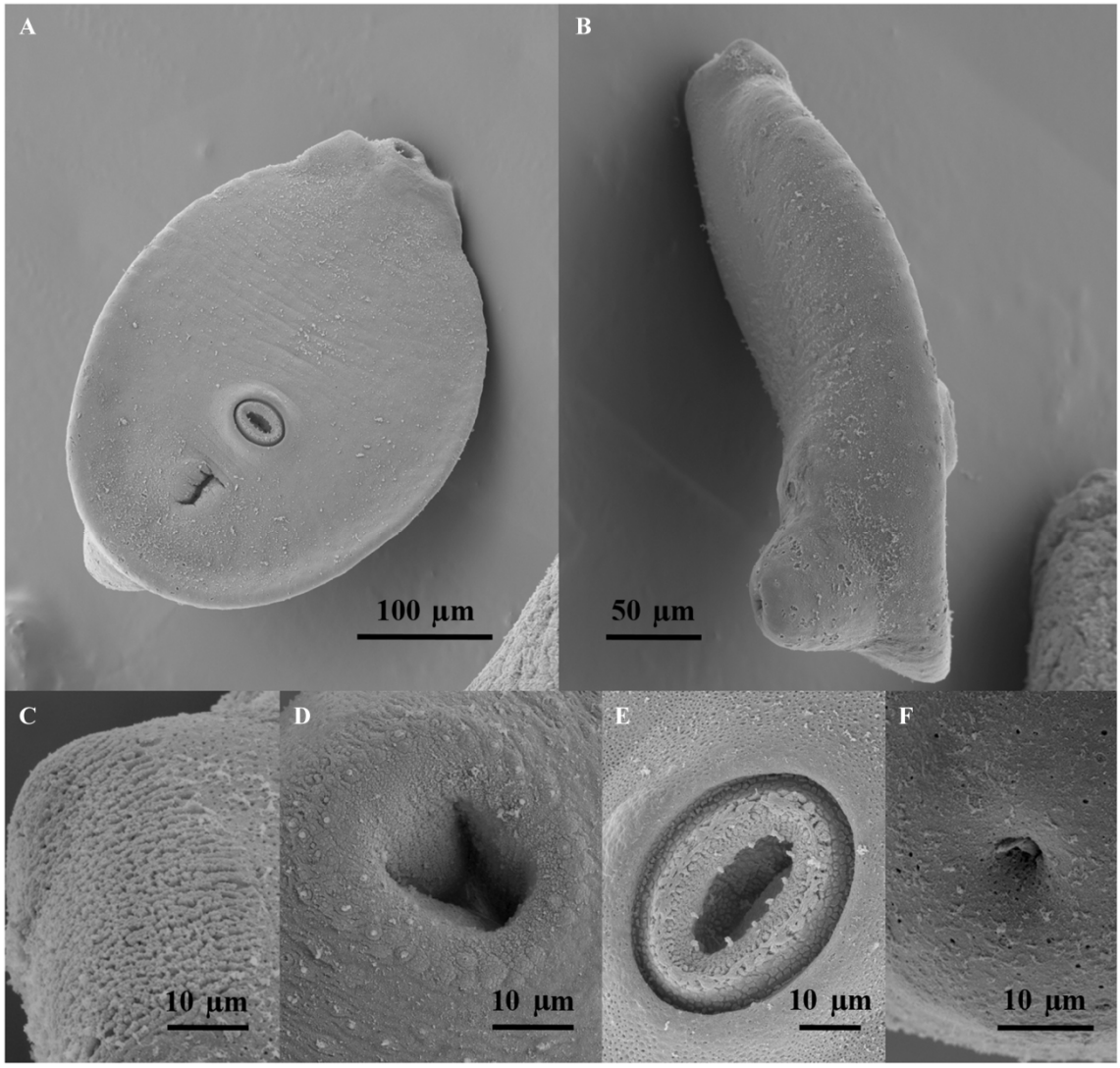

Fig. 5.3 – Ultrastructural aspects of the metacercaria of *Diplostomum* sp. isolated from the lens of the eye of the European flounder, *Platichthys flesus*, as revealed by scanning electron microscopy: A, Whole body, ventral surface; B, Whole body, dorsolateral surface; C Lappet region; D, Oral sucker; E, Ventral sucker; and F, Excretory pore.

The forebody (sensu Niewiadomska, 2002, p. 160) includes most of the worm, whereas the hindbody (sensu Niewiadomska, 2002, p. 160) is reduced to a small, postero-dorsal, conical eminence, at the tip of which it is possible to recognize the excretory pore. The ventral surface of the forebody is flat or slightly concave, and the dorsal surface is somewhat convex. Its tegument is unarmed and smooth, but somewhat irregular and porose in the region of the lappets. The mouth is ventrally subterminal. Two types of papillae were identified (Fig. 5.4 A, B): on the oral sucker, lappets and a region of the forebody anterior to the ventral sucker, the papillae were all of a similar structure, consisting of a round to elliptical base, a high tegumentary collar, and a short, cilium-like projection; on the ventral sucker, the papillae were particularly

conspicuous, lacked a visible tegumentary collar, and consist of a single, short, digitiform, cilium-like projection. A deep, longitudinal slit represents the aperture of the holdfast organ.

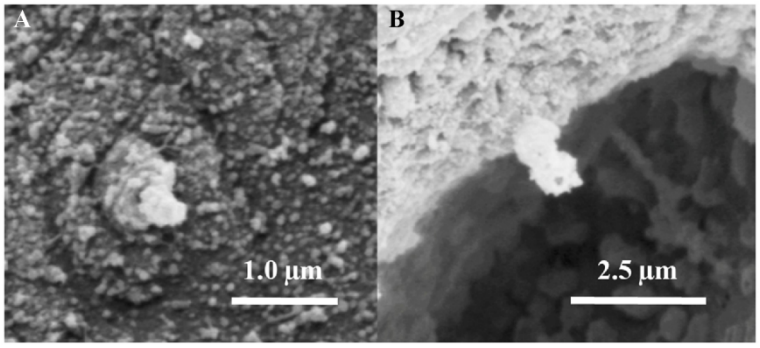

Fig. 5.4 – Ultrastructural view of the papillae found on A the oral sucker, lappets and forebody anterior to the ventral sucker and B the ventral sucker of *Diplostomum* sp. metacercariae from the lens of the eye of the European flounder, *Platichthys flesus.*

Molecular analysis

The PCR amplification of the 18S+ITS1+5.8S region of the rDNA from the two different forms of metacercariae found resulted in a single product of identical size, 810 nucleotides long. After the analysis of the PCR product, the first 132 bp were identified as corresponding to the 18S gene coding region. The following 607 bp were the ITS1 sequence, and the last 71 bp coded for the ribosomal 5.8S unit. The sequences obtained from the two metacercarial forms were identical.

A BLAST of the novel ITS1 sequence revealed the existence in GenBank of several high similarity sequences, all belonging to species of *Diplostomum*. In order to study the new sequence from the *Diplostomum* sp. obtained in our study, partial and complete ITS1 sequences for named (and assumed to be correctly identified) species of *Diplostomum* were retrieved from GenBank (Table 5.1). Pairwise alignments performed using the same partial ITS1 regions showed that the greatest similarity was found between *Diplostomum* sp. and *D. paracaudum*, as these exhibited few intraspecific differences (4/572 bp), i.e., 0.7% variation. *Diplostomum* sp. differed from *Diplostomum indistinctum* (Guberlet, 1923) at six sites (1.0%) including gaps; from *D. pseudospathaceum* at eight sites (1.4%); from *Diplostomum huronense* (La Rue, 1927) at 14 sites (2.5%); from *D. spathaceum* (samples from the UK) at 20 sites (3.5%); from *D. baeri* (samples from Canada) at 21 sites (3.7%); from *D. baeri* (samples from the

UK) at 22 sites (3.8%); from *D. spathaceum* and *D. parviventosum* Dubois, 1932 (samples from Poland) both at 25 sites (4.4%); from *D. mergi* at 30 sites (5.3%); and finally from *D. phoxini* (Faust, 1918) at 34 sites (6.0%).

The aligned sequences of the partial ITS1 region (572 nucleotides) of *Diplostomum* sp., *D. paracaudum*, *D. indistinctum*, *D. pseudospathaceum*, and *D. huronense* are presented in Fig. 5.5.

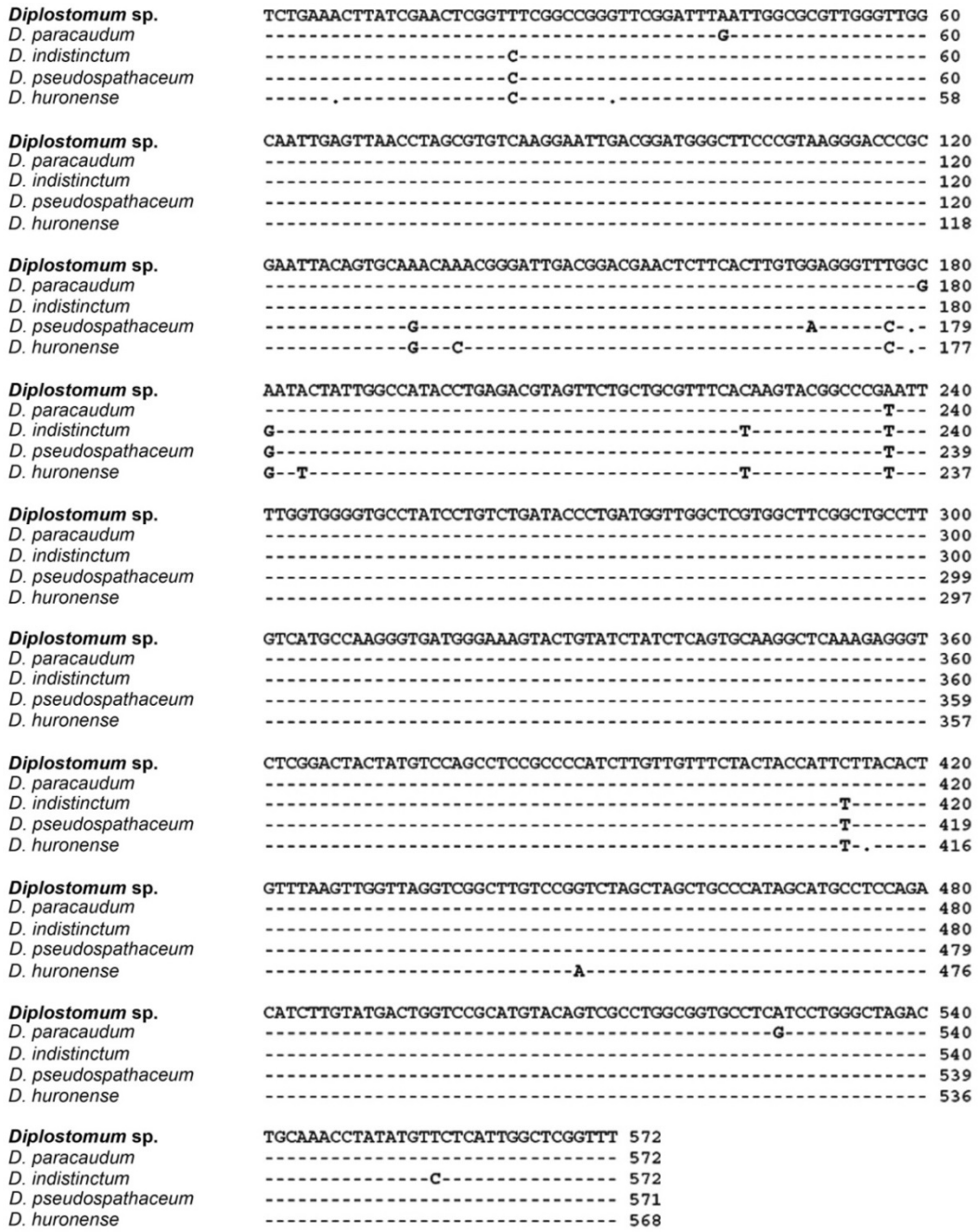

Fig. 5.5 – Partial alignment of the ITS1 rDNA region of *Diplostomum* sp. (present study), *D. paracaudum*, *D. indistinctum*, *D. pseudospathaceum*, and *D. huronense*. A *hyphen* indicates that the nucleotide, at that position, is identical to the top sequence belonging to *Diplostomum* sp. A *dot* indicates a gap in the alignment.

Phylogenetic analyses were conducted based on the alignment of partial and complete sequences of ITS1 rDNA using the NJ method. The resultant tree presented bootstrap consensus values of > 50% for almost all branches (Fig. 5.6). In addition, besides the expected positioning of the sequences of the out-groups, *Tylodelphys* sp. (Diplostomidae) and *I. erraticus* (Strigeidae), the cluster containing all of the *Diplostomum* spp. was clearly divided into two distinct clades (referred to as A and B). This observation was strongly supported by a high bootstrap value (99%). The species of *Diplostomum* whose metacercarial stage is described in this study was found in Clade A, branching with *D. paracaudum* (robustly supported by a 99% bootstrap value). The length of the branches, greater in Clade B than in Clade A, suggested closer phylogenetic relationships between the species of Clade A. The positioning of European and North American *Diplostomum* spp. does not indicate an evolutionary separation in terms of geography. Three European species (*Diplostomum* sp. from our study, *D. paracaudum* and *D. pseudospathaceum*) were closely associated with the material of three North American species (in Clade A), whereas the North American material of *D. baeri* fell within a clade composed of material of five European species (in Clade B).

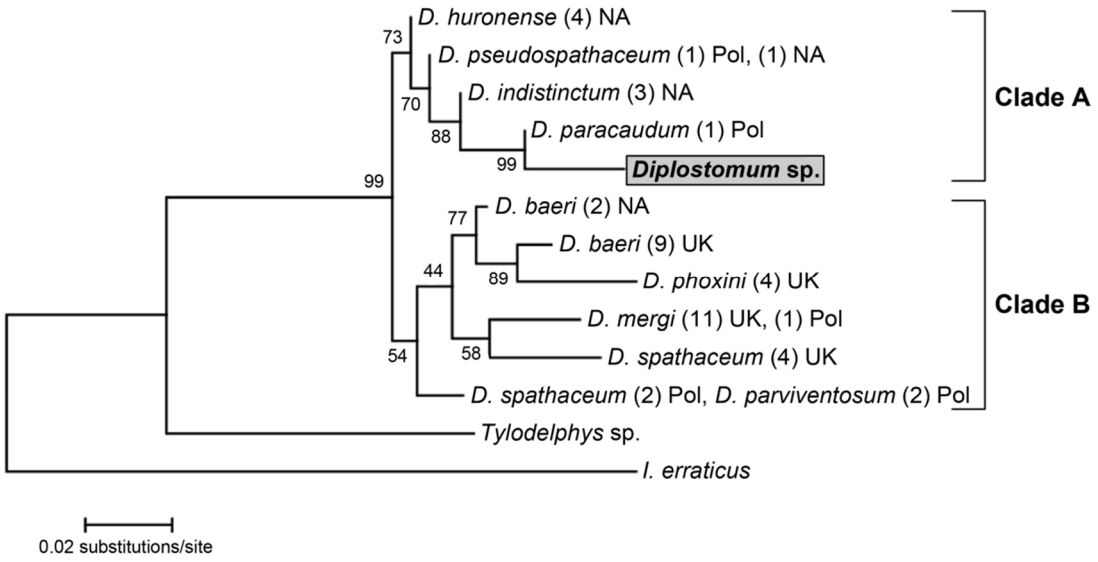

Fig. 5.6 – Phylogenetic tree depicting the relationships between *Diplostomum* spp., *Tylodelphys* sp., and *Ichthyocotylurus erraticus* as inferred from 48 ITS1 rDNA sequences using the NJ method. Numbers at the nodes represent the bootstrap values and where a clade of multiple sequences has been collapsed to a terminal branch, the numbers of sequences are in parentheses (abbreviations: NA, North America; Pol, Poland; and UK United Kingdom).

Morphometric analysis

Data on the metrical dimensions and indices are presented in Table 5.2. The coefficient of variation was highest for OL, HL, HW, and BL×BW/HL×HW.

Table 5.2 – Metric dimensions of characters and indices (mean, range, coefficient of variation, and limits of the 95% confidence interval for the population mean) for *Diplostomum* sp. metacercariae isolated from the lens of the eye of the European flounder, *Platichthys flesus*, caught off the northwest coast of Portugal (abbreviations: BL, Length of the Body; BW, Width of the Body; OL, Length of the Oral sucker; OW, Width of the Oral sucker; PHL, Length of the PHarynx; PHW, Width of the PHarynx; VL, Length of the Ventral sucker; VW, Width of the Ventral sucker; HL, Length of the Holdfast organ; HW, Width of the Holdfast organ; VD, Distance between the anterior extremity of the body and the center of the Ventral sucker; LL, Length of the Lappet; WaBI, Width of the body at the level of the Bifurcation of the Intestine; and WaO, Width of the body at the mid-length of the Oral sucker).

Character/Index	Mean	Range	Coefficient of variation (%)	Limits of the 95% confidence interval for the population mean
BL	465.2	293–569	14.7	440.6–489.7
BW	184.2	118–240	17.2	172.8–195.5
OL	53.2	22–66	19.6	49.5–56.9
OW	54.1	31–71	18.7	50.4–57.7
PHL	33.7	19–42	16.4	31.8–35.7
PHW	29.8	17–44	18.2	27.9–31.8
VL	42.9	27–57	17.9	40.1–45.6
VW	45.9	28–63	18.5	42.8–48.9
HL	78.8	48–113	19.9	73.1–84.4
HW	70.5	44–96	19.5	65.6–75.4
VD	295.9	180–361	15.1	279.9–311.9
LL (left)	46.7	25–58	17.3	43.8–49.6
LL (right)	46.3	28–57	16.9	43.5–49.1
WaBI	150.3	93–200	17.3	141.0–159.6
WaO	99.7	61–123	15.5	94.2–105.3
BW/BL (%)	39.6	32.7–46.4	8.4	38.4–40.8
BL×BW/HL×HW	16.6	10.2–38.8	40.9	14.1–19.0
BL×BW/VL×VW	45.3	28.0–78.1	26.3	41.1–49.6
OL×OW/VL×VW	1.5	0.8–3.0	36.6	1.3–1.8
HL×HW/VL×VW	3.0	1.3–6.9	39.2	2.6–3.4
OL×OW/PHL×PHW	3.0	0.9–5.0	32.2	2.7–3.3
VD/BL (%)	63.6	61.3–66.7	2.3	63.1–64.1
WaO/WaBI	0.7	0.5–0.8	10.1	0.6–0.7

The results of the principal component analysis revealed some degree of concordance with those obtained from the molecular phylogenetic analyses. The factorial model indicated that the variables mean WaO/WaBI, mean VL, mean HW,

mean OL×OW/PHL×PHW, and mean VW were those contributing most to the formation of factor 1, whereas mean PHL, mean OL, mean VD/BL (percent), and mean OL×OW/VL×VW were most influential in the formation of factor 2 (Fig. 5.7).

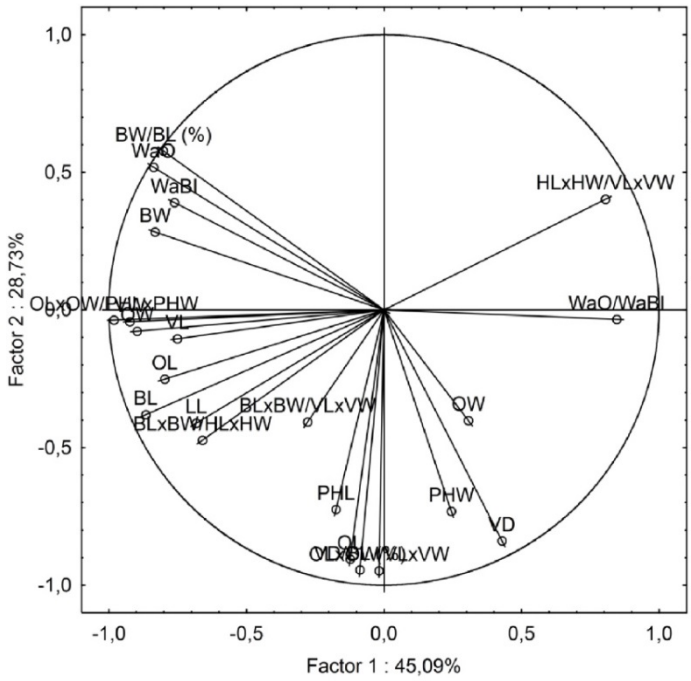

Fig. 5.7 – Variables factor map (*PCA*) for *Diplostomum* sp. – projection of the mean metric dimensions and indices on factor planes 1 and 2 (abbreviations: BL, Length of the Body; BW, Width of the Body; OL, Length of the Oral sucker; OW, Width of the Oral sucker; PHL, Length of the PHarynx; PHW, Width of the PHarynx; VL, Length of the Ventral sucker; VW, Width of the Ventral sucker; HL, Length of the Holdfast organ; HW, Width of the Holdfast organ; VD, Distance between the anterior extremity of the body and the center of the Ventral sucker; LL, Length of the Lappets; WaBI, Width of the body at the level of the Bifurcation of the Intestine; and WaO, Width of the body at the mid-length of the Oral sucker).

When considering the case projections in relation to factor 1, which explained 45.1% of the variability found, two groups could be identified, with *D. spathaceum* and *D. mergi* in opposition to the other three species. When considering the case projections in relation to factor 2, which explained 28.7% of the variability found, a group including all of the species, except for *Diplostomum* sp., was apparent (Fig. 5.8).

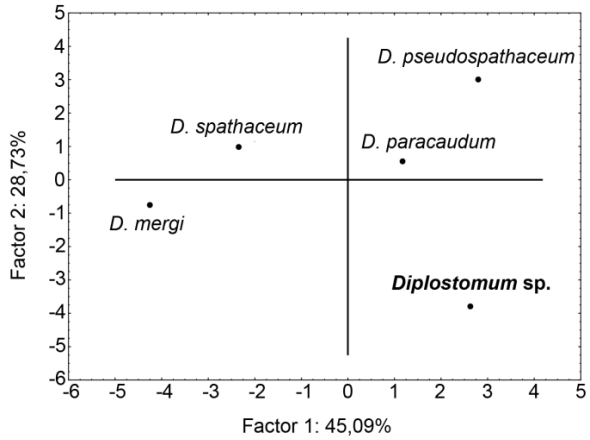

Fig. 5.8 – Principal components analysis – variable (*Diplostomum* sp., *D. paracaudum*, *D. pseudospathaceum*, *D. spathaceum*, and *D. mergi*) projection for factor planes 1 and 2.

A cluster of the morphometric data is shown in Fig. 5.9; this indicates that the five species in question can be classified according to their dimensions into two main groups, one of which appears divided in two subgroups and the other consists of a single species, i.e., *Diplostomum* sp. from flounders.

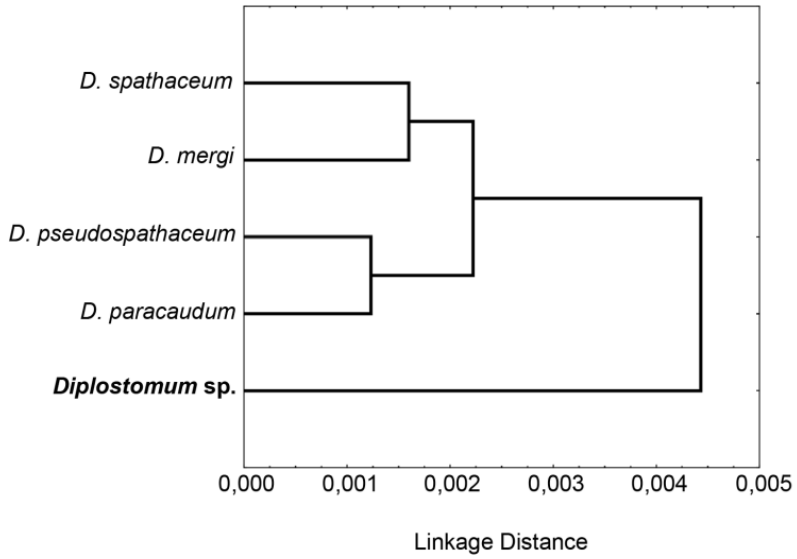

Fig. 5.9 – Cluster analysis (morphometrics) for *Diplostomum* sp. and genetically closely related species of *Diplostomum* – *D. paracaudum*, *D. pseudospathaceum*, *D. spathaceum* and *D. mergi*.

5.5. Discussion

Literature records of *Diplostomum* spp. in the European flounder include references to *D. baeri* (see Lüthen, 1988), *D. mergi* (see Chibani & Rokicki, 2004) and *D. spathaceum* (e.g., Brucko-Stempkowski, 1970; Kennedy et al., 1992; Dorovskikh, 1997; Køie, 1999; Palm et al., 1999). Such records are mainly from the Baltic region where the salinity is low. None of these corresponds to the metacercarial stage described in this study – assuming that the species were correctly identified and judging from both the phylogenetic analyses (i.e., all of these species are situated in clade B, whereas *Diplostomum* sp. from flounders is in clade A; Fig. 5.6) and the morphometric data. Furthermore, the species isolated does not correspond with any of those whose genetic characterization is available in GenBank. Since more than forty different species of *Diplostomum* exist (Niewiadomska, 1996), most of which have not been characterized in molecular terms, it is not possible to determine the identity of the material from flounders. Indeed, there also exists the possibility that it has been previously detected as *Diplostomum* sp. in flounders from the Baltic Sea (Engelbrecht, 1958; Chibani & Rokicki, 2004) in cases where authors have been unwilling to speculate on a specific identification.

Interestingly, the results of the molecular phylogenetic and morphometric analyses exhibit some degree of concordance with regard to differences between *Diplostomum* sp. and *D. spathaceum*, *D. mergi*, *D. pseudospathaceum*, and *D. paracaudum*, and also suggest that it is most similar to *D. paracaudum*. Nevertheless, the real value of the results derived from the morphometric analyses are a matter of contention (compare, for example, the works of A. A. Shigin and K. Niewiadomska on the same species). The implications of extensive morphometric variation in terms of the reliability of specimen identification to the species level have recently been pointed out by Chibwana & Nkwengulila (2009) in a work intended to discriminate between three closely related diplostomid species, i.e., *D. mashonense* Beverley-Burton 1963 and *Tylodelphys* spp. 1 and 2. Also, in another work, on opecoelid larvae, an extensive morphological measurement overlap was reported in three genetically different worms (Violante-González et al., 2009). According to the literature, different factors may act to produce significant variations in body dimensions. These include, among others, the host species, its size and age, the age of the metacercariae (e.g., Graczyk, 1991, 1992; Niewiadomska & Szymański, 1991, 1992), and the population size associated with intensity-dependent growth (Saldanha et al., 2009). Even taking into account other obvious factors, such as worm condition at fixation, the fixation technique, and other procedural variations, one can assume that, in this study, the variability found in body

shape represents different stages of metacercarial development, since the same lens was found infected with both 'round' and 'long' morphotypes.

By assessing the rDNA sequence data (partial ITS1 sequences) from adult forms, Galazzo et al. (2002) were able to demonstrate using phylogenetic analyses that North American and European species of *Diplostomum* include divergent groups – the American ones, i.e., *D. indistinctum*, *D. huronense*, and *D. baeri*, being basal to the European, i.e., *D. paracaudum*, *D. pseudospathaceum*, *D. spathaceum*, *D. parviventosum*, *D. mergi*, and *D. baeri*. These authors also showed that *D. baeri* from Europe was not conspecific with '*D. baeri*' from North America. The present study, which was conducted on specimens from a flatfish, *P. flesus*, whose geographical distribution is limited to European waters (Whitehead et al., 1986), lends further support to the idea of differences in the geographical distribution of species within the genus, since *D. paracaudum*, another European species, exhibited the greatest genetic similarity to the present material. It should be noted, however, that, although in this study the length of the ITS1 sequence (607 bp) equals that found by Galazzo et al. (2002) for *D. huronense*, *D. indistinctum*, and North American *D. baeri*, and differs from those found by Niewiadomska & Laskowski (2002) for *D. parviventosum*, *D. spathaceum*, and *D. paracaudum* (all 580 bp), *D. mergi* (579 bp), *D. pseudospathaceum* (578 bp), and European *D. baeri* (576 bp), it proved to be useful in discriminating between *Diplostomum* sp. and the other nine species of *Diplostomum* which have been characterized genetically. However, Niewiadomska & Laskowski (2002) found no molecular differences between *D. spathaceum* and *D. parviventosum*, although these species present apparent morphological differences at the cercarial, metacercarial, and adult stages. According to Galazzo et al. (2002), the North American and European species represent divergent groups within *Diplostomum*. Although the distribution of the molluscan hosts also needs to be taken into account, Locke et al. (2010) have suggested that the presence of these two groups do not support an evolutionary history associated with a geographical divergence of the species, given the mobility of the avian definitive hosts. This latter proposal is in accordance with our findings, which indicate that there does not appear to be an evolutionary separation of the European and North American species of *Diplostomum*. The results of the present work also reinforce the idea that different kinds of data should be considered for the accurate identification of diplostomid metacercariae at the specific level.

5.6. Acknowledgements

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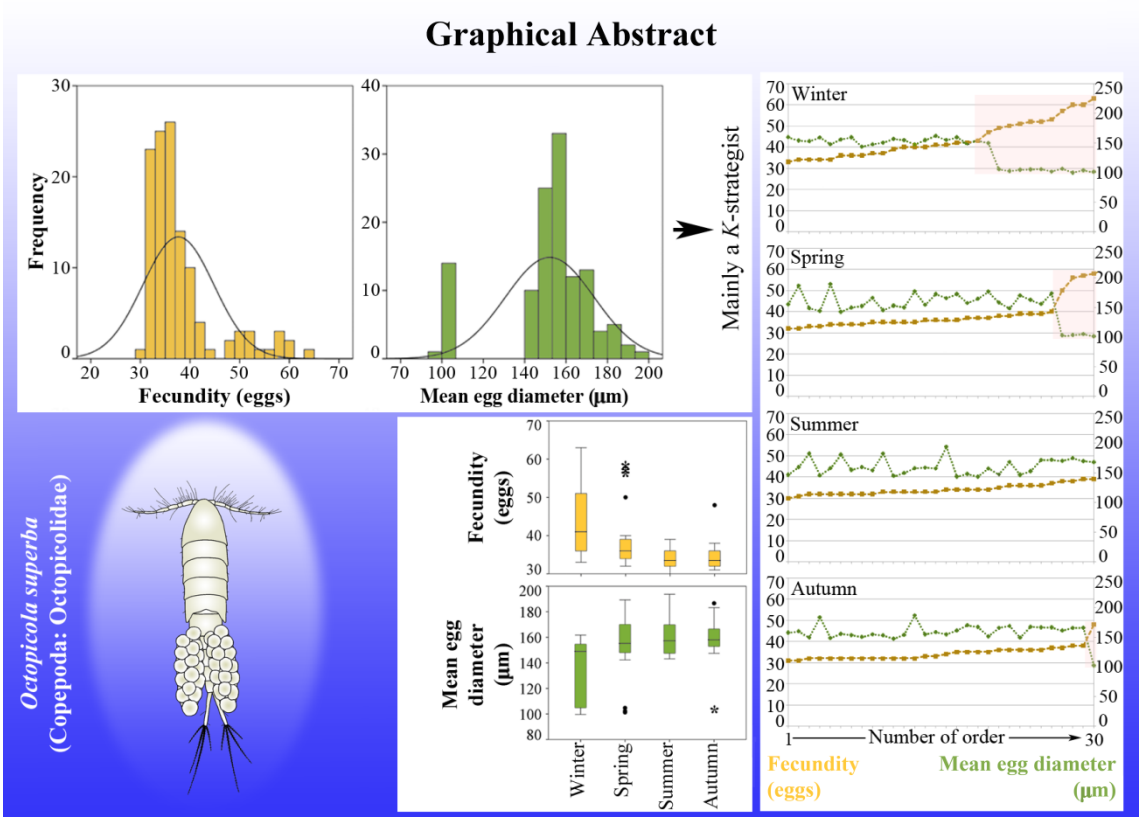
Chapter 6

Egg number-egg size: an important trade-off in parasite life history strategies



This chapter has been adapted from:

Cavaleiro, F. I., & Santos, M. J. (In Press). Egg number-egg size: an important trade-off in parasite life history strategies. *International Journal for Parasitology*.

6.1. Abstract

Parasites produce from just a few to many eggs of variable size, but our understanding of the factors driving variation in these two life history traits at the intraspecific level is still very fragmentary. This study evaluates the importance of performing multilevel analyses on egg number and egg size, while characterizing parasite life history strategies. A total of 120 ovigerous females of *Octopicola superba* (Copepoda: Octopicolidae) (one sample [*N* = 30] per season) were characterized with respect to different body dimensions (total length; genital somite length) and measures of reproductive effort (fecundity; mean egg diameter; total reproductive effort; mean egg sac length). While endoparasites are suggested to follow both an *r*- and *K*-strategy simultaneously, the evidence found in this and other studies suggests that environmental conditions force ectoparasites into one of the two alternatives. The positive and negative skewness of the distributions of fecundity and mean egg diameter, respectively, suggest that *O. superba* is mainly a *K*-strategist (i.e. produces a relatively small number of large, well provisioned eggs). Significant sample differences were recorded concomitantly for all body dimensions and measures of reproductive effort, while a generalised linear model (GLM) detected a significant influence of season*parasite total length in both egg number and size. This evidence suggests adaptive phenotypic plasticity in body dimensions and size-mediated changes in egg production. Seasonal changes in partitioning of resources between egg number and size resulted in significant differences in egg sac length but not in total reproductive effort. Evidence for a trade-off between egg number and size was found while controlling for a potential confounding effect of parasite total length. However, this trade-off became apparent only at high fecundity levels, suggesting a state of physiological exhaustion.

6.2. Introduction

Transition from a free-living existence to a parasitic mode of life impacted various life history traits, including fecundity (egg number) and egg size (see Poulin, 1995b; Calow, 1983). However, our theoretical framework is still lacking some important elements if we are to understand fully the mechanism of parasite egg production. These elements will allow us to evaluate the existence of general laws (i.e. patterns and processes) in parasite egg production. Furthermore, fitting the pieces of the puzzle together, namely the evidence from multilevel analyses on egg number and egg size, is crucial to elucidating parasite life history strategies.

Egg number and egg size are key concepts in parasite reproduction. For many years, our understanding of the former of these traits was largely based on the misconception that all parasites evolve toward extremely high egg output (Poulin, 1995a). There were different explanations for it: a high egg output represents the expected outcome of natural selection – according to the 'balanced mortality' hypothesis (Smith, 1954), parasites must compensate for the massive losses of infective stages that occur during the transmission phase of their life-cycles; a high egg output is the direct outcome of the conditions provided by the host environment (Jennings & Calow, 1975).

The strategy of egg production of a parasite is somewhere between two extremes (the *r*-end and the *K*-end) in a continuum of possibilities. It is the outcome of natural selection, representing the optimal compromise between egg number and egg size. In perfect *r*-strategist organisms, there are no density effects or competition; all available energy and matter are invested in reproduction, the smallest possible amount into each individual offspring. On the other hand, in perfect *K*-strategist organisms, the density effects are maximum and the competition is keen; the emphasis is on preserving the adult and only the remaining energy and matter are used in reproduction, i.e. in the production and maintenance of a small number of extremely fit offspring (Jennings & Calow, 1975). The way in which parasites of a species partition reproductive effort between egg number and egg size can however vary to some extent (see e.g., Ritchie et al., 1993), i.e. reflect adaptive phenotypic plasticity. This process enables individuals to accommodate changes in their environment, by making possible the rapid movement to a new fitness optimum (Price et al., 2003); however, unlike natural selection, it does not result in genetic adaptation, i.e. in changes in genotypic frequencies in the population (Poulin, 1996, 2007a).

The strategies of parasite egg production (egg number and egg size) have been addressed from broad perspectives in the literature (e.g. Gotto, 1962; Price, 1974; Jennings & Calow, 1975; Calow, 1983; Poulin, 1995b, 1996, 1997b, 2007a). Also, numerous studies have considered the egg production of parasites in different taxonomic groups, i.e. Monogenea, Digenea, Cestoda, Nematoda and Copepoda, in relation to different factors, i.e. adult size and longevity, maturation time or prepatency, temperature, photoperiod, salinity, season/time, sampling site, origin of the host (wild environment *vs* farm), host species, host size, number of parasites in the host, interactions between parasites in the host and number of treatments with anti-parasitic drugs (see e.g., Faust, 1949; Olsen, 1974; Anderson, 1982; Kennedy, 1983; Johnston & Dykeman, 1987; McGladdery & Johnston, 1988; Mehlhorn, 1988; Cable & Tinsley, 1991; Johnson & Albright, 1991; Tocque & Tinsley, 1991; Ritchie et al., 1993; Tully & Whelan, 1993; Roubal, 1994; Trouvé et al., 1998; Heuch et al., 2000; Rossin et al., 2005; Bravo et al., 2009; Bravo, 2010; González et al., 2012; Ruiz Daniels et al., 2013). Among these groups, the Copepoda is particularly suited to study parasite egg production for different reasons: firstly, copepods frequently occur in high prevalence and intensity levels year-round in their natural host populations; and secondly, unlike parasites in other taxonomic groups, copepods produce egg sacs which can be easily detached from the parasite and manipulated.

Causes of intraspecific variability in egg number and egg size can only be understood properly if the possible effects of factors at different levels, i.e. the macroenvironment, microenvironment, microhabitat (sensu Rohde, 1984) and parasite levels, are considered for analysis (see e.g. Timi et al., 2010; Loot et al., 2011). Moreover, unravelling how factors at these different levels interact with each other appears to be crucial to understanding how the mechanism of egg production works at the intraspecific level. For instance, evidence for developmental plasticity in size in response to water temperature (e.g. Nordhagen et al., 2000) and that individual parasites reach a size proportional to that of their hosts (e.g., Van Damme et al., 1993; Poulin, 1995a; Loot et al., 2011) is documented in the literature, while it is a general assumption that larger parasites tend to produce more eggs (Poulin, 2007a). Larger hosts likely provide parasites with a more permanent habitat (Poulin, 2007a). In this way, they may favour a delay in maturation and larger body sizes (Stearns, 1992; Roff, 1992; Poulin, 2007a), therefore interfering with parasite egg production. The effect of host body size on parasite egg production appears, however, to be controversial. Actually, Cole (1954) and Kennedy (1983) argued that a decrease in parasite maturation time should result in an increase of the reproductive potential, while the

same effect is expected to be seen in parasites with delayed maturity, as these should present larger body sizes. At the microhabitat level, the selective pressures affecting parasites relate to the food resources (quality and quantity) and the site of infection itself (i.e. its location within the host's body – the stress imposed on parasites living on body surfaces and in internal organs should vary greatly), as the host represents the source of food and the home simultaneously (Crompton, 1991; Castro, 1991; Combes, 1991). The nutrients available to parasites should also vary greatly with the number of conspecifics present at the site of infection, i.e. with the intensity of infection. According to the 'crowding effect' (Read, 1951), the larger the parasite burden, the more intense the competition for essential nutrients and, likely, the host immune response; in such a scenario, both the body size and the fecundity of the parasite are expected to be negatively affected. This type of effect has been documented for cestodes (Keymer et al., 1983; Dobson, 1986; Shostak & Dick, 1987; Heins et al., 2002), nematodes (Krupp, 1961; Khamboonruang, 1971; Michel et al., 1971, 1978; Szalai & Dick, 1989) and digeneans (Jones et al., 1989).

A phenotypic trade-off between egg number and egg size has already been demonstrated using data from different taxa of parasitic copepods (see Poulin, 1995b, 2007a), but its occurrence at the intraspecific level is less consensual (see e.g., Rossin et al., 2005; Timi et al., 2005). The trade-off appears to be influenced by a number of factors, i.e. the host quality (Rossin et al., 2005), the female body size (Herreras et al., 2007) and the site of infection (Loot et al., 2011), which should therefore be considered for analysis.

This study aimed to investigate how the mechanism of egg production works in parasites using a multilevel approach. Particular emphasis is given to the trade-off between egg number and egg size and the factors having a significant influence on these two life history traits. *Octopicola superba* (Copepoda: Octopicolidae), parasitic on the common octopus, *Octopus vulgaris* (recently suggested to represent a complex of species), was used as a model parasite. Data were assessed from mature, ovigerous females. The specific questions addressed were the following: (i) which strategy of egg production is followed by the parasite: is it mainly an *r*-strategist (i.e. produces a large number of small, poorly provisioned eggs) or a *K*-strategist (i.e. produces a small number of large, well provisioned eggs) species; (ii) was there an influence of season, site of infection, host body size, number of conspecifics present at the site of infection and parasite body size (or of interactions between these variables) in egg number and/or egg size; and (iii) was there a phenotypic trade-off between egg number and egg size, while controlling for a potential effect of confounding variables? While

considering each of these questions, the existence of general trends was evaluated on the basis of the information available in the literature.

6.3. Materials and Methods

The parasite

Octopicola superba is a parasite of *O. vulgaris*, endemic to European waters (Humes, 1957; Deboutteville et al., 1957; Bocquet & Stock, 1960; Cavaleiro et al., 2013). It most likely has a single host life-cycle. Indeed, according to our findings, both copepodites and adults are commonly found on *O. vulgaris*. Information on this parasite is scarce concerning its behaviour (Deboutteville et al., 1957) and infection levels (Bocquet & Stock, 1960). Associated disease is not documented in the literature, which suggests that *O. superba* might not be pathogenic for the natural population of octopus; otherwise, the lack of records on associated disease is likely a consequence of the low number of studies so far conducted on this parasitic infection. According to our findings, ovigerous females of *O. superba* are present at high prevalence and intensity and year-round on *O. vulgaris* off northwestern Portugal. The parasite can be easily isolated from the sediment obtained from the washings of the octopus' body surface and mantle cavity. All of these aspects make it ideally suited to study the mechanism of egg production in parasites. Besides, species associated with marine invertebrates seem to be particularly suited to studies of the impact of the type, habitat and behaviour of the host in the number and size of the eggs laid by the copepod (see Gotto, 1962).

Host sampling and parasitological survey

Sampling of octopuses was conducted seasonally during 2010. Octopuses were caught in marine waters off the northern Portuguese town of Matosinhos (41º10'N, 8º42'W) (pot catches), collected by a boat which regularly fishes these waters for *O. vulgaris* exclusively, and individually placed in plastic bags to prevent loss of parasites. Seasonal samples of octopuses (winter sample, collecting date: 2 March; spring sample, 24 and 31 May; summer sample, 7 September; and autumn sample, 22 November) consisted of 30 specimens each. Shortly after being delivered to the fresh fish market, at the harbour in Matosinhos, the octopuses were transported to the laboratory, at the campus of University of Porto, Portugal; all of them were kept frozen

(≈ -20 ºC) until they could be scanned for parasites. During examination, the total length of the octopus' body was measured; the sex and stage of sexual maturity were identified. Octopicolid copepods were isolated from the sediment from the saline solution (3.5%) used to wash the different organs (for further details, see Cavaleiro et al., 2013) and identified as *O. superba* according to the identification key in the latter work. Data on egg production were obtained from mature females (N_{total} = 120, 30 from each season) having two intact egg sacs and selected at random, one from each infrapopulation (sensu Bush et al., 1997).

Measurements and statistical analysis of data

Ovigerous females were mounted in 90% lactic acid on cavity glass slides, and observed under a compound optical microscope (using a ×25 phase contrast objective in the case of the whole specimens and the individual egg sacs and a ×100 phase contrast objective in the case of the individual eggs). Eggs were examined for signs of non-viability, such as a dark colour and irregular shape, and they all seemed equally viable. Two body dimensions (used as reference measures of size) were recorded while examining parasites under the compound optical microscope (Fig. 6.1): the parasite total length (μm) (excluding setae on caudal rami) and the length of the genital somite (μm).

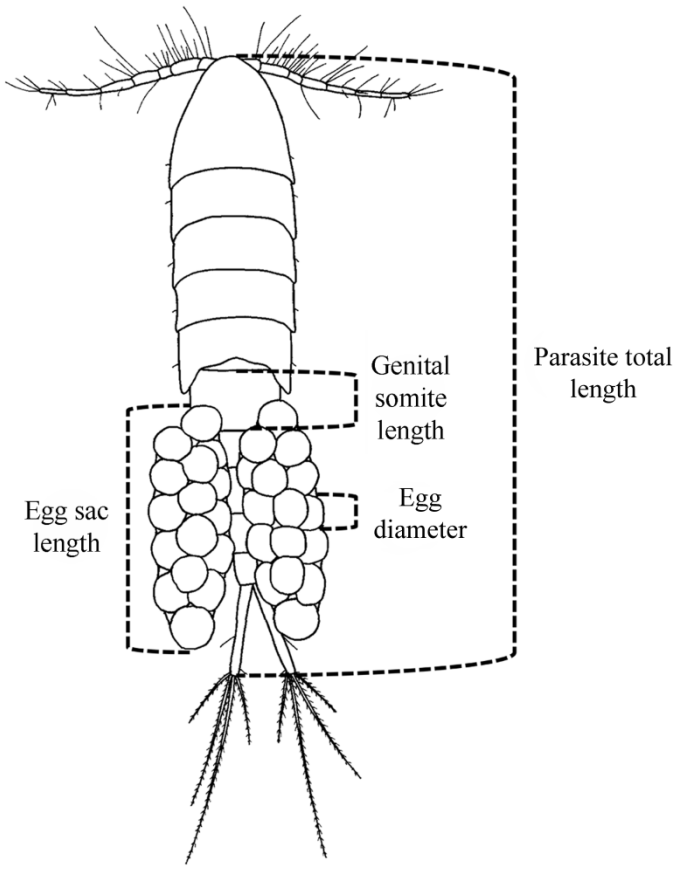


Fig. 6.1 – Morphometric measurements taken from the mature, ovigerous females of *Octopicola superba* (modified from Humes, 1957).

Beyond these two measures, the following variables were determined as part of the effort to characterize the mechanism of egg production: egg sac length (μm) (assessed for the two sacs); fecundity; egg diameter (μm) (assessed for all eggs making up the clutch); and total reproductive effort (a measure of the total resources invested in one clutch). Fecundity was assessed as the total number of eggs in the two sacs; mean egg sac length as the average length of the two egg sacs; mean egg diameter as the average diameter of the eggs in the two sacs; and total reproductive effort by multiplying fecundity by mean egg volume (Caley et al., 2001). Mean egg volume was determined using the records for mean egg diameter and the formula for the volume of a sphere (mean egg volume = $\frac{4}{3}\times\pi\times[\frac{\text{mean egg diameter}}{2}]^3$), since the egg is nearly spherical.

In order to evaluate the parasite life history strategy, the total sample of female parasites was characterized considering the two sets of variables, i.e. each body dimension (parasite total length and genital somite length) and measures of

reproductive effort (fecundity, mean egg diameter, total reproductive effort and mean egg sac length) (mean, SD, range interval [RI, minimum-maximum], coefficient of variation [CV], limits of the 95% confidence interval [CI] for the population mean and distribution [one sample Kolmogorov-Smirnov's test]). Furthermore, multiple factorial analysis (MFA; extraction method: principal components) was used to analyse the structure of the data. The skewness and kurtosis values for the distributions of fecundity and mean egg diameter were determined, in order to evaluate which strategy of egg production (*r*-strategy or *K*-strategy) is preferentially followed by the parasite. Host total length was characterized with respect to the same statistical parameters used in the case of the other studied variables and considering the total sample of octopuses.

A potential influence of season on egg production was considered by characterizing the seasonal samples of female parasites with respect to the two sets of variables (i.e. by considering their distribution). Values for each body dimension (parasite total length and genital somite length) and measures of reproductive effort (fecundity, mean egg diameter, total reproductive effort and mean egg sac length) were compared between the four seasons of the year (Kruskal-Wallis' test); pairwise sample comparisons were performed (Mann-Whitney's *U* test) only when the Kruskal-Wallis' test yielded a statistically significant difference. The same strategy was followed to detect a potential influence of site of infection on egg production. Discriminant function analysis (DFA; method: independent variables entered together) was conducted to obtain a general picture of the differences between the females of different seasons. The two sets of variables were considered in the analysis. A general linear analysis model with type III sum of squares (GLM multivariate analysis) was then used to evaluate the constraints of egg production in *O. superba*. In a preliminary analysis, only the main effects were assessed. Fecundity and mean egg diameter were considered, simultaneously, as the dependent variables; fixed factors included season exclusively; and covariates included host total length, number of conspecifics present at the site of infection and parasite total length. According to the results obtained in this analysis, the following interaction terms were then considered for analysis (using the same dependent variables, fixed factor and covariates): season*parasite total length; host total length*parasite total length; and number of conspecifics present at the site of infection*parasite total length.

An independent regulation of egg number and egg size was investigated using two strategies. The first of these consisted of plotting on the same graph, data for fecundity and mean egg diameter (the specimens were first arranged by ascending

fecundity). Each seasonal sample was considered separately for analysis. The second strategy consisted of evaluating whether there was a phenotypic trade-off between egg number and egg size. A non-parametric partial rank correlation test, conducted separately for each season of sampling, evaluated the existence of a significant negative correlation between the two life history traits while controlling for a potential confounding effect of the variables shown by the GLM multivariate analysis to have a significant influence on fecundity or/and egg size.

Measurements from female parasites were taken under a compound optical microscope (Carl Zeiss Axiophot Photomicroscope) and using the digital image processing software AXIOVISION of Carl Zeiss, version 4.6.3 (Carl Zeiss Microimaging Inc., Thornwood, NY, USA). Data for body dimensions and measures of reproductive effort for the different seasons of sampling/sites of infection were depicted as box-and-whisker plots. In these, the minimum and maximum values encompass 95% of the data; outliers and extremes appear outside this range interval. Statistical tests, multivariate analyses and graphical representations of data were performed using Statistical Package for Social Sciences (SPSS) for Windows, version 21.0 (SPSS Inc., Chicago, Illinois, USA), STATISTICA for Windows, version 10.0 (StatSoft Inc., Tulsa, USA) and Microsoft Excel 2010 (Microsoft, Redmond, WA, USA). Non-parametric partial rank correlation analyses were defined in the syntax editor window of SPSS, according to the instructions available at the IBM website (http://www01.ibm.com/support/docview.wss?uid=swg21474822). The significance level considered was $P < 0.05$, except in the case of the pairwise sample comparisons. In this case, the Bonferroni correction set significance at $P < 0.008(3)$. In this way, it was possible to account for the Type I error.

6.4. Results

The females of *O. superba* considered in the analyses were isolated from the body skin ($N = 66$), gills ($N = 26$), mesentery covering the gonad ($N = 13$), mantle musculature ($N = 11$), eyes ($N = 2$) and funnel ($N = 2$). Data recorded from the parasite and host samples (total samples) are given in Table 6.1.

Table 6.1 – Body dimensions and measures of reproductive effort (mean ± SD (RI) [Range Interval], CV [Coefficient of Variation] (%), limits of the 95% CI [Confidence Interval] for the population mean and results of the Kolmogorov-Smirnov's test) recorded for the total samples (*N* = 120) of mature, ovigerous females of *Octopicola superba* and *Octopus vulgaris*.

Character **Characteristic**	**Mean ± SD** **(RI)**	**CV (%)**	**Limits of the 95% CI for the population mean**	**Kolmogorov-Smirnov's test** ***Z*; *P***
Body dimension				
Parasite total length[a] (μm)	1,846.1±97.2 (1,612.6–2,268.1)	5.3	1,828.7–1,863.5	2.655; < 0.0001[c]
Genital somite length[a] (μm)	277.1±18.1 (229.0–317.9)	6.5	273.9–280.3	0.965; 0.309
Host total length[b] (cm)	67.7±9.5 (50.2–90.1)	14.1	66.3–69.1	1.098; 0.179
Measure of reproductive effort				
Fecundity[a] (eggs)	37.7±7.2 (30–63)	19.0	36.4–39.0	2.496; < 0.0001[c]
Mean egg diameter[a] (μm)	152.1±21.5 (99.6–193.7)	14.1	148.3–155.9	2.239; < 0.0001[c]
Total reproductive effort[a] ($/10^6$) (μm^3)	70.0±22.3 (27.0–129.4)	31.9	66.0–74.0	0.890; 0.407
Mean egg sac length[a] (μm)	681.9±80.1 (560.6–930.0)	11.7	667.6–696.2	2.186; < 0.0001[c]

[a]Parasite

[b]Host

[c]Significant result ($P < 0.05$).

According to these data, the distribution of parasite total length, fecundity, mean egg diameter and mean egg sac length did not fit the normal distribution. The most fecund females produced more than twice the number of eggs than the less fecund ones (63/30 = 2.1); also, there were females producing eggs approximately twice as large as those produced by others (193.7/99.6 = 1.9). The structure of the data recorded from the female parasites is depicted in the variables factor map shown in Fig. 6.2. Projecting the arrows onto the first dimension (which accounted for most of the variability found, i.e. 79.5%), it can be seen that the variables genital somite length, parasite total length and mean egg diameter are most important for the first principal component. The vectors of parasite total length and mean egg diameter are on a straight line; accordingly, these two variables were highly correlated, i.e. large body sizes were strongly correlated with small egg sizes. Genital somite length and mean egg diameter were also negatively correlated, while a positive correlation is observed for parasite total length and genital somite length.

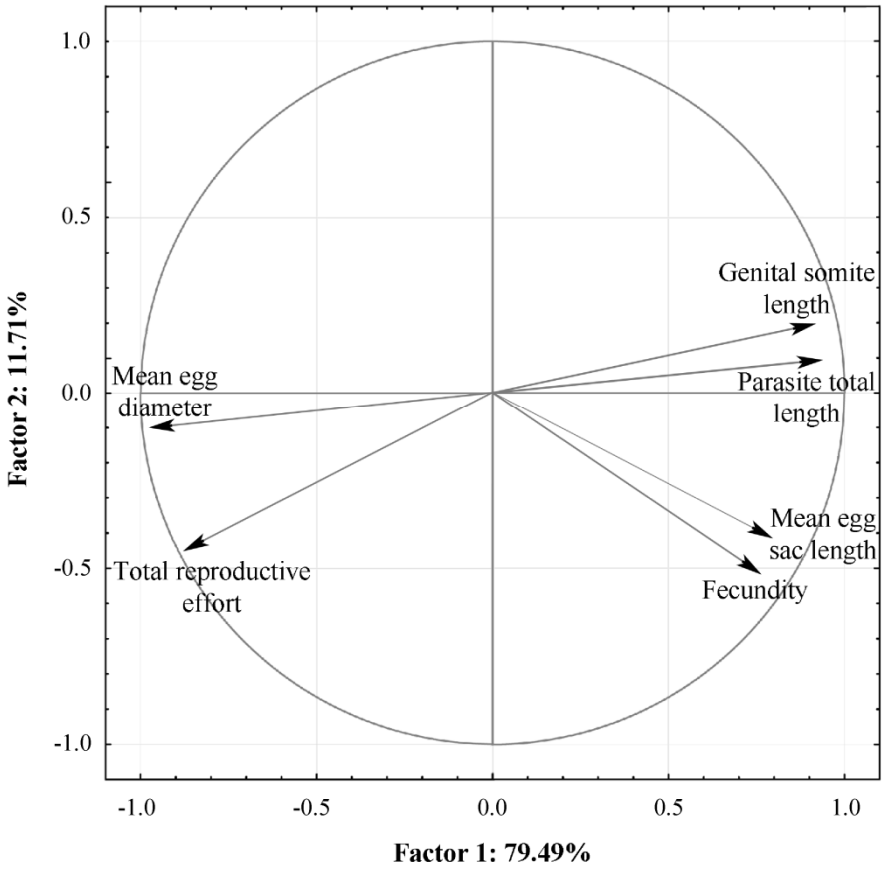


Fig. 6.2 – Graphical depiction of the projections of the body dimensions and measures of reproductive effort on the principal multiple factorial analysis plane. Percentage values are for the variability explained by each factor.

The distributions of fecundity (g_1 = 1.874) and mean egg diameter (g_1 = -1.143) were positively and negatively skewed, respectively; both distributions were leptokurtic (fecundity: g_2 = 2.902; mean egg diameter: g_2 = 1.142) (Fig. 6.3).

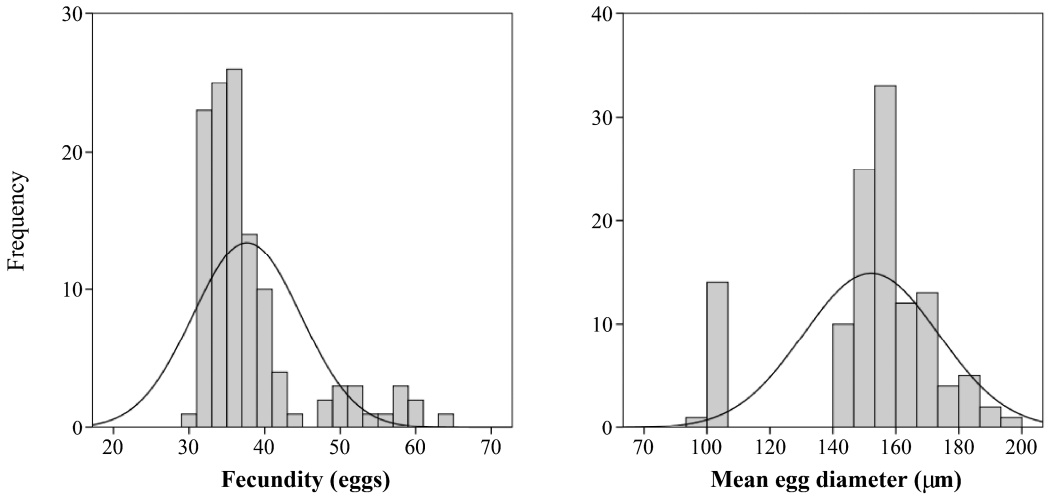


Fig. 6.3 – Distributions of fecundity and mean egg diameter for the total sample of female *Octopicola superba*.

The females in the total sample belonged to one of two strategies, one closer to the *r*-end of the continuum of possibilities of egg production and the other closer to the *K*-end. Actually, the partition of reproductive effort between egg number and egg size varied from season to season. More specifically, the investment in egg number and egg size tended to vary in opposite ways from one season to the next – i.e. when the investment in egg number decreased that in egg size increased. On average, the winter females were longer, had longer genital somites and egg sacs and produced a larger number of eggs and smaller eggs compared with spring, summer and autumn females; opposite trends were observed for the summer females (Fig. 6.4).

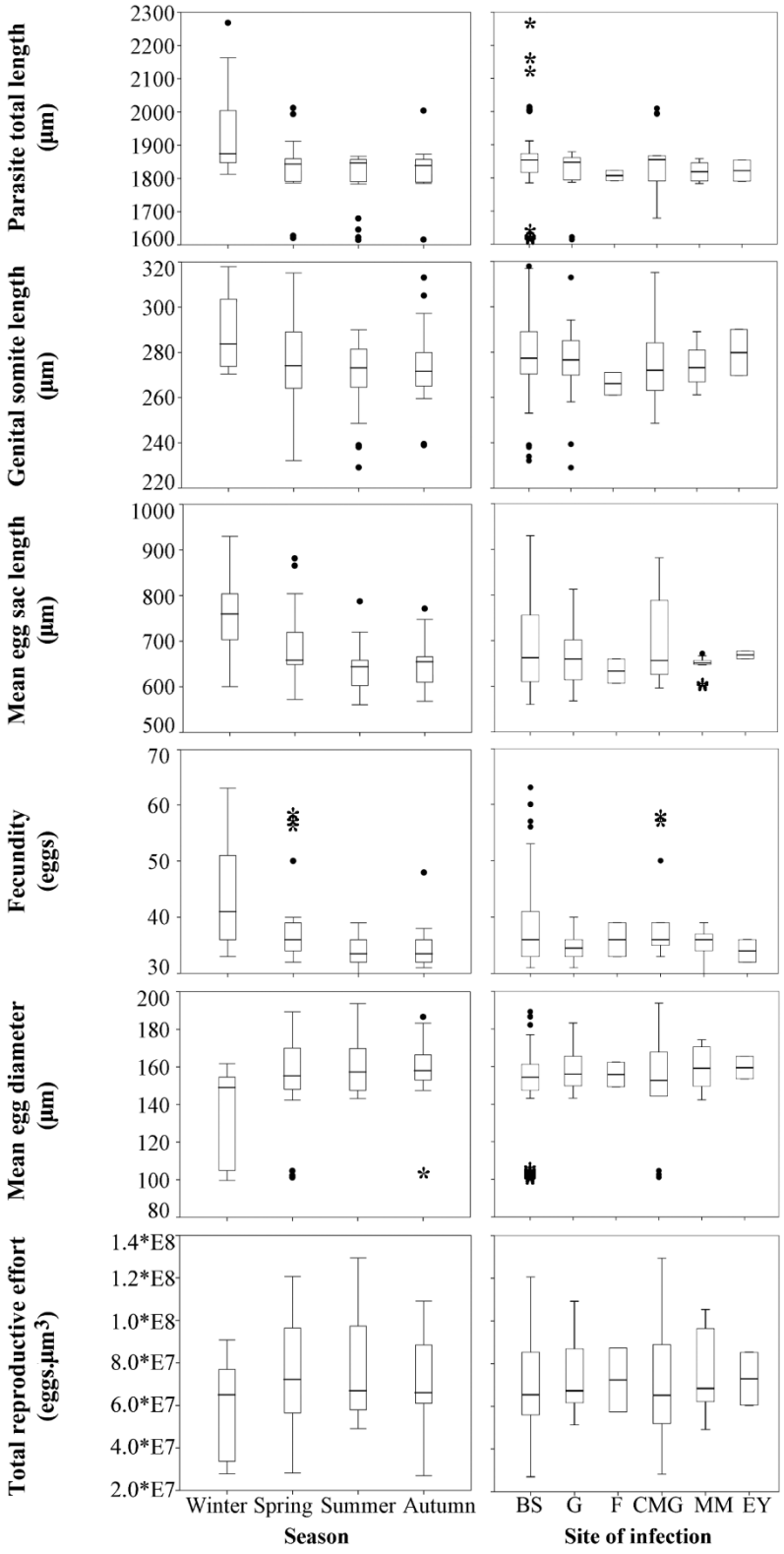


Fig. 6.4 – The distribution of parasite total length, genital somite length, mean egg sac length, fecundity, mean egg diameter and total reproductive effort values for each of the seasonal samples of mature, ovigerous females of *Octopicola superba*/sites of infection (abbreviations: BS, Body Skin; CMG, Covering Mesentery of Gonad; EY, EYes; F, Funnel; G, Gills; and MM, Mantle Musculature).

The marked difference between the winter females and those of the remaining seasons is also clear in the two-dimensional DFA plot (Fig. 6.5). In this analysis, only discriminant function 1 was statistically significant (axis 1: Wilks' Lambda = 0.596, χ^2 = 58.986, DF = 18, P < 0.0001; axis 2: Wilks' Lambda = 0.931, χ^2 = 8.120, DF = 10, P = 0.617); however, it accounted for 88.5% of the variance among seasons. The winter females accumulated on the positive end of that function.

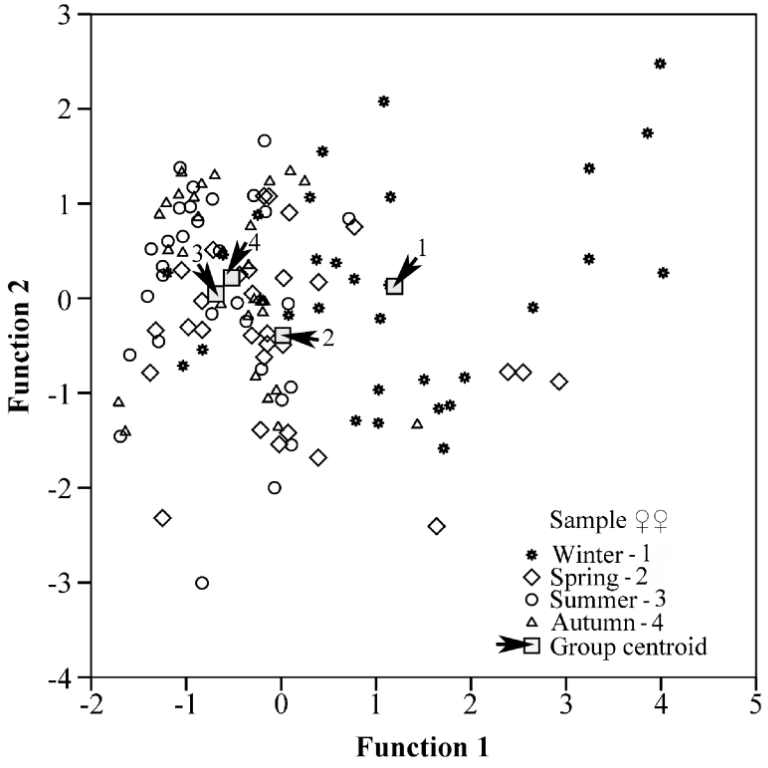


Fig. 6.5 – Discriminant function analysis of the four seasonal samples of mature, ovigerous female *Octopicola superba* – projection of the cases on discriminant functions 1 and 2.

Statistically significant sample differences for multiple sample comparisons were detected for all variables except total reproductive effort (Table 6.2); most differences for pairwise sample comparisons were sample pairs including the winter sample (Table 6.3).

Table 6.2 – Multiple sample comparisons of body dimensions and measures of reproductive effort for mature, ovigerous females of *Octopicola superba* (results of the Kruskal-Wallis' test).

Character Characteristic	Season (*K*; *P*)	Site of infection (*K*; *P*)
Body dimension		
Parasite total length	26.250 < 0.0001[a]	7.172 0.208
Genital somite length	17.408 0.001[a]	2.838 0.725
Measure of reproductive effort		
Fecundity	40.136 < 0.0001[a]	4.706 0.453
Mean egg diameter	16.382 0.001[a]	2.992 0.701
Total reproductive effort	5.470 0.140	2.077 0.838
Mean egg sac length	31.843 < 0.0001[a]	3.125 0.681

[a]Significant result ($P < 0.05$).

Table 6.3 – Pairwise sample comparisons of body dimensions and measures of reproductive effort for mature, ovigerous females of *Octopicola superba* (results of the Mann-Whitney's *U* test).

Character Characteristic	Winter *vs* Spring	Winter *vs* Summer	Winter *vs* Autumn	Spring *vs* Summer	Spring *vs* Autumn	Summer *vs* Autumn
Body dimension						
Parasite total length	199.0 < 0.0001[a]	157.0 < 0.0001[a]	157.0 < 0.0001[a]	422.0 0.679	398.5 0.446	435.0 0.824
Genital somite length	284.5 0.014	198.0 < 0.0001[a]	199.0 < 0.0001[a]	395.5 0.420	407.0 0.525	450.0 1.000
Measure of reproductive effort						
Fecundity	256.0 0.004[a]	99.0 < 0.0001[a]	114.5 < 0.0001[a]	233.0 0.001[a]	245.0 0.002[a]	437.0 0.845
Mean egg diameter	281.0 0.012	230.5 0.001[a]	187.5 < 0.0001[a]	408.0 0.535	403.5 0.492	445.0 0.941
Mean egg sac length	230.0 0.001[a]	126.0 < 0.0001[a]	155.0 < 0.0001[a]	270.0 0.008[a]	377.0 0.280	330.0 0.076

[a]Significant result ($P < 0.008(3)$).

With respect to the site of infection (Fig. 6.4), the Kruskal-Wallis' test yielded a non-statistically significant difference for all studied variables (Table 6.2). Accordingly, an effect of site of infection on egg production was not considered further in analyses. The results of the GLM multivariate analysis are presented in Table 6.4. Concerning the main effects, statistically significant results were detected for season (for fecundity) and parasite total length (for fecundity and mean egg diameter). As for the interaction terms, significant results were detected only for season*parasite total length (for fecundity and mean egg diameter).

Table 6.4 – Results of the general linear model (GLM multivariate analysis) with fecundity and mean egg diameter of *Octopicola superba* as dependent variables, season as fixed factor and host total length, parasite total length and number of conspecifics present at the site of infection as covariates.

Level Main effect	DF	MS Fecundity Mean egg diameter	*F*	*P*
Macroenvironment				
Season	3	111.340	4.745	0.004[a]
	3	45.451	0.467	0.706
Microenvironment				
Host total length	1	41.583	1.772	0.186
	1	1.256	0.013	0.910
Microhabitat				
Number of conspecifics present at the site of infection	1	34.649	1.477	0.227
	1	31.538	0.324	0.570
Parasite				
Parasite total length	1	1458.629	62.163	< 0.0001[a]
	1	32476.933	333.432	< 0.0001[a]
Error	113	23.465		
	113	97.402		
Interaction term				
Season*parasite total length	4	454.004	19.813	< 0.0001[a]
	4	7847.421	80.729	< 0.0001[a]
Host total length*parasite total length	1	44.239	1.931	0.167
	1	0.089	0.001	0.976
Number of conspecifics present at the site of infection*parasite total length	1	39.445	1.721	0.192
	1	27.075	0.279	0.599
Error	113	22.914		
	113	97.207		

[a]Significant result ($P < 0.05$).

Frequently, females with similar fecundity differed more or less markedly in the size of their eggs, i.e. mean egg diameter (Fig. 6.6). It is worth noting that, above ≈ 43 eggs, the fecundity increased markedly while the mean egg diameter decreased reaching the minimum value, i.e. ≈ 100 μm.

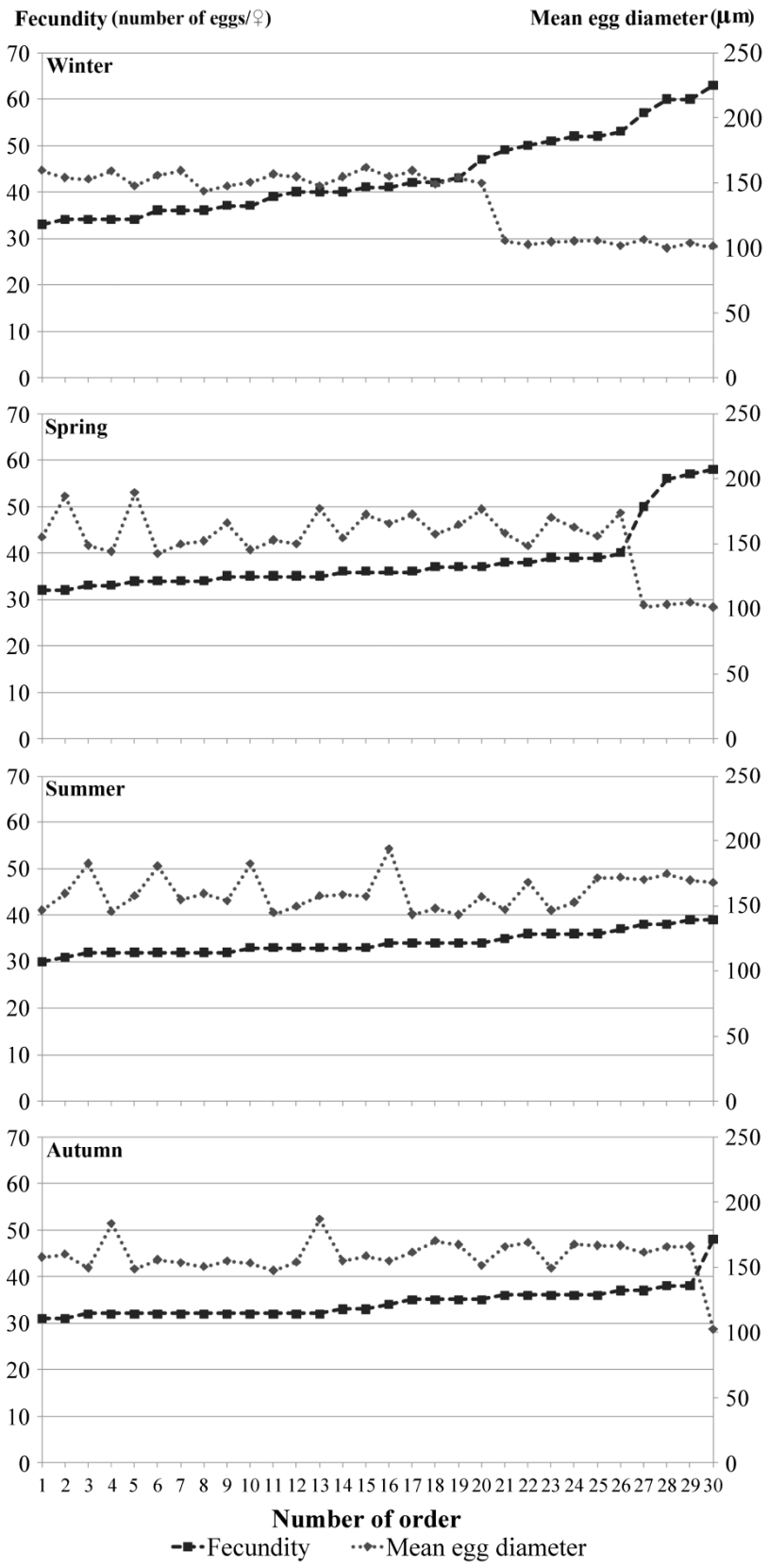


Fig. 6.6 – Variability in fecundity and mean egg diameter recorded for each of the seasonal samples of mature, ovigerous female *Octopicola superba* (specimens arranged by ascending fecundity).

The results of the non-parametric partial rank correlation test are shown in Table 6.5. According to this, significant negative relationships were recorded for the winter, spring and autumn samples of females, while controlling for the potential confounding effect of parasite total length.

Table 6.5 – Results for the correlation between fecundity and mean egg diameter evaluated for the different seasonal samples of *Octopicola superba* using a non-parametric partial rank correlation test.

	Season			
Control variable	**Winter**	**Spring**	**Summer**	**Autumn**
Parasite total length	-0.620	-0.570	0.400	-0.457
	< 0.0001[a]	0.001[a]	0.031[a]	0.013[a]

[a]Significant result ($P < 0.05$).

6.5. Discussion

Egg number and egg size are two important reproductive traits which are crucial to understanding parasite life history strategies.

In this study, the importance of the trade-off between egg number and egg size was first addressed by considering the question of whether the parasite is more an *r*-strategist or a *K*-strategist, i.e. how natural selection acted upon egg number and egg size. According to Jennings & Calow (1975), endoparasites follow both an *r*- and *K*-strategy at the same time, which is made possible by the stable, nutrient-rich environment provided by the host. Actually, evolutionary theory predicts that all species would follow an *r*- and *K*-strategy simultaneously, had it not been for macroenvironmental conditions, which invariably force them into one of the alternatives. In the case of the ectoparasites, they live on more unstable microhabitats (i.e. on external body surfaces) than endoparasites, a situation which should, therefore, have implications in terms of their reproductive strategy. Indeed, it has been argued that in ectoparasitism, the premium on preserving the adult is greater than that on producing a large number of eggs (Jennings & Calow, 1975), i.e. that ectoparasites are mainly *K*-strategists. This positioning of ectoparasites more towards the *K*-end of the *r*-*K* spectrum of life history strategies goes against the 'balanced mortality' hypothesis, as this assumes massive egg production (see Smith, 1954; Price, 1974; Stunkard, 1975; Kennedy, 1976; Combes, 1995), and is supported by the results here reported for *O. superba*. Indeed, the marked difference between the skewness of the distributions of fecundity and mean egg diameter, the fact that both of these distributions exhibited high peaks around the mean and the relative position of

fecundity and mean egg diameter in the variables factor map can all be understood as evidence that the females of *O. superba* do not follow both an *r*- and *K*-strategy at the same time, contrary to that argued for endoparasites. Moreover, the positive and negative skewness of the distributions of fecundity and mean egg diameter, respectively, might be understood as an indication that females are particularly committed to producing a relatively small number of large, well provisioned eggs. This is because the skewness measures the level of symmetry of the distribution of each of the two life history traits around the mean. As stated, in *K*-strategist organisms the emphasis is on preserving the adult; the remaining energy and matter are used in the production and maintenance of a small number of extremely fit offspring (Jennings & Calow, 1975). Such preservation of the adult (i.e. of the parent) would be very beneficial in the case of the host-parasite system studied here. More specifically, in this particular system, host-to-host transmission of *O. superba* is impaired by a combination of factors regarding the host's and parasite's ecology: octopuses are typically sedentary animals with sparsely distributed populations, while *O. superba* is highly host-specific and monoxenous (see Cavaleiro et al., 2013). Hence, the preservation of the adult is beneficial, enhancing the lifetime reproductive success of individual females by increasing the temporal spread of egg production. Besides, the production of large, well provisioned eggs might also be beneficial once they result in infective stages that are better equipped to seek out 'new' hosts or that have a better chance of reaching areas where 'new' hosts may be found (Gotto, 1962). Earlier findings had already suggested that sedentary hosts select for large egg sizes in parasitic copepods (see Poulin, 1995b) and that ectoparasites, including monogeneans (Roubal, 1994) and copepods (Ritchie et al., 1993), do not follow an *r*- and *K*-strategy at the same time. It is worth noting that the coefficient of variation was lower for mean egg diameter than for fecundity, which suggests that egg size is under a tighter regulation than egg number.

The second question addressed egg production by considering the possibility of influences of different variables and variable interactions on the number of eggs produced and their size. While there was no evidence for an influence of site of infection on egg production, it was found that both egg number and egg size varied according to season, in accordance to earlier evidence for copepods ectoparasitic on fish (see Ritchie et al., 1993). The cause of the seasonal variation in reproductive strategy can only be determined in an additional experimental study. Remarkably, despite the variability observed in mean egg sac length, the total reproductive effort did not vary significantly with the season of sampling. This means that, despite the significant sample differences in fecundity and egg size, the total amount of resources

invested in one clutch did not vary to a significant extent between samples. Significant sample differences were recorded concomitantly for all body dimensions and measures of reproductive effort (Table 6.3), with the seasonal differences found being in accordance with earlier evidence for parasitic copepods (e.g. Ritchie et al., 1993). This evidence suggests adaptive phenotypic plasticity in body dimensions and size-mediated changes in egg production. Actually, a significant influence of the interaction term season*parasite total length on fecundity and mean egg diameter is supported by the results of the GLM multivariate analysis. Moreover, larger specimens tended to produce more eggs (see Fig. 6.4), which is in accordance with earlier findings for monogeneans (Kearn, 1985), cestodes (Shostak & Dick, 1987), nematodes (Mössinger & Wenk, 1986; Sinniah & Subramaniam, 1991; Rossin et al., 2005; Herreras et al., 2007), copepods (Tedla & Fernando, 1970; Ritchie et al., 1993; Van Damme et al., 1993; Timi et al., 2005), bopyrid isopods (Wenner & Windsor, 1979) and ticks (Honzáková et al., 1975; Iwuala & Okpala, 1977). This association between body size and egg number could have been related to the fact that larger females tended to have larger genital somites (see Fig. 6.2). Actually, one can speculate that the genital somite was not fully grown in smaller females and, therefore, that the space available for the oocysts and yolk was smaller in these females. A significant influence of host body size and number of conspecifics present at the site of infection (i.e., the ‘crowding effect’) on egg number and size was not detected, which might be related to the fact that the host is incomparably larger than the parasite, providing it with virtually infinite resources.

With respect to the existence of a phenotypic trade-off between egg number and egg size, there were some interesting findings. To begin with, the seasonal distributions of fecundity and mean egg diameter revealed that a negative association between the two traits might only become apparent at high levels of fecundity, with the mean egg diameter dropping to the minimum level. Unlike free-living organisms, parasites do not experience shortages of food, having more than enough resources to maintain a high rate of egg production (Poulin, 2007a). Nonetheless, it is likely that the female physiology sets a limit to reproduction (and to clutch dimension – females cannot carry infinitely large clutches). By this we mean that, beyond a certain fecundity level (in the study case, ≈ 43 eggs), the females eventually become physiologically exhausted due to the large energetic investment already made in egg production. As a consequence, smaller amounts of yolk are produced and allocated to each individual egg. The occurrence of the trade-off is reinforced by the results of the non-parametric partial rank correlation test, as a significant negative correlation between the two life history traits was detected in three of the cases. The fact that previous studies on

parasite egg production found no evidence for this negative association to occur at the intraspecific level (e.g. Timi et al., 2005; Rossin et al., 2005), might indicate that the fecundity levels recorded were just too low for it to be observed. The results for the summer season (i.e. the significant positive correlation between egg number and egg size) are not easy to explain but, also in this case, they might be related, at least to some extent, with the fact that fecundity did not reach the threshold level (≈ 43 eggs) above which the trade-off was observed. A close look at the data in Fig. 6.6 also reveals that it is not possible to predict, with any confidence, the mean egg size that we can expect to observe based on fecundity. This suggests an independent regulation of egg number and egg size.

In conclusion, the findings of this research suggest that multilevel analyses of egg number-egg size data sets are crucial to gain accurate knowledge on how the mechanism of egg production works in parasites. The analysis of the data set recorded for *O. superba* suggests that although the parasite tends to produce a relatively small number of large, well provisioned eggs, the strategy followed is somewhat flexible throughout the year, the changes in fecundity and egg size probably being determined by an effect of season on the maturation time (i.e., body size) of the parasite. The occurrence of a trade-off between egg number and egg size appears to be the consequence of factors at the parasite level exclusively. Experimental infections in the laboratory will be important to characterize it further, namely by evaluating the existence of lifetime variation in egg production.

6.6. Acknowledgements

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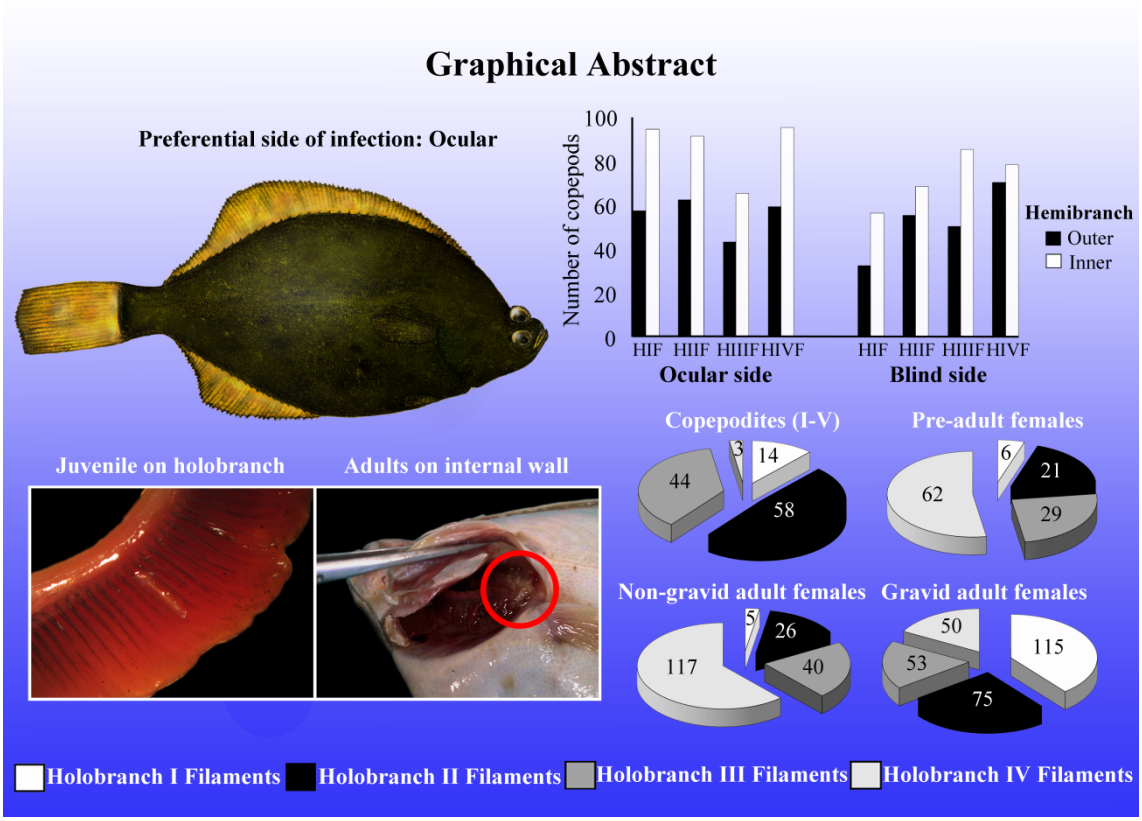
Chapter 7

Site selection of *Acanthochondria cornuta* (Copepoda: Chondracanthidae) in *Platichthys flesus* (Teleostei: Pleuronectidae)



This chapter has been adapted from:

Cavaleiro, F. I., & Santos, M. J. (2011). Site selection of *Acanthochondria cornuta* (Copepoda: Chondracanthidae) in *Platichthys flesus* (Teleostei: Pleuronectidae). *Parasitology*, *138*, 1061–1067.

7.1. Abstract

Acanthochondria cornuta (Copepoda: Chondracanthidae) (*N* = 4841; prevalence: 80.0%; mean ± SD [range] intensity: 28.8±24.0 [1–110] parasites) infected the branchial chambers of the European flounder, *Platichthys flesus* (L.), (*N* = 210) according to an established spatial pattern. This was independent of host size. Higher intensities resulted, most frequently, in higher numbers of infection sites, probably due to increased intraspecific competition. Preferential infection of the ocular side was supported by the recorded abundance data and reflected, probably, the fish's bottom-dwelling behaviour. As the parasite develops from one stage into another, it seems to migrate towards different sites: the copepodites and pre-adult females occurred, mainly, in the holobranchs; the adults preferred the internal wall (non-gravid/post-gravid females; adult males) or the pseudobranchs (gravid females). The ventilating water current along with the blood supply are suggested as two major factors in determining parasite spatial distribution within the chamber. Parasite crowding in a restricted and narrow space of the posterior region of the internal wall was recorded frequently and resembled that previously reported for the plaice. Differences to other host-parasite systems previously studied should relate with the anatomy of the respiratory apparatus. Bigamous females are reported for the first time.

7.2. Introduction

Parasites are usually given the opportunity to choose between a variety of unique sites on or in their hosts. Indeed, the current knowledge allows us to say that site selection is universal among them, though varying between the species and groups (Rohde, 1979). Each given site constitutes a unique microhabitat – due to its very own abiotic and biotic features – determining, to some extent, the parasite's choice.

Site selection is well documented in the literature for the parasitic copepods of fish (see for example, Geets et al., 1997; Lo & Morand, 2001; Scott-Holland et al., 2006; Timi et al., 2010). In particular, among the chondracanthid copepods, the members of the genus *Acanthochondria* Oakley, 1927 were described to infect, specifically, the branchial chambers of flatfish (see for example, Kabata, 1959, 1979, 1992). These latter constitute a unique and protected environment that offers the parasites, a number of different possible sites for attachment. Also, they allow blood-feeding species to feed abundantly, particularly when they infect the filaments of the holobranchs and pseudobranchs. In accordance with Llewellyn (1956), variability in the volumes of water that pass by the four holobranchs might lead to different infection levels, once the larval specimens are given different opportunities to attach on each of them. Such variability was demonstrated, for instance, for the brown trout (*Salmo trutta* forma *fario* L.), with the volume of water passing over holobranchs II and III being significantly greater than that passing over holobranchs I and IV (Paling, 1968). Besides this, several intrinsic factors of the parasites may also determine how they distribute among the different holobranchs (Ramasamy et al., 1985; Geets et al., 1997).

In recent years, several studies have documented high infection levels of a species of *Acanthochondria*, i.e., *A. cornuta* (Müller, 1776), in European flounder, *Platichthys flesus* (Linnaeus, 1758) (Teleostei: Pleuronectidae), of North Sea (Schmidt et al., 2003) and Atlantic (Marques et al., 2006; Cavaleiro & Santos, 2007, 2009) waters and considered issues like the spatial and seasonal occurrence of infection. Notwithstanding, several aspects of the species 'niche volume' (see Rohde, 1994), including, among others, parasite spatial distribution on the host and food, remain still to be elucidated.

The main aim of this paper is to describe in both qualitative and quantitative terms, the pattern of site selection of *A. cornuta* in the European flounder. The possible driving forces of the observed pattern of spatial distribution are discussed.

7.3. Materials and Methods

Host sampling

In total, 210 European flounder were collected: in September 2005, off four localities (Viana do Castelo, Matosinhos, Aveiro and Figueira da Foz) of north-central Portugal (41°40′N, 8°50′W to 40°8′N, 8°52′W), eastern North Atlantic (Fig. 7.1); and during one year (once per season) in the geographical locality where flounders were most infected, i.e., Matosinhos, between September 2005 and May 2006. Additional details on sampling are provided in the papers by Cavaleiro & Santos (2007, 2009).

Fig. 7.1 – Geographical location of the four sampling areas in the north-central Portuguese coast, eastern North Atlantic.

Parasite survey

Ocular and blind branchial chambers were examined for *Acanthochondria* parasites. In each of them, 17 possible infection sites were considered for analysis. They were: (i) the operculum (internal surface); (ii) the branchiostegal pocket (delimited by the operculum and the branchiostegal membrane); (iii) the urohyal; (iv) the internal wall of the chamber; (v-xvi) the four holobranchs, in each of which, three sites were considered for analysis – i.e., the bony part (raker) and the inner and outer hemibranchs (filaments); and (xvii) the pseudobranch (filaments). The opercula and holobranchs were first dissected out of the fish and only then examined for parasites. Both the ocular and blind holobranchs were numbered from I to IV. The one nearest the operculum was assigned as holobranch I, whereas the innermost was assigned as holobranch IV. Besides the branchial chambers, examined sites on the fish's body

surface included also the skin, fins, eyes, nostrils and mouth cavity. Observations were carried out under a stereo dissecting microscope at 30× magnification. Chondracanthids did not detach from the tissues to which they were attached during the host manipulation. Consequently, they could be localized (and counted) with precision on the host's body. Their identification to the species level, that is, as representatives of *A. cornuta*, was made following the description of Kabata (1979, 1992). As for the life-cycle stages i.e. copepodite (I-V), pre-adult female or adult female/male, they were identified in accordance with Heegaard (1947). Different stages of sexual maturity were considered for the adult female: (i) non-gravid, i.e. female without egg sacs; (ii) gravid, i.e. female with intact egg sacs; and (iii) post-gravid, i.e. female with remnants of egg sacs.

Analysis of parasite site selection

Infection levels, that is, prevalence, intensity and abundance, were estimated in accordance with Bush et al. (1997). Also, the mean, standard deviation (SD) and range of variation were calculated (for intensity and abundance) and recorded whenever necessary. Statistical analyses were conducted on SPSS for Windows, version 17.0 (SPSS Inc., Chicago, Illinois), with the significance level set at $P < 0.05$. The different levels of analysis considered were as described below.

General site selection. To characterize the spatial distribution of *A. cornuta* on the host's body, the mean intensity was computed for each class of fish including specimens with the same number of infection sites (sites on the ocular and blind sides of the fish's body were considered separately). The existence of a significant relationship between the two variables, that is, intensity and number of infection sites, was evaluated by the Spearman's rank correlation test. Also, the existence of a significant difference in infection occurrence among the ocular and blind sides was investigated. Moreover, the infection prevalence in the two sides was compared using the McNemar's test for matched-pairs of dichotomous variables. The abundance levels recorded for the two sides were compared using the Wilcoxon's matched-pairs signed ranks test. Non-parametric tests were preferred over parametric ones owing to the non-normal distribution of both intensity and abundance data (Kolmogorov-Smirnov's test: $1.598 \leq Z \leq 2.494$; $0.000 < P \leq 0.012$).

Site selection within the branchial chambers. Prevalence and intensity (mean ± SD) levels were assessed for each infection site in the ocular and blind branchial chambers. Next, the existence of particular spatial distributions inside them was investigated. Moreover, significant differences in parasite abundance among the filaments of the inner and outer hemibranchs of a given holobranch were evaluated by the Wilcoxon's matched-pairs signed ranks test, whereas differences in abundance among the four holobranchs and among the internal wall of the chamber, the holobranch (I-IV) filaments and the pseudobranch filaments were tested by the Friedman's rank sum test. All these analyses were conducted separately for the ocular and blind chambers. The number of specimens in different stages of development and sexual maturity i.e., copepodites (I-V), pre-adult females, non-gravid adult females, gravid adult females, post-gravid adult females and adult males, was quantified for each site of attachment in the branchial chamber. In particular, the distribution of copepodites (I-V), pre-adult females, non-gravid adult females and gravid adult females was assessed for the four holobranchs. Finally, an analysis of the number of copepods at different infection sites in the branchial chamber was performed for fish of different size. The classes considered in this analysis were as follows: class 1: < 25.0 cm (N = 43); class 2: [25.0–30.0[cm (N = 107); class 3: [30.0–35.0[cm (N = 39); and class 4: ≥ 35.0 cm (N = 21).

7.4. Results

The overall infection levels of a total of 4841 specimens of *A. cornuta* were: prevalence = 80.0%; intensity (mean ± SD [range]) = 28.8±24.0 (1–110) parasites.

The branchial chamber constituted, as expected, the preferred site of attachment. However on rare occasions, i.e. in three fish recording several infection sites, a few parasites were also isolated from the skin (N = 2) and fins (N = 5). Internally, no parasite was found attached to the bony parts of the holobranchs, urohyal and operculum, and within the branchiostegal pocket. Also, no specimen was found infecting the eyes, nostrils and mouth cavity. The recorded data support the existence of a positive correlation between the intensity and the number of infection sites (Spearman's rank correlation test: r_s = 0.795, N = 168, P < 0.0001) (Fig. 7.2).

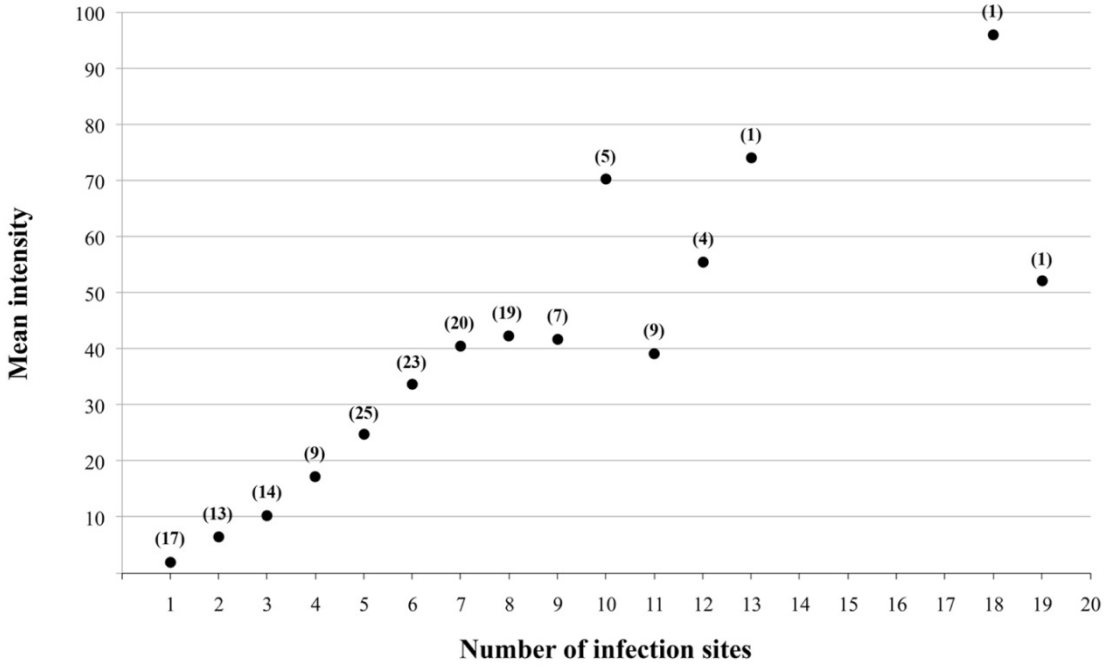


Fig. 7.2 – Relationship between the mean intensity of *Acanthochondria cornuta* and the number of infection sites on the host's body (the numbers of fish are given in parentheses).

Besides this, the existence of a side bias in infection was partially supported by the results of the performed statistical analyses, i.e., for abundance but not for prevalence (Wilcoxon's matched-pairs signed ranks test: Z = -5.099, N = 210, P < 0.0001; McNemar's test: N = 210, P = 0.134; respectively), with the parasites preferring the ocular side (mean ± SD abundance: 13.1±14.4 parasites) to the blind side (mean ± SD abundance: 10.0±11.2 parasites) of the fish's body.

Similar infection trends were found when the sites of attachment on the ocular and blind branchial chambers were considered separately. Moreover, the internal wall was the site most frequently/heavily parasitized, followed closely by the filaments of the pseudobranchs. The infection levels recorded for the filaments of the holobranchs (I-IV) were comparatively lower (Table 7.1).

Table 7.1 – Infection levels – prevalence (%) and intensity (mean ± SD) – of *Acanthochondria cornuta* recorded for the different sites of attachment in the ocular and blind branchial chambers of the European flounder, *Platichthys flesus* (L.) (abbreviations: IWC, Internal Wall of the Chamber; HIF, Holobranch I Filaments; HIIF, Holobranch II Filaments; HIIIF, Holobranch III Filaments; HIVF, Holobranch IV Filaments; and PF, Pseudobranch Filaments).

Fish's body side	**Ocular**						**Blind**					
Infection site	**IWC**	**HIF**	**HIIF**	**HIIIF**	**HIVF**	**PF**	**IWC**	**HIF**	**HIIF**	**HIIIF**	**HIVF**	**PF**
Prevalence (%)	59.5	29.1	29.5	25.7	34.8	58.1	55.2	20.0	24.3	24.3	23.8	52.4
Intensity (mean ± SD)	9.4±8.8	2.5±2.1	2.5±2.0	2.0±1.3	2.1±1.4	8.2±7.4	7.5±6.8	2.1±1.4	2.4±1.9	2.7±1.9	3.0±3.3	6.8±6.2

It is worth noting that, when infecting the internal wall, the parasites were frequently crowded in the posterior region, moreover, in a restricted and narrow space between the end of holobranch I and the pseudobranch. As for the variability in parasite distribution among the inner and outer hemibranchs, significant differences were found only for the ocular side, that is, for holobranchs I, II and IV, with the parasites accumulating, to a greater extent, on the inner hemibranch (Fig. 7.3 and Table 7.2).

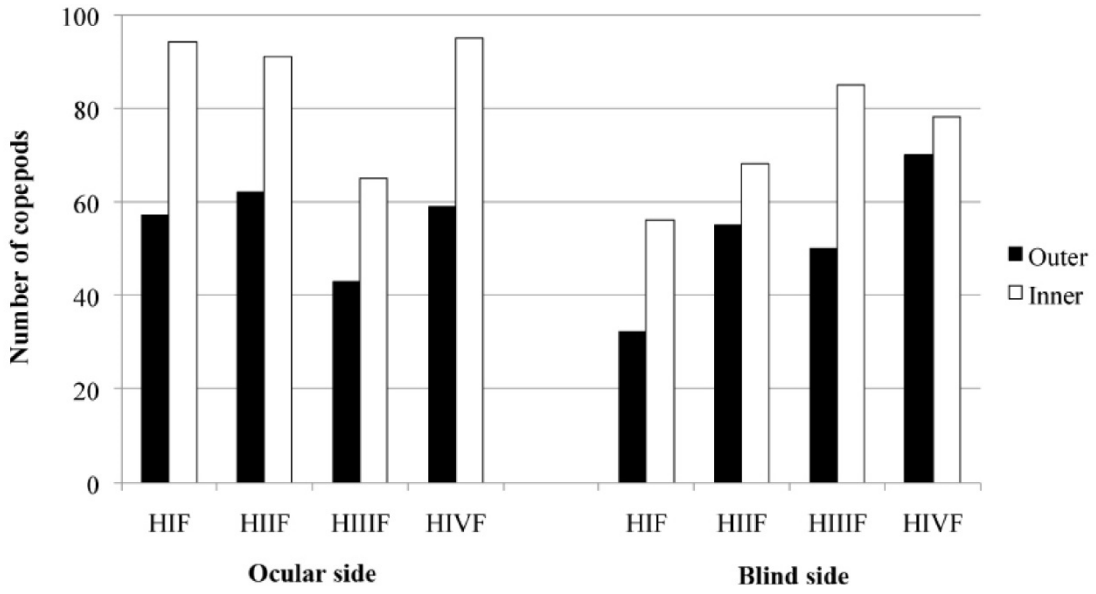


Fig. 7.3 – Spatial distribution of *Acanthochondria cornuta* among the inner and outer hemibranchs of the ocular and blind holobranchs (I-IV) of the European flounder, *Platichthys flesus* (L.) (abbreviations: HIF, Holobranch I Filaments; HIIF, Holobranch II Filaments; HIIIF, Holobranch III Filaments; and HIVF, Holobranch IV Filaments).

Table 7.2 – Results for the Wilcoxon's matched-pairs signed ranks test, which compared between parasite abundance on the inner and outer hemibranchs of holobranchs (I-IV) (abbreviations: HIF, Holobranch I Filaments; HIIF, Holobranch II Filaments; HIIIF, Holobranch III Filaments; and HIVF, Holobranch IV Filaments).

Side	HIF	HIIF	HIIIF	HIVF
Ocular	Z = -2.791 P = 0.005[a]	Z = -2.211 P = 0.027[a]	Z = -1.345 P = 0.179	Z = -2.151 P = 0.031[a]
Blind	Z = -1.764 P = 0.078	Z = -0.833 P = 0.405	Z = -1.869 P = 0.062	Z = -1.521 P = 0.128

[a]Significant result (P < 0.05).

The internal wall (mean ± SD abundance [ocular and blind together]: 9.7±13.2 parasites) was preferred over the pseudobranch (mean ± SD abundance [ocular and blind together]: 8.3±11.3 parasites), and this over the holobranchs (I-IV) together (mean ± SD abundance [ocular and blind together]: 5.1±6.9 parasites), with significant

differences in infection abundance recorded between the three sites, both for the ocular and blind sides (Friedman's rank sum test: χ^2 = 15.356, DF = 2, P < 0.0001 [ocular side] and χ^2 = 11.548, DF = 2, P = 0.003 [blind side]). The parasite exhibited no preference for any given holobranch, for either the ocular or blind sides (Friedman's rank sum test: χ^2 = 4.429, DF = 3, P = 0.219 [ocular side] and χ^2 = 3.482, DF = 3, P = 0.323 [blind side]). Some interesting trends on the spatial distribution of the different stages of development and sexual maturity inside the chamber were noticed. Moreover, the younger stages i.e., the copepodites (I-V) and pre-adult females, dominated on the filaments of the holobranchs (I-IV); instead, the adults were mainly found infecting the filaments of the pseudobranchs – in the case of the gravid adult females – and the internal walls of the chambers – in the case of the post-gravid females and adult males. As for the non-gravid adult females, they distributed fairly equally among the internal wall, the filaments of the holobranchs (I-IV) and the filaments of the pseudobranchs. Their distribution is, however, remarkable in that it resembles that of the adult males (Fig. 7.4).

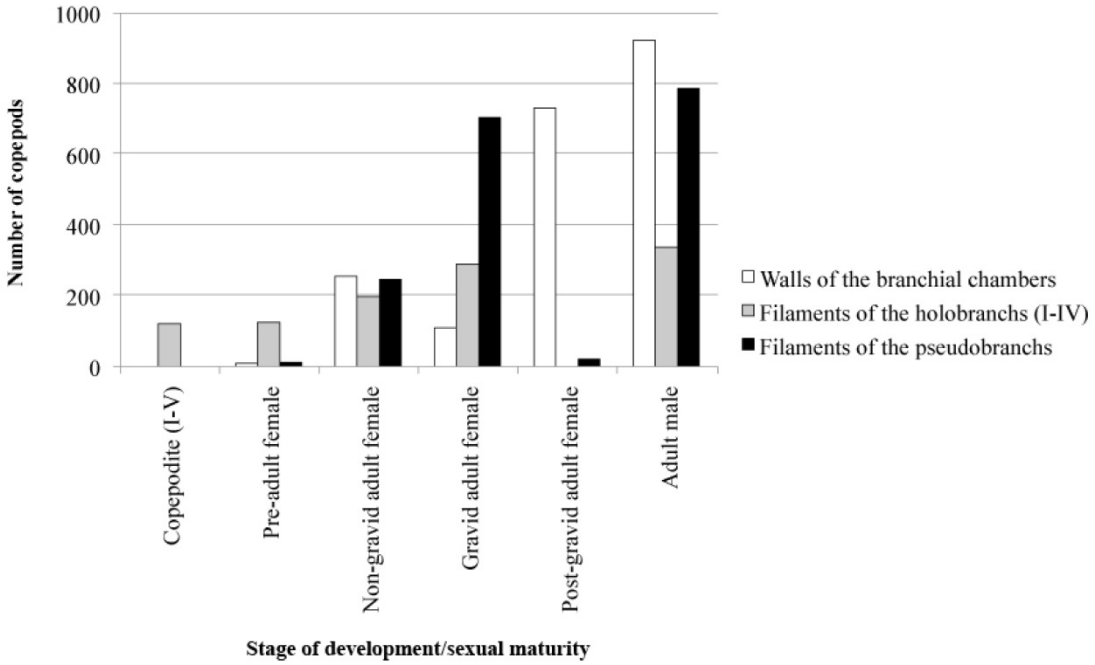


Fig. 7.4 – Site distribution of the different stages of development and sexual maturity of *Acanthochondria cornuta* inside the branchial chamber of the European flounder, *Platichthys flesus* (L.).

Concerning the distribution among the four holobranchs, the copepodites (I-V) were dominant on holobranchs II and III, the pre-adult and non-gravid adult females on holobranch IV and the gravid adult females on holobranch I (Fig. 7.5).

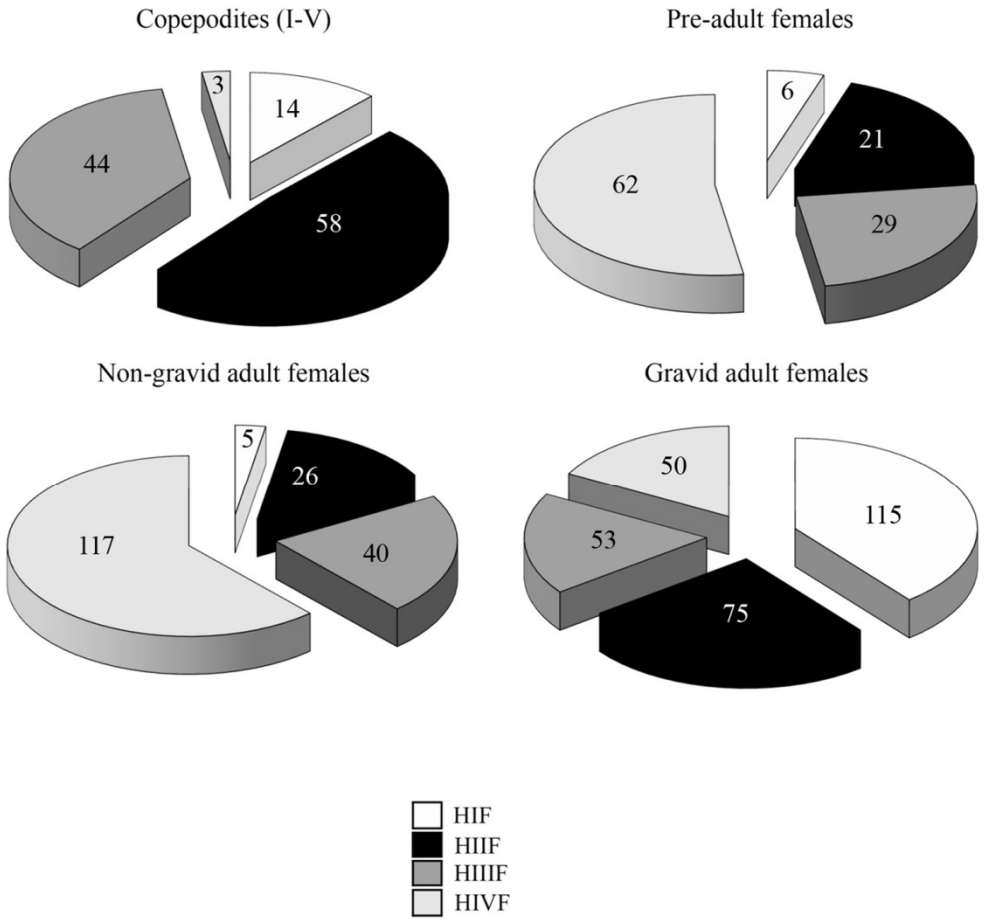


Fig. 7.5 – Site distribution of *Acanthochondria cornuta* (copepodites (I-V), pre-adult females, non-gravid adult females and gravid adult females) on the holobranchs (I-IV) of the European flounder, *Platichthys flesus* (L.) (abbreviations: HIF, Holobranch I Filaments; HIIF, Holobranch II Filaments; HIIIF, Holobranch III Filaments; and HIVF, Holobranch IV Filaments).

Site selection seems not to depend on host size (Fig. 7.6).

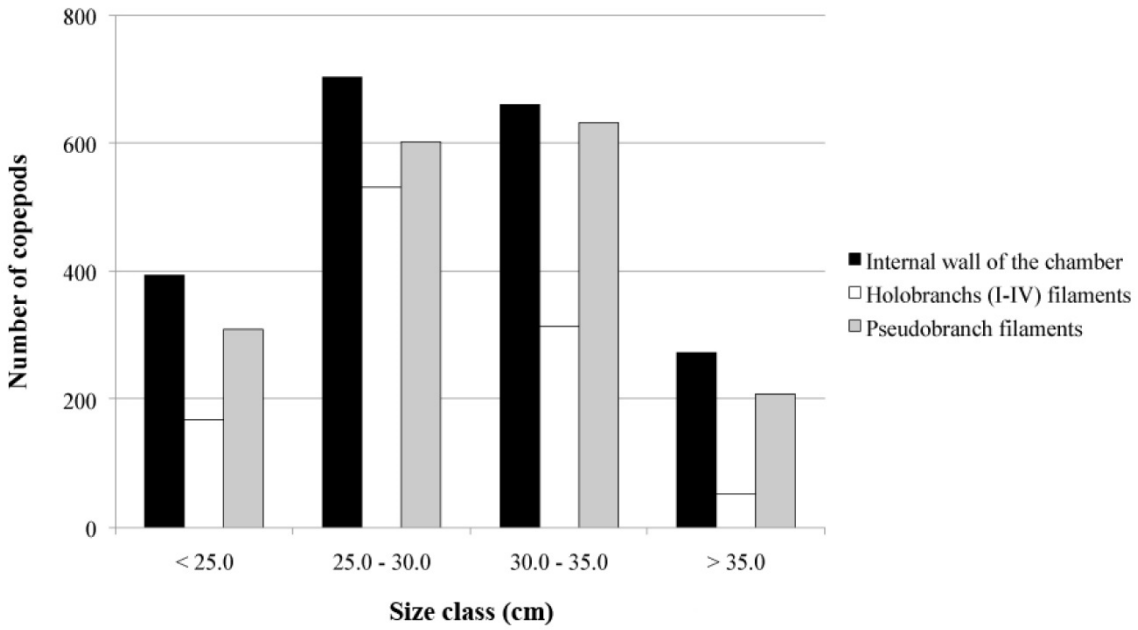


Fig. 7.6 – Site distribution of *Acanthochondria cornuta* recorded for four size classes of European flounder, *Platichthys flesus* (L.).

The site of attachment of the male parasites was always the female genito-abdomen, as indeed typical for the male chondracanthids. However, it was found that three of the isolated females were bigamous, that is, were coupled with two males, each of which attached to a different nuptial organ.

7.5. Discussion

Overall, the evidence found in this work suggests that *A. cornuta* distributes according to a particular pattern in the European flounder's branchial chambers. The intensity is indicated as an important regulator of its spatial distribution, with high levels resulting, sometimes, in the infection of 'atypical' sites, such as the skin and fins. This might be due to increased intraspecific competition, once *A. cornuta* is a large body-size species, compared to the amount of free space under the operculum, that tends to occur in high numbers in the European flounder, as reported in this study and others (Schmidt et al., 2003; Marques et al., 2006). The side bias in infection abundance

might reflect the fact that the fish spends most of its lifetime buried in the bottom sediment. This means that the ocular side offers less resistance to the respiratory water flow, being therefore, comparatively better ventilated. As a consequence, most of the infective stages carried by the ventilating current will be directed to it. Despite the previous efforts to characterize the ecology of *Acanthochondria* infections in flatfish (see Kabata, 1959), no similar bias in parasite abundance was recorded. This latter author reported, however, the occurrence of parasite crowding at the same region of the internal wall of the plaice, *Pleuronectes platessa* Linnaeus, 1758. As for the differences found in other host-parasite systems e.g., sites of attachment and overall infection levels, they should relate, at the least in part, to differences in the anatomy of the respiratory apparatus, that of the European flounder being of the 'open' type (Kabata, 1959). As *A. cornuta* develops from one stage into another, it seems to migrate to different sites within the chamber, with the copepodites (I-V) located mostly in the central holobranchs, and the remaining stages in sites at some distance from the chamber's centre. Moreover, the intensity trend found for the copepodites (I-V), that is II-III-I-IV, along with the preferential infection of the inner hemibranchs and the crowding in the posterior region of the internal wall suggest that the ventilating water current is a major driving force of spatial distribution. Another possible constraint of site selection is the blood supply (probably a main food source) since (i) the parasite was absent from the operculum and branchiostegal membrane, both of which are poorly vascularized and (ii) the filaments of the holobranchs (I-IV) and pseudobranch together accounted for most of the parasite records. Less easy to explain is the variability in the main sites of attachment of the gravid and post-gravid females. On the one hand, the pseudobranchs seem to constitute an adequate place for the egg sac development, since they are not under the direct effect of the main direction of the ventilating current. On the other hand, while in the internal wall, the ovigerous females can liberate their eggs easily to the surrounding environment, therefore becoming post-gravid. Competition (for space and nourishment) with other branchial parasites present in the examined fish i.e., *Nerocila orbignyi* (Guérin-Méneville, 1832) (Isopoda: Cymothoidae) and gnathiid pranizae (Isopoda: Gnathiidae) (see Cavaleiro & Santos, 2007, 2009), should have been a less important factor in shaping *A. cornuta* spatial distribution. This is because the infection levels recorded for those two taxa were very low.

In conclusion, the results found in this work seem valuable in providing new insights on the behaviour of the chondracanthid copepods. Moreover, in the parasitic copepods, site selection has been suggested as being highly dependent on the species ability to move freely over the host's body surface e.g., *Lepeophtheirus pectoralis*

(Copepoda: Caligidae) on the European flounder (see Scott, 1901) and, to a lesser extent, on both morphological and physiological factors (Lo & Morand, 2001). However, in the case of *A. cornuta* (and all other gill copepods), the parasite was expected to remain stationary, that is, attached to the initial infection site. This is because it lives in a perpetual waterfall needing, therefore, to be securely fastened, a situation that argues against mobility (Kabata, 1982). Such an expectation is not supported by our data, which suggest instead, a displacement of the parasite along its successive stages of development and sexual maturity. This behaviour might be advantageous since it will reduce the competition among the different stages. The latter might become particularly problematic in the summer season, when the number of adult females increases approximately 3–7 times, as compared with other seasons of the year (Cavaleiro & Santos, 2009). The condition of having more than one male attached is not new to female chondracanthids. Indeed, it was previously reported for *Rhynchochondria longa* Ho, 1967 by Ho (1967) and for *Juanettia cornifera* Wilson, 1921 by Ho (1970). To the best of our knowledge, however, no report on such a condition exists for *A. cornuta*. In this species, the female is provided with paired nuptial organs (Østergaard & Boxshall, 2004), which suggests that coupling with more than one male may constitute a relevant aspect of its reproductive biology. Notwithstanding, bigamous females were rare in this study.

7.6. Acknowledgements

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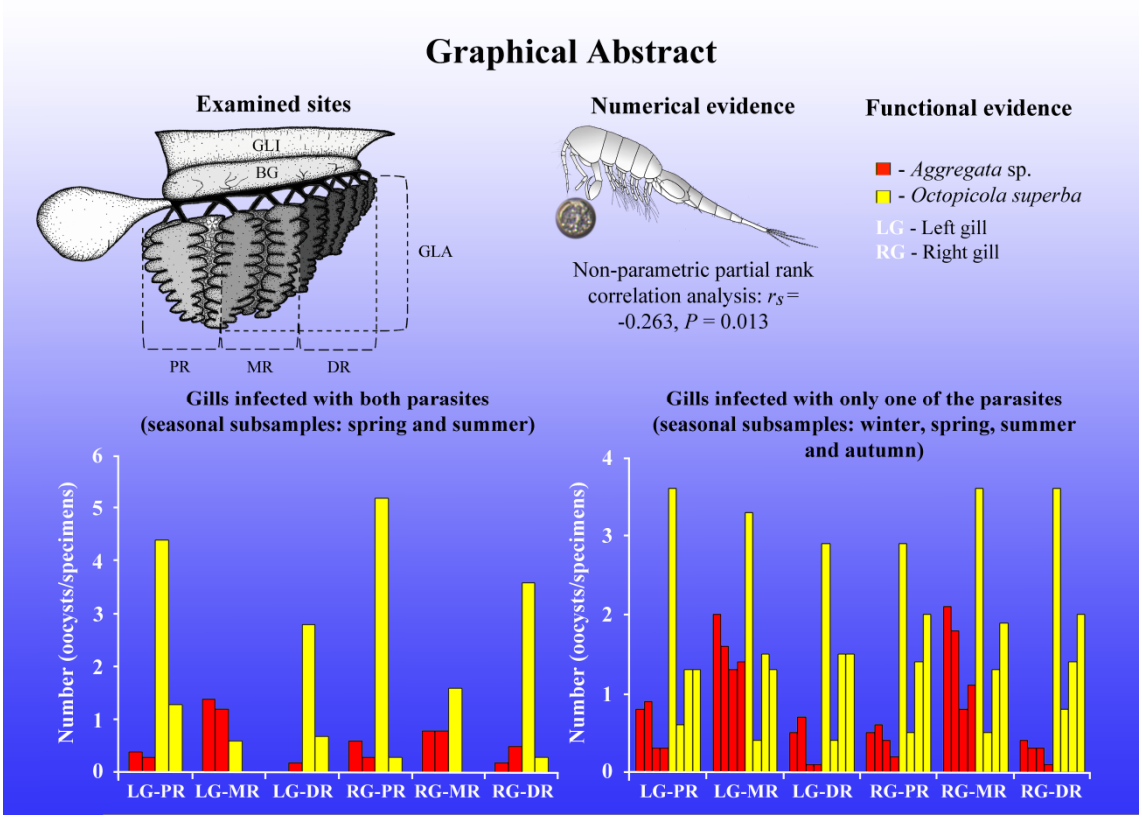
Chapter 8

Numerical and functional responses to the presence of a competitor – the case of *Aggregata* sp. (Apicomplexa: Aggregatidae) and *Octopicola superba* (Copepoda: Octopicolidae)



This chapter has been adapted from:

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8.1. Abstract

Evidence of interference competition between the eimeriorin coccidian *Aggregata* sp. and the octopicolid copepod *Octopicola superba* at the level of the gills of naturally infected *Octopus vulgaris* is evaluated. Numerical and functional responses are considered for analysis, and the fundamental and realized spatial niches (FSNs and RSNs) are measured as part of the study. While it was not possible to measure the FSN of *Aggregata* sp., the analysis of the infection levels of *O. superba* recorded for non-concomitantly and concomitantly infected hosts suggests that the gills and body skin constitute, respectively, the main and accessory sites of infection of the parasite. According to the evidence found, the gills function mainly as an accessory site of infection of *Aggregata* sp., in specimens in which the caecum and intestine are massively infected. Evidence for a negative interaction between *Aggregata* sp. and *O. superba* has been found while controlling for a potential confounding effect of host size. Furthermore, the presence of *O. superba* on gill lamellae appears to have been negatively affected by the presence of *Aggregata* sp., while this latter remained mostly undisturbed. The mean number of oocysts of *Aggregata* sp. in the gills was higher in spring and summer, which were also the seasons presenting the broadest RSN for *O. superba*.

8.2. Introduction

The common octopus, *Octopus vulgaris* Cuvier, 1797 (Cephalopoda: Octopodidae), acts as host of parasites of different taxonomic groups. Among them, two, the eimeriorin coccidian *Aggregata octopiana* (Schneider, 1875) Frenzel, 1885 (Apicomplexa: Aggregatidae) and the octopicolid copepod *Octopicola superba* Humes, 1957 (Copepoda: Octopicolidae), are highly host specific and were reported to occur in high prevalence (Pascual et al., 1996) and abundance (Bocquet & Stock, 1960) in samples of *O. vulgaris* from different geographical regions. Both of them were reported to infect the gills (e.g. Hochberg, 1983; Gestal et al., 2002; Mladineo & Jozić, 2005; Pascual et al., 2006; Mladineo & Bočina, 2007), but the occurrence of concomitantly infected hosts – that is, the simultaneous infection of *A. octopiana* and *O. superba* in *O. vulgaris* – and the possibility of interspecific interference competition at the level of the gills have not yet been addressed in any study. The gill infection with eimeriorin coccidians presumably impairs the octopicolid copepods' ability to physically establish on gill tissue resulting, therefore, in interspecific interference competition. Indeed, a complete substitution of the epithelial and connective tissues by cysts and developmental stages of *A. octopiana*, resulting in necrosis and desquamation, has already been documented for *O. vulgaris* (Mladineo & Bočina, 2007).

Evidence of interspecific competition is best documented for helminth parasites (see e.g. Poulin, 2007a; Randhawa, 2012). It can be numerical or functional and both types are equally convincing (see Poulin, 2001, 2007a). When searching for numerical evidence of interspecific competition in concomitantly infected hosts, one must test for the existence of a negative relationship between the numbers of parasites of the two species. Furthermore, a potential confounding effect of variables at the host and environment levels on parasite populations and communities (see e.g. Thomas et al., 2005) must be accounted for, if such a relationship is to be properly detected. In turn, functional evidence of competition concerns a change in how a parasite uses a given host resource, in response to the presence of another parasite. This type of evidence is most frequently detected as a slight shift in the site of infection. Accordingly, it can be derived by characterizing the ecological niches (sensu Hutchinson, 1957) of parasites, or more specifically, by considering their spatial dimension. Both the fundamental spatial niche (FSN) and the realized spatial niche (RSN) must be considered for analysis (see Poulin, 2007a). The former refers to the potential distribution of a parasite in the host's body, that is, the range of sites in which a parasite species can develop, while the latter concerns the actual niche occupied by a parasite, which is determined by the interactions it establishes with other parasites. The FSN can only be measured if

data from specimens harbouring single species infections are available (e.g. Holmes, 1961; Patrick, 1991). In summary, the interspecific competition can result in changes in numbers of parasites and/or in changes in the spatial distribution of parasites in the host's body.

The gills of octopuses constitute an atypical site of infection of eimeriorin coccidians, as these are usually transmitted trophically, that is, through predation of crustaceans, the usual intermediate hosts (Hochberg, 1990). Nonetheless, they might be found infected with them in cases of massive infection, as documented for *O. vulgaris* and the genus *Aggregata* (e.g. Mladineo & Jozić, 2005; Pascual et al., 2006). An association between the infection of the gills and the infection of the gastrointestinal tract, the usual site of infection, has, however, not yet been tested.

This study follows on from a survey on the parasite fauna of wild-caught *O. vulgaris*, during the course of which both eimeriorin coccidians (i.e. *Aggregata* sp., most likely *A. octopiana*; it was not possible to measure the sporozoite dimensions to unequivocally ascertain the identity of the species) and octopicolid copepods (i.e. *O. superba*, European subspecies [*O. s. superba*]) were observed at the gills. Its aims were as follows: first, to characterize, in numerical terms, the occurrence of *Aggregata* sp. and *O. superba* in the body and gills of the wild-caught specimens of *O. vulgaris*; second, to characterize the FSNs and RSNs of *Aggregata* sp. and *O. superba*; and third, to search for numerical and functional evidence of interference competition between *Aggregata* sp. and *O. superba* at the level of the gills.

8.3. Materials and Methods

Octopus vulgaris *sampling and parasitological examination*

The samples of *O. vulgaris* examined for parasites consisted of 30 specimens each and were collected seasonally during 2010 (winter sample: 2 March; spring sample: 24 and 31 May; summer sample: 7 September; and autumn sample: 22 November) off Matosinhos (41°10′N, 8°42′W), northwest Portuguese coast, northeast Atlantic Ocean. Each octopus was characterized with respect to different variables, which included the total body length, sex and stage of sexual maturity (determined according to Dia & Goutschine, 1990); the Kruskal-Wallis' test evaluated whether octopuses in different samples were of comparable size (i.e. total length). The body skin and connective tissue of arms were washed with saline solution (35‰) to remove the ectoparasites

present and, after dissection, all organs were examined for the presence of parasites. The occurrence of lesions in the body skin and connective tissue of arms, namely of areas of exfoliation with discernible coccidian oocysts in the epidermis, was evaluated. The observations were first carried out under a stereo dissecting microscope and then under a compound microscope (mucus and skin scrapings and smears of all organs). The infection parameters (i.e. prevalence and abundance) were determined according to Bush et al. (1997). In order to properly address the issue of interspecific interference competition, different sites were considered for analysis in each gill (Fig. 8.1): the Gill LIgament (GLI); the Branchial Gland (BG); the Gill LAmellae (GLA); the band of connective tissue joining the dorsal and ventral lamellae (indicated with a white *); and the stalks joining the primary lamellae to the BG (indicated in black). Furthermore, three lamellar regions – the proximal, middle and distal lamellar regions of the left and right gills – were analysed separately. Each of these extends along 1/3 of the gill axis length.

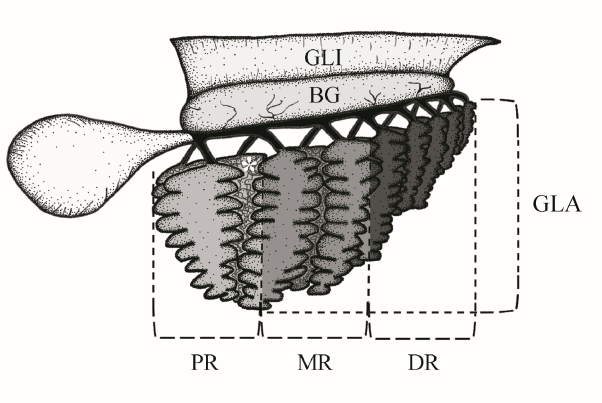

Fig. 8.1 – The different sites considered for analysis in each gill (abbreviations: BG, Branchial Gland; GLA, Gill LAmellae; GLI, Gill LIgament; PR, Proximal Region; MR, Middle Region; and DR, Distal Region; in black are the stalks joining the primary lamellae to the branchial gland, while the white * marks the band of connective tissue joining the dorsal and ventral lamellae) (modified from Budelmann et al., 1997).

Occurrence of Aggregata *sp. and* O. superba *in the body and gills of* O. vulgaris

In order to get a general picture of the occurrence of the two parasites in the surveyed octopuses (*N* = 120), the number and percentage of specimens infected with (i) each of them and (ii) *Aggregata* sp. and *O. superba* were determined. Concerning the occurrence of the two parasites at the gills, in particular, we evaluated the number and percentage of specimens infected with (i) *Aggregata* sp. but not with *O. superba*, (ii) *O. superba* but not with *Aggregata* sp., (iii) *Aggregata* sp. and *O. superba*, (iv) *Aggregata* sp., regardless of whether or not *O. superba* had been detected on the body of *O.*

vulgaris; and (v) *O. superba*, regardless of whether or not *Aggregata* sp. had been detected in the body of *O. vulgaris*. Beyond that, the number of oocysts of *Aggregata* sp. and specimens of *O. superba* were assessed (mean and SE) for the gills of non-concomitantly and concomitantly infected octopuses. Although other parasites were found infecting the examined octopuses, only these two were found frequently (were component taxa – prevalence for the total sample of octopuses > 10% [sensu Bush et al., 1990]) and in high numbers. Hence, the occurrence of other parasites and the possibility of interspecific competition between other pairs of parasites were disregarded.

Characterization of the ecological niches of Aggregata *sp. and* O. superba

The characterization of the ecological niches of *Aggregata* sp. and *O. superba* focused on the spatial dimension of the niche exclusively and considered both the FSN and the RSN. The seasonal samples of octopuses were considered separately for analysis, so that seasonal patterns of parasite occurrence and abundance could not interfere with the results and it was possible to evaluate whether or not the observed niche configuration was consistent between samples. The FSN of *Aggregata* sp. could not be measured once *O. superba* was found infecting all the examined octopuses. The RSN of *Aggregata* sp. and the fundamental and RSNs of *O. superba* were characterized by quantifying the differences in parasite occurrence and abundance between the sites of infection. In the case of the RSNs, only the octopuses infected with *Aggregata* sp. and *O. superba* were considered for analysis. The infection parameters assessed for each site of infection included the number and percentage of octopuses in which the site was found infected with a particular parasite and parasite counts (mean ± SD [range]). Concerning *Aggregata* sp., it is not possible to determine the true number of parasites (that is, the exact number of sporozoites) present in a given site. A reliable estimate of this infection parameter could however be obtained by counting the oocysts visible to the naked eye, as those octopuses which were more heavily infected usually presented both more oocysts (enclosing many sporocysts) and sporocysts (enclosing several sporozoites). The oocyst counting was performed in tissue sections of about 1.0 cm^2 (caecal wall, intestinal wall and proximal, middle and distal lamellar regions of gills) – a measure henceforth referred to as ‘density of coverage of *Aggregata* sp.’; only the oocysts visible on the surface were counted. This procedure could be adopted since, as a rule, the oocysts were regularly distributed throughout the infected tissues. The total numbers for the gastrointestinal tract and gills were obtained by summing the

counts for the different sites of infection, that is, the densities of coverage for the lamellar regions and the counts for the stalks and band of connective tissue in the case of the gills, and the densities of coverage for the caecal and intestinal walls in the case of the gastrointestinal tract. The Levins' measure of niche breadth (*B*) was assessed (following Geets et al., 1997; see also Šimková et al., 2000) for each infrapopulation (sensu Bush et al., 1997) and standardized afterwards (B_A). The mean and SD levels of *B* and B_A were determined for both types of niches (fundamental and RSNs). *B* and B_A were assessed as follows:

$$B = \frac{1}{(\sum[p_j{}^2])}$$

where p_j is the proportion of specimens of a parasite found on infection site *j*.

$$B_A = \frac{B-1}{N-1}$$

where *B* is the Levins' measure of niche breadth and *N* the number of infection sites. The existence of a relationship between the infection of gills and gastrointestinal tract was evaluated using the total numbers of oocysts recorded for the two sites (Spearman's rank order correlation test). The overlap between RSNs was measured using the percentage overlap measure, also known as the Renkonen's index (*P*) (following Geets et al., 1997; see also Šimková et al., 2000):

$$P = 1 - (\sum\frac{[p_{ia} - p_{ja}]}{2})$$

where p_{ia} is the proportion of parasites of taxon *i* found on infection site *a* and p_{ja} the proportion of parasites of taxon *j* found on infection site *a*.

Evaluation of numerical and functional evidence of interference competition

An influence of season and host sex and stage of sexual maturity in the distribution of the two parasites across the different lamellar regions of the gills was evaluated considering the total sample of octopuses. Moreover, the counts recorded for the different seasons of sampling, sexes and stages of sexual maturity were plotted together and the existence of substantial differences was evaluated. Afterwards, numerical evidence of interspecific interference competition at the level of the gills was evaluated by running a non-parametric partial rank correlation analysis in SPSS. This

analysis tested the existence of a significant negative relationship between the counts recorded for the two parasites, while controlling for a potential confounding effect of host body size (i.e. total length) in the results. Since there is no direct way to conduct it in SPSS, the analysis was specified in a syntax editor window, in accordance with the instructions provided at the IBM website (http://www01.ibm.com/support/docview.wss?uid=swg21474822). Only the octopuses infected with at least one of the two parasites at the gills were considered for analysis. Functional evidence of competition was evaluated by characterizing the occurrence of each parasite (number and percentage of octopuses in which the site was found infected with a particular parasite and density of coverage/parasite counts (mean ± SD [range])) in each of the three lamellar regions. This characterization was performed separately for the seasonal subsamples of octopuses infected with (i) both parasites at the gills and (ii) only one of the two parasites at the gills and for the left and right gills. A change in the infection levels of one parasite recorded for different lamellar regions, which could have been determined by the presence of the other parasite, was evaluated.

Statistical analysis of data

Data were analysed using SPSS for Windows, version 19.0 (SPSS Inc., Chicago, Illinois). The significance level was set at $P < 0.05$. Non-parametric tests were used because the abundance data (sensu Bush et al., 1997) for *O. superba* did not fit the normal distribution (one-sample Kolmogorov-Smirnov's test: $Z = 1.353$, $P = 0.051$, $N = 120$ [*Aggregata* sp.]; and $Z = 2.032$, $P = 0.001$, $N = 120$ [*O. superba*]) (Zar, 1996).

8.4. Results

Characterization of the seasonal samples of O. vulgaris

The data recorded for the seasonal samples of *O. vulgaris* were as follows: winter sample: 69.8±8.2 (56.6–86.0) cm, 13 ♀♀ and 17 ♂♂ and 16 immatures and 14 matures; spring sample: 68.3±10.9 (53.4–88.7) cm, 15 ♀♀ and 15 ♂♂ and 16 immatures and 14 matures; summer sample: 65.8±10.8 (50.2–90.1) cm, 17 ♀♀ and 13 ♂♂ and 19 immatures and 11 matures; and autumn sample: 66.9±7.9 (53.4–89.1) cm, 11 ♀♀ and 19 ♂♂ and 15 immatures and 15 matures. The octopuses in different samples were of comparable size (Kruskal-Wallis' test [for total body length]: χ^2 =

3.755, DF = 3, *P* = 0.289). No area of exfoliation with discernible coccidian oocysts was ever seen in body skin and connective tissue of arms.

Occurrence of Aggregata *sp. and* O. superba *in the body and gills of* O. vulgaris

Fifteen (12.5%) out of the 120 examined octopuses were infected only with *O. superba*, while none was infected with *Aggregata* sp. exclusively; the two parasites co-occurred in 105 (87.5%) octopuses. In 39 octopuses (32.5%), the gills were infected with *Aggregata* sp. but not with *O. superba*; in 40 (33.3%), they were infected with *O. superba* but not with *Aggregata* sp.; and in 11 (9.2%), they were infected with both parasites. When disregarding whether the other parasite had also been detected in the octopus' body, it was found that *Aggregata* sp. and *O. superba* occurred at the gills of 50 (41.7%) and 51 (42.5%) octopuses, respectively. The number of specimens of *O. superba* recorded for the gills was smaller, on average, for the subsample of concomitantly infected octopuses ($N_{O.\ vulgaris}$ = 105), compared with that recorded for the subsample of non-concomitantly infected octopuses ($N_{O.\ vulgaris}$ = 15). However, this result was clearly not statistically significant. In this respect, no consideration is made for *Aggregata* sp., as none of the octopuses was infected with it exclusively (Fig. 8.2). Figures 8.3 and 8.4 show the oocyst and specimen counts for the gills of the examined octopuses. A non-linear relationship between the counts for the two parasites is evident (Fig. 8.3). Single and concomitant infections occurred in female and male octopuses, as well as in immature and mature octopuses (Fig. 8.4).

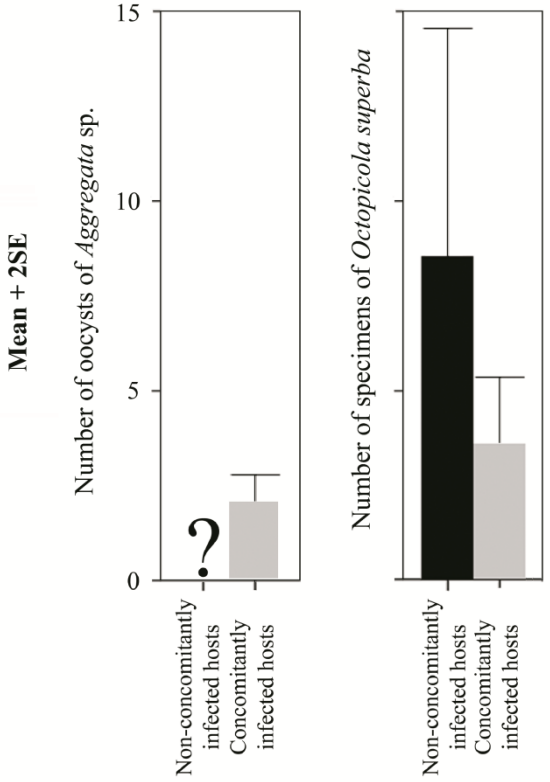

Fig. 8.2 – Mean (+ 2 SE) number of oocysts of *Aggregata* sp. and specimens of *Octopicola superba* recorded for the gills of non-concomitantly ($N_{O.\ vulgaris}$ = 15) and concomitantly ($N_{O.\ vulgaris}$ = 105) infected hosts.

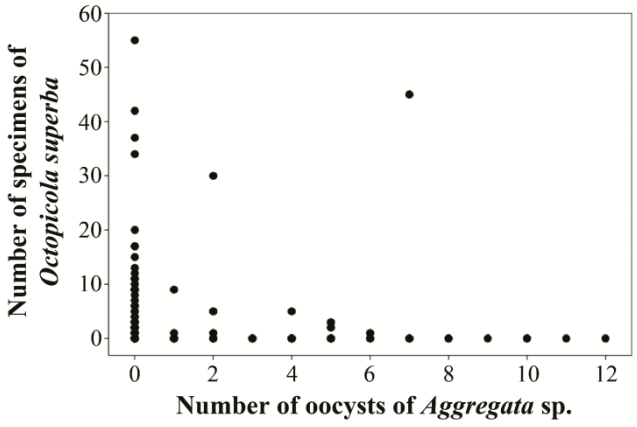

Fig. 8.3 – Number of oocysts of *Aggregata* sp. and specimens of *Octopicola superba* recorded for the gills of the examined octopuses ($N_{O.\ vulgaris}$ = 120).

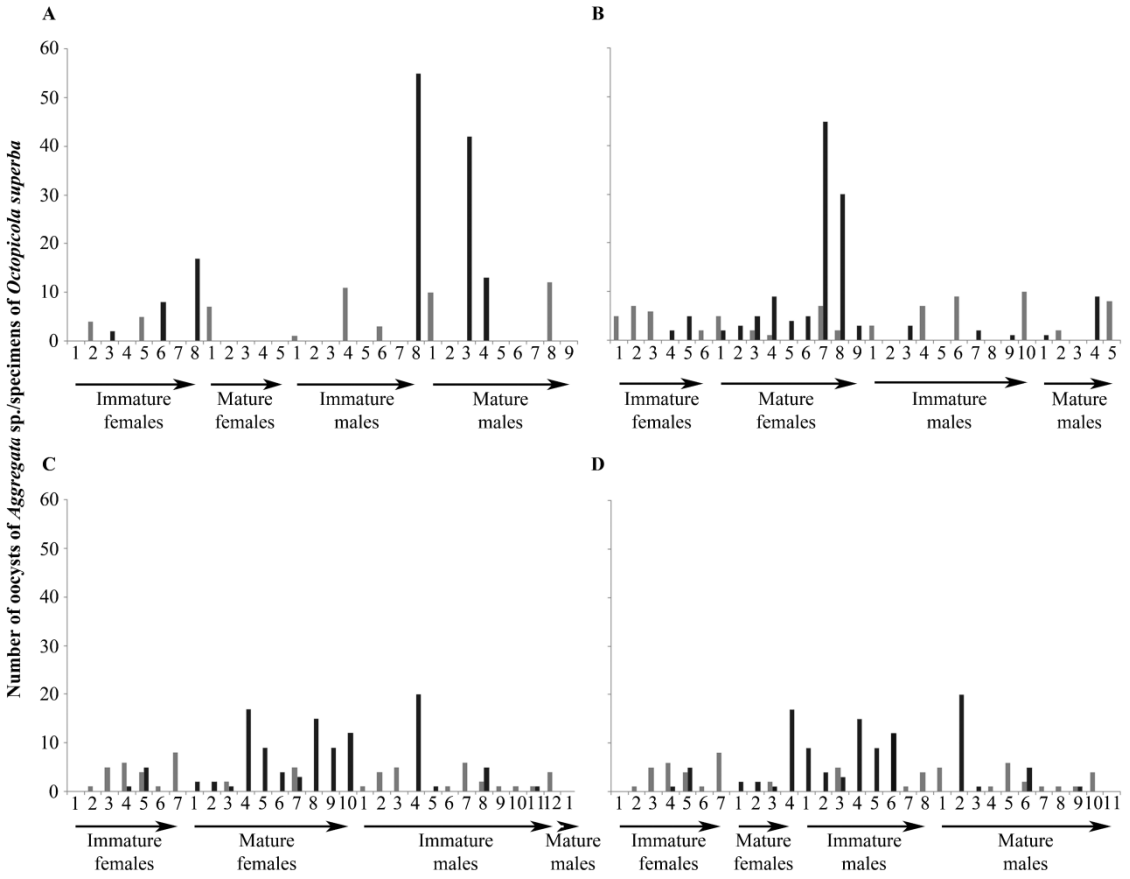

Fig. 8.4 – Counts of oocysts of *Aggregata* sp. (in grey) and specimens of *Octopicola superba* (in black) for the gills of each of the examined octopuses (ordered by ascending total length in each group – immature females, mature females, immature males and mature males): A, winter sample; B, spring sample; C, summer sample; and D, autumn sample.

Characterization of the ecological niches of Aggregata *sp. and* O. superba

The RSN of *Aggregata* sp. consisted of two sites in all seasonal samples of octopuses: the gastrointestinal tract and the gills. The infection levels recorded for each of these sites and the values for the measures of niche breadth (i.e. B and B_A), are given in Table 8.1 for each seasonal sample. According to this table, in concomitantly infected hosts, the highest and lowest infection levels were recorded for the gastrointestinal tract and gills, respectively.

Table 8.1 – The Realized Spatial Niche (RSN) of *Aggregata* sp. (as determined for the seasonal subsamples of *Octopus vulgaris* infected with *Aggregata* sp. and *Octopicola superba*): infection levels – number of octopuses/percentage of octopuses; and oocyst counts (mean ± SD [range]) – recorded for the different sites and Levins' (*B*) and standardized (B_A) measures (mean ± SD) of niche breadth.

	RSN			
Season ($N_{O. vulgaris}$)	**Winter (30)**	**Spring (30)**	**Summer (30)**	**Autumn (15)**
Host site				
Gastrointestinal tract	30/100 31.4±11.7 (18–60)	30/100 29.4±11.9 (3–59)	30/100 26.1±12.3 (2–53)	15/100 28.1±7.7 (19–45)
Gills	8/26.7 1.8±3.6 (0–12)	15/50.0 2.6±3.3 (0–10)	13/43.3 2.1±2.6 (0–8)	9/60.0 2.0±1.9 (0–5)
Niche breadth				
B	1.1±0.1	1.2±0.3	1.2±0.3	1.1±0.1
B_A	0.1±0.1	0.2±0.3	0.2±0.3	0.1±0.1

Regarding *O. superba*, the FSN of the parasite consisted, also, of two sites, that is, the body skin and gills, but this could only be determined for the autumn sample of octopuses (Table 8.2). The mean parasite count was markedly higher in the gills than in the body skin. As for the RSN of the parasite, it consisted of two to six sites, which varied according to season of sampling and included the body skin, mantle musculature, gills, covering mesentery of gonad, eyes and funnel. The highest infection levels were recorded for the body skin in all seasonal samples. According to the standardized values of niche breadth (B_A), in autumn, the FSN of the parasite was, in average, broader than the RSN.

Table 8.2 – The Fundamental (FSN) (as determined for the seasonal subsample of *Octopus vulgaris* infected only with *Octopicola superba*) and Realized (RSN) (as determined for the seasonal subsamples of *O. vulgaris* infected with *Aggregata* sp. and *O. superba*) Spatial Niches of *O. superba*: infection levels – number of octopuses/percentage of octopuses; and specimen counts (mean ± SD [range]) – recorded for the different sites and Levins' (*B*) and standardized (B_A) measures (mean ± SD) of niche breadth.

	FSN	**RSN**			
Season ($N_{O. vulgaris}$)	**Autumn (15)**	**Winter (30)**	**Spring (30)**	**Summer (30)**	**Autumn (15)**
Host site					
Body skin	15/100 1.0±0.0 (1)	30/100 62.5±22.7 (18–108)	30/100 58.6±76.5 (1–198)	30/100 83.2±59.7 (5–198)	15/100 7.4±7.7 (2–32)
Mantle musculature	-	-	7/23.3 0.8±2.1 (0–8)	12/40.0 4.0±7.2 (0–32)	-
Gills	11/73.3 8.5±11.7 (0–37)	6/20.0 4.6±12.7 (0–55)	16/53.3 4.3±9.6 (0–45)	16/53.3 3.6±5.6 (0–20)	2/13.3 0.1±0.4 (0–1)
Covering mesentery of gonad	-	-	12/40.0 4.0±6.9 (0–30)	15/50.0 9.8±13.6 (0–48)	-
Eyes	-	-	4/13.3 0.1±0.3 (0–1)	3/10.0 0.1±0.3 (0–1)	-
Funnel	-	-	2/6.7 0.1±0.4 (0–2)	2/6.7 0.1±0.4 (0–2)	-
Niche breadth					
B	1.3±0.3	1.1±0.3	1.3±0.4	1.5±0.5	1.1±0.2
B_A	0.3±0.3	0.1±0.3	0.1±0.3	0.2±0.3	0.1±0.2

A significant positive correlation was detected between the oocyst counts recorded for the gills and gastrointestinal tract (Spearman's rank order correlation test: $r_s = 0.370$, $P < 0.0001$, $N = 105$). The overlap between the RSNs of the two parasites (*P*) was 0.3.

Numerical and functional evidence of interference competition

An influence of season and host sex and stage of sexual maturity in the distribution of the two parasites across the different lamellar regions of the gills could be excluded after analysing the corresponding plots (Fig. 8.5 A and B).

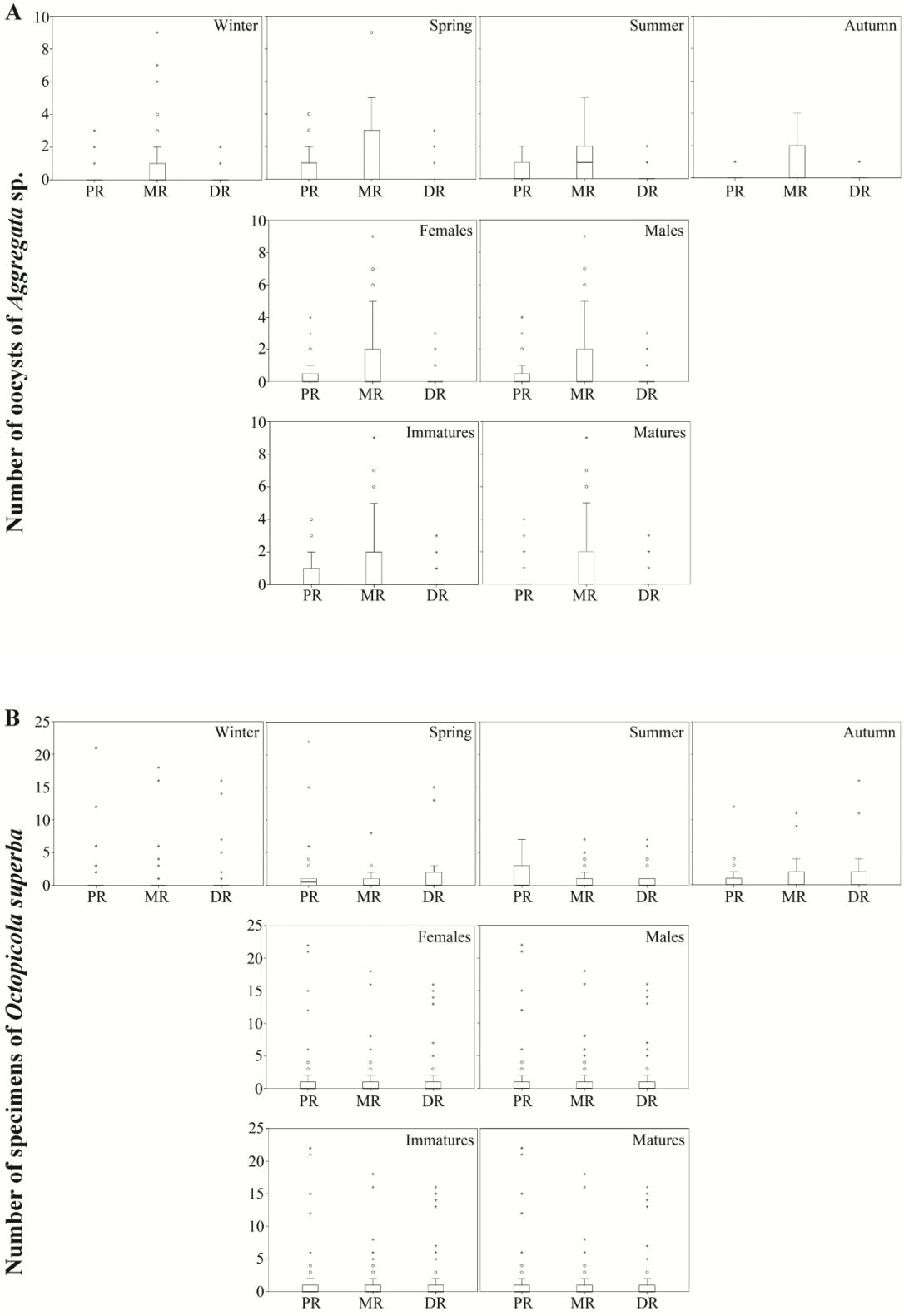

Fig. 8.5 – Distribution of parasites (number of oocysts/specimens) across the different lamellar regions according to season of sampling and host sex and stage of sexual maturity: A, *Aggregata* sp.; and B, *Octopicola superba* (abbreviations: PR, Proximal Region; MR, Middle Region; and DR, Distal Region).

Statistical support for a significant negative relationship between the two parasites has been found (non-parametric partial rank correlation analysis: r_s = -0.263, P = 0.013, N = 90). The sites of infection of *Aggregata* sp. in the gills included the stalks joining the primary lamellae to the BG (1/0.8%, 0.0±0.1 [0–1] oocysts), the band of connective tissue joining the dorsal and ventral lamellae (2/1.7%, 0.0±0.1 [0–1] oocysts) and the lamellae (50/41.7%, 1.8±2.8 [0–12] oocysts); the gill ligament and the BG were never found infected. *Octopicola superba* was found on the gill lamellae exclusively. According to the infection levels in Table 8.3, which respects the seasonal subsamples of octopuses whose gills were infected with the two parasites, *Aggregata* sp. was more frequent and found in higher numbers in the middle lamellar regions of the left and right gills, whereas *O. superba* was more frequent and found in higher numbers on the proximal and distal lamellar regions of both gills. These trends were consistent between spring and summer seasons.

Table 8.3 – Infection levels of *Aggregata* sp. and *Octopicola superba* – number of octopuses/percentage of octopuses; oocyst/specimen counts (mean ± SD [range]) – recorded for the Proximal (PR), Middle (MR) and Distal (DR) lamellar Regions of the Left (LG) and Right (RG) Gills (the seasonal subsamples considered for analysis consisted of those octopuses whose gills were infected with both parasites).

Site	***Aggregata* sp.**						***Octopicola superba***					
	LG			**RG**			**LG**			**RG**		
	PR	**MR**	**DR**	**PR**	**MR**	**DR**	**PR**	**MR**	**DR**	**PR**	**MR**	**DR**
Season ($N_{O.\ vulgaris}$)												
Spring (5)	2/40.0	5/100	0/0	2/40.0	3/60.0	1/20.0	4/80.0	2/40.0	3/60.0	5/100	2/40.0	4/80.0
	0.4±0.5 (0–1)	1.4±0.5 (1–2)	0.0±0.0 (0–0)	0.6±0.9 (0–2)	0.8±0.8 (0–2)	0.2±0.4 (0–1)	4.4±3.0 (0–7)	0.6±0.9 (0–2)	2.8±3.1 (0–7)	5.2±6.3 (1–15)	1.6±2.6 (0–6)	3.6±4.3 (0–10)
Summer (6)	2/33.3	5/83.3	1/16.7	2/33.3	4/66.7	2/33.3	5/83.3	0/0	3/50.0	2/33.3	0/0	1/16.7
	0.3±0.5 (0–1)	1.2±0.8 (0–2)	0.2±0.4 (0–1)	0.3±0.5 (0–1)	0.8±0.8 (0–2)	0.5±0.8 (0–2)	1.3±1.0 (0–3)	0.0±0.0 (0–0)	0.7±0.8 (0–2)	0.3±0.5 (0–1)	0.0±0.0 (0–0)	0.3±0.8 (0–2)

No major difference in the spatial distribution of *Aggregata* sp. was found when considering the subsamples of octopuses whose gills were infected with it exclusively. However, when considering the subsamples of octopuses whose gills were infected only with *O. superba*, no clear trend of spatial distribution could be identified (see Table 8.4).

Table 8.4 – Infection levels of *Aggregata* sp. and *Octopicola superba* – number of octopuses/percentage of octopuses; oocyst/specimen counts (mean ± SD [range]) – recorded for the Proximal (PR), Middle (MR) and Distal (DR) lamellar Regions of the Left (LG) and Right (RG) Gills (the seasonal subsamples considered for analysis consisted of those octopuses whose gills were infected with only one of the two parasites).

Site	***Aggregata* sp.**						Site	***Octopicola superba***					
	LG			**RG**				**LG**			**RG**		
	PR	**MR**	**DR**	**PR**	**MR**	**DR**		**PR**	**MR**	**DR**	**PR**	**MR**	**DR**
Season ($N_{O.\ vulgaris}$)							**Season ($N_{O.\ vulgaris}$)**						
Winter (8)	4/50.0	6/75.0	4/50.0	4/50.0	7/87.5	3/37.5	Winter (6)	5/83.3	5/83.3	5/83.3	6/100	6/100	5/83.3
	0.8±0.9 (0–2)	2.0±1.7 (0–5)	0.5±0.5 (0–1)	0.5±0.5 (0–1)	2.1±1.4 (0–4)	0.4±0.5 (0–1)		3.6±5.3 (0–15)	3.3±4.2 (0–12)	2.9±3.5 (0–10)	2.9±3.7 (0–10)	3.6±4.6 (0–13)	3.6±4.2 (0–11)
Spring (10)	5/50.0	7/70.0	5/50.0	5/50.0	7/70.0	3/30.0	Spring (11)	6/54.5	4/36.4	4/36.4	6/54.5	5/45.5	6/54.5
	0.9±1.1 (0–3)	1.6±1.3 (0–4)	0.7±0.8 (0–2)	0.6±0.7 (0–2)	1.8±1.9 (0–6)	0.3±0.5 (0–1)		0.6±0.7 (0–2)	0.4±0.7 (0–2)	0.4±0.7 (0–2)	0.5±0.5 (0–1)	0.5±0.7 (0–2)	0.8±0.9 (0–2)
Summer (12)	4/33.3	8/66.7	1/8.3	5/41.7	7/58.3	3/25.0	Summer (10)	5/50.0	7/70.0	7/70.0	8/80.0	8/80.0	7/70.0
	0.3±0.5 (0–1)	1.3±1.4 (0–4)	0.1±0.3 (0–1)	0.4±0.5 (0–1)	0.8±0.8 (0–2)	0.3±0.5 (0–1)		1.3±1.6 (0–4)	1.5±1.4 (0–4)	1.5±1.5 (0–4)	1.4±1.4 (0–4)	1.3±1.3 (0–4)	1.4±1.4 (0–4)
Autumn (9)	3/33.3	5/55.6	1/11.1	2/22.2	5/55.6	1/11.1	Autumn (13)	10/76.9	10/76.9	8/61.5	9/69.2	8/61.5	8/61.5
	0.3±0.5 (0–1)	1.4±1.6 (0–4)	0.1±0.3 (0–1)	0.2±0.4 (0–1)	1.1±1.2 (0–3)	0.1±0.3 (0–1)		1.3±1.2 (0–4)	1.3±1.1 (0–3)	1.5±1.7 (0–5)	2.0±3.0 (0–9)	1.9±2.5 (0–8)	2.0±3.4 (0–12)

8.5. Discussion

The eimeriorin coccidians of the genus *Aggregata* can develop in different sites of the body of *O. vulgaris*, including the body skin, connective tissue of arms, mantle musculature, gills, covering mesentery of digestive gland, covering mesentery of gonad and different sections of the gastrointestinal tract (oesophagus, crop, caecum and intestine) (Gestal, 2000; Gestal et al., 2002; Mladineo & Jozić, 2005; Pascual et al., 2006; Mladineo & Bočina, 2007). These cited studies focused on the eimeriorin coccidians, and failed to mention the occurrence of other parasites which, being present, could have influenced the spatial occurrence pattern of *Aggregata*. In this way, the available literature cannot be used to characterize the actual FSN of the parasite. The only consideration that can be made is that the RSN of the parasite consisted of two of the infection sites mentioned in the literature. In the case of *O. superba*, the FSN and RSN consisted of the same two sites in autumn; nonetheless, according to the recorded B_A values, the FSN was broader, on average, than the RSN. By definition, the RSNs are subsets of the FSNs, which means that they comprise only some of the sites in which a parasite species can develop. Moreover, in cases where interactions with other parasite species are unimportant – that is, have no significant effect on any of the parasites – they represent the optimal sites within the FSN, whereas in cases where interactions are actually important, they represent the sites of the FSN which are available to the parasite (Poulin, 2007a). According to these ideas, it is possible to conclude that the FSN of *O. superba* is not characterized in full in this study. Furthermore, it excludes some of the sites in which the parasite can develop (i.e. mantle musculature, covering mesentery of gonad, eyes and funnel). A possible cause for this situation may be the number of octopuses infected with *O. superba* but not with *Aggregata* sp. Moreover, this was too low (i.e. $N_{O.\ vulgaris} = 15$) to characterize it in full. The infection levels recorded for the FSN of *O. superba* are interesting, inasmuch the mean parasite count was higher for the gills than for the body skin. Furthermore, while comparing the infection levels recorded for the RSN with those recorded for the FSN, it was found that lower and higher levels were recorded, respectively, for the gills and body skin. These findings suggest that the gills constitute the preferred site of infection of *O. superba*. Also, they might be understood as preliminary functional evidence of interspecific interference competition. A preference for the gills is not surprising, once these provide parasitic copepods with suitable food, that is, epithelial cells, mucus and blood. The body skin also provides them with epithelial cells and mucus constituting, therefore, an adequate alternative site of infection. When the gills are infected with eimeriorin coccidians, the octopicolid copepods' ability to physically establish on them

is probably impaired. As a consequence, they may have to move to other sites of the host's body, most likely the body skin, as suggested by the infection levels recorded for the RSN of *O. superba*. The infection with *Aggregata* sp. can also affect the spatial distribution of *O. superba* on the host's body by leading to changes in the octopus' behaviour, as those found by Mladineo and Jozić (2005) – specimens of *O. vulgaris* became excited, left their shelters and swam and became inactive inside their shelters a few days before dying. The reason for this is two-fold: on the one hand, in addition to crawling, the octopuses move by jet propulsion, and changes in their locomotory behaviour (and ultimately, in the respiratory water flow through the gills) can affect the distribution of *O. superba* on the gills, as this probably moves while under the dislodging action of the respiratory water current; on the other hand, a prolonged stay inside a shelter can affect the spatial distribution of *O. superba*, as this was reported to exhibit a circadian behavioural rhythm, inhabiting the mantle cavity of *O. vulgaris* during daytime and moving out along its arms, mantle and head after dark (Deboutteville et al., 1957). The significant positive correlation between the numbers of oocysts recorded for the gills and the gastrointestinal tract can be understood as evidence that the gills function mainly as an accessory site of infection in octopuses in which the main sites of absorption along the gastrointestinal tract (that is, the caecum and intestine) are massively infected. The Renkonen's index (*P*) ranges from 0 (no overlap between niches) to 1 (complete overlap), which means that the overlap between the RSNs of the two parasites was low. Such a low level can be understood as preliminary evidence for interactive site segregation (see Holmes, 1973; Poulin, 2007a), that is, of adjustments in the infection site of *O. superba* in response to the presence of *Aggregata* sp. in the gills. Moreover, although the gills seem to function mainly as an accessory site of infection of *Aggregata* sp., they were found infected with the coccidian in 41.7% of the examined octopuses, while they seem to constitute the preferred site of infection of *O. superba* but were only infected with the copepod in 42.5% of the examined octopuses. The standardization of the Levins' values of niche breadth (*B*) resulted in low values, once the Levins' standardized measure of niche breadth (B_A) ranges from 0 to 1. Such low values indicate that the spatial niches are dominated by few sites or, more precisely, that the two parasites are specialists with respect to the sites they infect.

Numerical evidence of a negative interaction between the two parasites at the level of the gills was given by the non-parametric partial rank correlation analysis. Furthermore, this analysis could demonstrate the existence of a significant negative relationship between the counts recorded for the two parasites, while controlling for a

potential confounding effect of host body size (i.e. total length) in the results. It is worth noting, that the mean number of oocysts of *Aggregata* sp. in the gills was higher in spring and summer and that these were also the seasons for which the RSN of *O. superba* consisted of more sites, that is, was broader. These data suggest, therefore, a negative effect of *Aggregata* sp. on *O. superba*. The characterization of the spatial distribution of the two parasites at the level of the gills further suggested the existence of such a negative effect. On the one hand, the spatial distribution patterns of the two parasites were complementary in octopuses whose gills were infected with both of them; on the other hand, the spatial distribution pattern of *Aggregata* sp. was consistent between octopuses whose gills were infected with the two parasites and with it exclusively (contrary to that found for *O. superba*). Despite the evidence underpinning the existence of a negative interaction between *Aggregata* sp. and *O. superba*, the non-linear relationship between the oocyst and specimen counts for the gills suggests that both parasites occurred aggregated among hosts. This aggregated distribution of parasites, where a few hosts harboured many parasites while most harboured none or just a few, was first noted by Crofton (1971), being consistent with one of the few general laws in parasite ecology (Shaw & Dobson, 1995; Poulin, 2007b). A possible cause of the aggregation of *Aggregata* sp. could have been the differential exposure and susceptibility of the octopuses to the parasite. Furthermore, *Aggregata* sp. is a trophically transmitted parasite, and aggregation could have resulted from the uneven distribution of the infective stages in the population of first intermediate hosts. Besides, the octopuses were of different size and host body size has been recognized as a reliable proxy for different factors closely related with susceptibility to infection (see Poulin, 2013). In the case of *O. superba*, the aggregation might not only be related with the different size of the octopuses; indeed, it might also be the result of the combined effect of a series of factors usually associated with the octopodid cephalopods (i.e. sedentarism and solitary behaviour) and the octopicolid copepods (i.e. direct life-cycle and high host specificity).

In conclusion, this study's findings suggest that the octopicolid copepods are able to detect changes in the gills resulting from infection with eimeriorin coccidians, and that their behaviour is mobile enough to allow them to adjust the site of infection.

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Chapter 9

Concluding Remarks

final notes

future research

9.1. Final Notes

The parasitological data recorded for the common octopus, *Octopus vulgaris* (Cephalopoda: Octopodidae) and the European flounder, *Platichthys flesus* (Linnaeus, 1758) (Actinopterygii: Pleuronectidae) (see also the previous works of Cavaleiro, 2007; Cavaleiro & Santos, 2007, 2009) are interesting from a comparative perspective for the following reasons:

- First, the recorded infection levels (i.e. prevalence and intensity) suggest that copepods are the most common ectoparasites occurring on those two species of hosts in Portuguese coastal waters. The infection with parasitic copepods can become particularly problematic in aquaculture systems since (i) animals are usually kept at high densities in tanks and (ii) copepods usually have monoxenous life-cycles. Furthermore, since *O. vulgaris* is presently considered a candidate species for marine aquaculture (Estefanell et al., 2013) and since Portugal has all conditions necessary to implement aquaculture systems for this cephalopod, it is important to evaluate whether *Octopicola superba* Humes, 1957 (Copepoda: Octopicolidae) is pathogenic for the natural population of octopuses (by analysing the occurrence of associated histopathology) and also to characterize its life-cycle in detail, including the macro- and microenvironmental factors involved.

- Second, the recorded seasonality trends were similar for three of the species of parasitic copepods present on the two hosts, i.e. *O. superba*, *Acanthochondria cornuta* (Müller, 1776) (Copepoda: Chondracanthidae) and *Lepeophtheirus pectoralis* (Müller, 1777) (Copepoda: Caligidae), and it may be that all parasitic copepods present at the studied geographic area follow the same seasonality trend. Furthermore, all those parasites seemed to be influenced by variations in macroenvironmental factors, i.e. seawater temperature and photoperiod, which indicates that the season of the year is an important proximate cause of niche restriction. This type of information is crucial to define effective control and management methods in aquaculture systems, but should be complemented with data from laboratory experiments.

- Third, the metazoan ectoparasite communities of *O. vulgaris* and *P. flesus* consisted of a few species only, most of which were species of copepods. Furthermore, the high

intensity levels recorded for the three species of copepods more commonly isolated from those two hosts (i.e. *O. superba*, *A. cornuta* and *L. pectoralis*) suggest that they are highly adapted to them. Actually, and as concerns *O. superba*, this parasite presumably has a monoxenous life-cycle, and since octopuses are typically sedentary, solitary and short-living, strong adaptation is, indeed, very likely. Moreover, the intensity of *O. superba* was significantly greater in female than in male octopuses (i.e., the sex of the host appears to be an important proximate cause of niche restriction in *O. superba*), and such difference in intensity levels can reflect part of the strategy that ensures the long-term survival of the species.

- Fourth, it should be noted that, despite the large variety of microhabitats provided by the host, the copepods ectoparasitic on *O. vulgaris* and *P. flesus* were found to exhibit a clear preference for a few, well-defined sites on the body of their hosts. Presumably, the type of food available at a given microhabitat is likely an important proximate cause of niche restriction in these parasites. The case of *A. cornuta* is particularly remarkable. This parasite was mainly found in the branchial chambers of *P. flesus*, which can be related with the fact that, while on them, it has easy access to virtually unlimited blood. However, the preference for this site can also reflect avoidance of interspecific competition (with *L. pectoralis*, present on the body skin and fins of *P. flesus*), avoidance of predators (the large dimension of the females makes them easily noticeable by predators present in the macroenvironment) and facilitation of mating (mate finding by males should be enhanced in a more confined microhabitat). *O. superba* shows a preference for the gills but may have become less restrictive with respect to the site of infection during the course of evolution, as a result of the competition with *Aggregata* sp.

- And fifth, the parasite fauna recorded for *O. vulgaris* and *P. flesus* is remarkable, inasmuch as it reflects the ecology of the host, i.e. the feeding ecology in the case of *O. vulgaris* (*Aggregata* sp., the second most frequent parasite, is transmitted trophically, i.e. by predation of the crustaceans intermediate hosts), and the migratory behaviour between different salinity environments in the case of *P. flesus* (the marine situation for larval *Diplostomum* is unusual, and the infection with *Diplostomum* sp. was probably acquired while the flounder stayed at low salinity environments). Therefore, the evidence found indicates that parasitological studies help us better understand animal life.

9.2. Future Research

The aims of future research are the following:

1) To characterize further the parasite fauna of *O. vulgaris* from Portuguese waters (infections with coccidians and mesozoans).

2) To evaluate whether *O. superba* is pathogenic for the natural population of *O. vulgaris* and, if so, which type of histopathological lesions are associated with the infection.

3) To evaluate the exact effect of temperature and photoperiod on life-cycle progression of *O. superba* (laboratory experiments).

4) To characterize, for the first time, the basic life-cycle pattern of octopicolid copepods and to describe the evolution of the population age structure along the year, using the collection of parasites isolated from *O. vulgaris*.

5) To establish a hypothesis on how *O. superba* ensures its host-to-host transmission.

6) To elucidate further the systematics of octopicolid copepods, by unraveling the phylogenetic position of Octopicolidae in the lichomolgoidean complex of families (following Humes & Boxshall, 1996) through molecular data analysis.

7) To characterize the larval parasites isolated from *O. vulgaris* and the latter in molecular terms, and to evaluate the existence of differences in the parasite fauna of different species in the *O. vulgaris* complex.

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