Ecological and histopathological aspects of Didymodiclinus sp. (Trematoda: Didymozoidae) parasite of the dusky grouper, Epinephelus marginatus (Osteichthyes: Serranidae), from the western Mediterranean Sea

~~Infection demography, host-parasite interaction~~Ecological and histopathological ~~description~~aspects of *Didymodiclinus* sp. (Trematoda: Didymozoidae) ~~on~~parasite of the ~~gills and pseudobranchs of~~ dusky ~~groupers~~grouper, *Epinephelus marginatus* (Osteichthyes: Serranidae), from the western Mediterranean Sea

Running title: *Didymodiclinus* sp. of dusky grouper

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Acknowledgements

S. Mele received a post-doctoral grant from the Regione Autonoma della Sardegna (Master and Back 2012–2013 PRR-MAB-A2013-17 605).

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Abstract

The dusky grouper *Epinephelus marginatus* (Lowe) is an ecologically and commercially important fish species of the Atlantic and Mediterranean coastal rocky habitats. ~~While~~Despite records of didymozoid infections ~~have been documented~~ in several grouper species, the identification and pathogenesis of these parasites in *E. marginatus* are lacking. The aim of this study is to ~~describe~~characterise the didymozoids of *E. marginatus*, particularly their mechanisms of infection and histopathological features. Dusky groupers (*n*=205) were caught off Majorca Island (western Mediterranean Sea) and examined for parasites. Of the fish sampled, 45% ~~had~~were infected with white and yellow didymozoid capsules and brown nodules ~~attached to~~, found on the gills and pseudobranchs. Parasite abundance had a strong positive relationship with the fish length; only fish larger than 20 cm were infected, suggesting infection via consumption of an intermediate host, for which *E. marginatus* size was a limiting factor. The capsules contained two convoluted viable adult trematodes, identified as *Didymodiclinus* sp., in close contact with host capillary vessels, with no evidence of the tissue inflammatory response. Conversely, nodules containing ~~degenerated~~degraded parasites were surrounded by an intense inflammatory infiltrate. The ~~immunomodulatory role of~~findings suggest that *Didymodiclinus* sp. ~~on~~have the ~~host~~potential to evade the host's immune system ~~was subsequently discussed, offering a new insight into host-parasite interaction and the impact on fish welfare~~by inhibiting the inflammatory response.

Keywords:

Epinephelus marginatus, *Didymodiclinus* sp., Didymozoidae, histopathology, Balearic Islands

Introduction

The dusky grouper, *Epinephelus marginatus* (Lowe) (Osteichthyes: Serranidae), is a hermaphroditic, long-lived, slow-growing species, commonly found in the tropical and temperate Atlantic and Mediterranean rocky habitats, down to the approximate depth of 50 m (de Almeida Rodrigues Filho ~~et al.,~~, Gomes Sanches, de Oliveira Garcia, Viana Pannuti, Figueiredo Sebastiani & Guimarães Moreira 2009). It is one of the largest top predators in the western Mediterranean littoral ecosystems, that like other grouper species, shows an ontogenetic change in diet composition and an expansion of the trophic niche, with juveniles feeding primarily on Brachyura crustaceans and adults on cephalopods and fishes (Reñones, Polunin & Goñi~~,~~ 2002; Linde, Grau, Riera, & Massutí-Pascual~~,~~ 2004).

In addition to its key role in coastal ecosystem food webs, the dusky grouper has a high economic value. It is a target species of the commercial artisanal fishery and an important species in maintenance of recreational activities, including scuba diving and ~~spear fishing~~spearfishing (Reñones, Piñeiro, Mas & Goñi~~,~~ 2007). *Epinephelus marginatus* is classified endangered as its population size has decreased along the entire geographic distribution range (de Almeida Rodrigues Filho *et al*., 2009). ~~Its~~ Biological (e.g., hermaphroditism, late sexual maturity, slow growth and longevity) and behavioural (e.g., site fidelity, inquisitive character) features, coupled with high recreational fishing pressure, ~~mainly recreational,~~ are the main ~~contributors~~causes of the ~~reduction~~decline of ~~the~~this species ~~population~~.

Dusky grouper populations can be affected by different parasitic diseases (Santos, Buchmann & Gibson~~,~~ 2000; Moravec, Glamuzina, Marino, Merella & Di Cave 2003; Merella, Reñones & Garippa~~,~~ 2005; Roumbedakis ~~et al.,~~, Marchiori, Paseto, Gonçalves, Luque, Cepeda, Sanches & Martins 2013, Roumbedakis ~~et al.,~~, Marchiori, Garcia, Pereira Junior, Castro & Martins 2014; Mahmoud, Alhindy & Fahmy~~,~~ 2015). Natural outbreaks associated with gnathiid isopod larvae have been observed in wild and captive *E. marginatus* (Marino ~~et al.,~~, Giannetto, Paradiso, Bottari, De Vico & Macrì 2004; Genc~~,~~ 2007). Recently, skin lesions and dermatitis, most likely associated with tissue parasites, have been observed in dusky groupers from the Libyan coast (Rizgalla ~~et al.,~~, Bron, Shinn, Herath, Paladini & Ferguson 2016). White capsules, tightly attached to the gills, pseudobranchs and orobranchial cavity,

have been reported on ~~free-ranging~~wild dusky groupers in the Adriatic Sea (Canestri-Trotti, Fioravanti, Patarnello~~,~~ & Restani~~,~~ 1994) and eastern Atlantic Ocean (Gijón-Botella & López-Román~~,~~ 1987), as well as on captive specimens in public aquaria and aquaculture facilities (Padrós, personal observation). These parasites ~~were broadly~~have been identified as ~~didymozoids~~members of the didymozoid family (Trematoda: Didymozoidae). ~~The~~ Reñones previously observed presence of encapsulated parasites on the gills ~~was also observed in~~ of dusky grouper specimens collected ~~for the~~in a separate study ~~Reñones, Polunin & Goñi (2002) on the~~of population dynamics of the species in ~~the Balearic Islands (~~western Mediterranean~~)~~ Sea (Reñones~~, personal observation~~ *et al*., 2002). Widespread occurrence of these parasites in dusky grouper populations highlights the magnitude of potential implications and the ~~immediate~~ need for further research into the mechanisms of infection and the parasite itself.

The aim of this study was to describe the ~~demography of infection~~didymozoid population, host-parasite relationship and histopathological features of the didymozoids ~~observed~~found on the gills of the *E. marginatus* western Mediterranean population.

Materials and methods

Host and parasite collection

Two hundred and five dusky groupers caught off Majorca Island (Balearic Islands, western Mediterranean Sea), were sampled between 1998-99 (n=94) and 2000-03 (n=111) ~~were sampled~~ (Fig. 1). Specimens were obtained from the artisanal bottom longline and trammel net commercial captures, ~~spear fishing~~spearfishing competitions and experimental fishing carried out in order to acquire individuals under the minimum landing size established for the species in the area ~~(~~, i.e. 45 cm in total length (TL) for the commercial fishery and 2 kg in total weight (TW) in the ~~spear fishing).~~spearfishing.

In the laboratory, fish were measured (TL~~, total length~~ in cm) and weighed (TW~~, total weight~~ in grams), gills and gill chambers were ~~visually~~ examined for the presence of parasites, and otoliths and gonads ~~extracted~~removed for age and sex determination, respectively. The individuals sampled ranged from 6.6 cm to 103.5 cm ~~in~~ TL and from 0 to 60 years ~~in age~~ (Table 1). Of the 205 specimens

examined, 22 could not be sexed due to the small size (< 16 cm TL). Ninety four individuals were examined for didymozoid abundance (samples collected in 1998-99). In these specimens, following the examination of the gills and gill chambers, randomly selected left or right gills and pseudobranch were ~~extracted~~removed, fixed in 10% buffered formalin and stored individually. For the 2000-03 samples only the presence/absence of didymozoid capsules was recorded. Four additional sample individuals were captured in 2014 and 2016 (Table 1) and their infected gills and pseudobranchs were in part fixed in 10% formalin for histopathology, and in part dissected in fresh to ~~extract~~recover live parasites for morphological examination. These samples were not included in the ~~infection data~~statistical analysis.

~~**Histopathology**~~

~~Formalin-fixed samples were decalcified in 8% formic acid for 48 h, dehydrated through a graded alcohol series and xylene, using an automatic tissue processor and paraffin-embedded according to standard techniques. Sections of 3µm were obtained with a microtome (RM2245, Leica Biosystems). Sections were stained with hematoxylin and eosin and Masson's trichrome with automatic multistainer (ST5020, Leica Biosystems). Sections were observed under a light microscope (Nikon Eclipse 80i) and photographed.~~

Morphological analysis

Parasites, ~~extracted under the stereomicroscope~~excised from the capsules, were stained with iron acetocarmine, washed in several changes of distilled water, counter-stained in copper phthalocyanine Astra blue dye solution, differentiated in 5% hydrochloric solution, dehydrated through a graded ethanol series (70-100%), cleared in dimethyl phthalate and mounted in Canada balsam. Micrographs were made with an Olympus DP72 camera attached to a light microscope Olympus BX41 with Nomarski differential interference contrast. Measurements were taken with the Cell ~~B~~digital~~B~~ digital image analysis software (Olympus, Japan).

~~**Demography of infection**~~

Histopathology

Formalin-fixed samples were decalcified in 8% formic acid for 48 h, dehydrated through a graded alcohol series and xylene, using an automatic tissue processor, and paraffin-embedded according to standard techniques. Sections of 3 µm thickness were obtained with a microtome (RM2245, Leica Biosystems, Germany). Sections were stained with hematoxylin and eosin and Masson's trichrome with automatic multistainer (ST5020, Leica Biosystems). Sections were examined under a light microscope (Nikon Eclipse 80i) and photographed.

Statistical analysis of the parasite population

Prevalence (P%), abundance (A), mean abundance (MA) and mean intensity (MI) were calculated according to Bush, Lafferty, Lotz~~.~~ & Shostak (1997). Confidence intervals (CI) of the indices were calculated with the Sterne's exact method and the bias-corrected and accelerated Efron-Tibshirani's bootstrap, using the free software Quantitative Parasitology web 1.0.9 (Reiczigel, Rózsa & Reiczigel~~, 2013).~~ 2013). Prevalence was calculated on all 1998-2003 samples and on the 1998-1999 subsamples, whereas A, MA and MI were calculated only on the 1998-1999 subsamples.

Generalised linear models (GLMs) were used to assess the possible effects of ~~host biological parameters~~TL on prevalence and abundance of the parasite (dependent variables). Prior to the GLM analysis, the collinearity between explanatory variables (total length, total weight and age) was checked with the Spearman rank correlation test (ρ). ~~As~~ Fish length was positively correlated with fish weight (all samples 1998-2003: $\rho = 1$, $p < 0.01$; subsamples 1998-1999: $\rho = 1$, $p < 0.01$) and age (all samples 1998-2003: $\rho = 0.97$, $p < 0.01$; subsamples 1998-1999: $\rho = 0.95$, $p < 0.01$), thus only TL was considered in the analysis of prevalence and abundance. In addition, since *E. marginatus* is a protogynous hermaphrodite, sex related differences could have not been assessed separately from size-related differences. The prevalence of infection ~~is a binary response variable (1= presence, 0= absence), and therefore it~~ was modelled using a binomial distribution with logit link function (Zuur, Ieno, Walker, Saveliev & Smith~~,~~ 2009). Since parasite abundance is a count data, the Poisson and the negative binomial distribution were considered as alternative possible models. Initially, a Poisson

GLM model was constructed and the dispersion parameter (Ø) estimated. For Ø value <7 the Poisson distribution, corrected for over-dispersion was considered, whereas for larger Ø values the response variable was assumed to follow a negative binomial distribution (Zuur~~, Ieno, Walker, Saveliev & Smith~~, *et al.*, 2009). Both distributions were fitted using the log-link function. The GLM models were performed with R statistical software version 3.2.2 (www.R-project.org/, R Development Core Team 2015).

Results

Macroscopic findings~~ and histopathology~~

Pedunculated capsules with a broad base, attached to the inner margin of the primary gill and pseudobranchial lamellae, were observed on the infected dusky groupers (Fig. 2). These structures were classified in three types based on their colour: white~~,~~ (3.414-7.285 x 1.779-6.040 mm), yellow (4.985-9.370 x 2.615-6.370 mm) and brown~~.~~ (1.553-5.359 x 1.359-3.325 mm). White capsules had an irregularly ovoid shape and were smaller than the globular yellow ones. Brown structures had an opaque and wrinkled surface and were the smallest of the three types.

White and yellow capsules displayed a cystic arrangement with bright and transparent walls containing didymozoid ~~worms~~trematodes (Fig. 2A).

Morphological analysis

Each capsule contained a pair of parasites with convoluted posterior ends (Fig. 2). The parasites were gonochoristic, expressing weak sexual dimorphism, with underdeveloped male and female organs in both, female and male partners, respectively (Figs 2B-C). Measurements (given in micrometres unless otherwise indicated) and description of the female shown in Fig 2B and 2D are as follows: length 77,680, oral sucker 62 x 47, pharynx 45 x 34 and ventral sucker 103. Pharynx, oesophagus and proximal part of caeca covered by gland cells. Ovary and vitellarium simple, anterior and posterior to genital junction, respectively (Fig. 2D). Mehli's glands present, uterus with one loop. Genital pore ventral to the oral sucker. Mature eggs 15-17 x 10-11. Measurements and description of the male specimen shown in Fig. 2C are as follows: length 53,861, oral sucker 54 x 57, pharynx 52 x

38 and ventral sucker 117 (Fig. 2C). Pharynx, oesophagus and proximal part of caeca covered by gland cells. Two parallel testes. Uterus filled with few eggs 15 x 10.

Histopathology

Microscopic examination of the three capsule types indicated that their colour was due to the colour of the capsular tissues and presence of the yellow egg in the uterus of parasites. White capsules were characterised by a white wall containing early mature stages of adult parasites, which had few eggs in the uterus. Yellow capsules, also of a white wall, contained late mature stages, with the egg-filled uterus. Finally, the colour of the brown capsules was most likely due to the mix of the wall and the egg remains infiltrate of haematin and the necrotic cells. The transversal sections of ~~these structures~~white and yellow capsules revealed the presence of multiple encapsulated, apparently viable parasites (Fig. 3A). Capsules were attached to the connective tissue of the gill filament, close to the efferent artery (Fig. 3A) and appeared to distort the lamellar profile without affecting the cartilage growth. ~~A number of likely newly formed~~Several small vessels were also observed in this location. Two convoluted ~~worms~~didymozoids were surrounded by a thick capsule of host origin, derived from the connective tissue of the gill filament (Fig. 3A). An external layer of a moderately cellular collagen ~~constituted~~formed the connective capsule wall, with the presence of few capillaries, and an internal layer of compact collagen fibres, arranged in a ~~circular~~concentric pattern (Fig. 3B). The capsule wall was of variable thickness, ranging from 150 µm on the lateral sides of the parasite attachment site, to 400 µm on the opposite part. In the thinner portion, the wall was mostly composed by the compact collagen layer. The free portion of the connective capsule was covered by a moderately hyperplastic pluristratified epithelium containing mucous cells. An increased number of mucous cells, compared to gill epithelium, was observed in the capsule areas of higher thickness (Fig. 3B). Several thin fibrovascular septa departed from the inner portion of the connective capsule ~~numerous thin fibrovascular septa departed~~, dividing the parasite coils and gradually reducing their thickness toward the centre of the cyst (Fig. 3C). Most internal sections of the capsules were divided only by thin blood capillaries, allowing a direct contact between the vascular endothelial cells and the parasite surface (Fig. 3D). No signs of inflammatory response were observed around viable parasites.

Brown capsules contained ~~degenerated worms~~degraded parasites, surrounded by a thin connective layer of compact collagen (150-200 µm). Internal structures, with the exception of shelled eggs, were no longer visible and parasite remnants were mixed with necrotic debris and few inflammatory cells (Fig. 3E). A mixed inflammatory infiltrate was observed around the collagen capsule of ~~degenerated~~degraded parasites (Fig. 3F).

~~**Morphological analysis**~~

~~Each capsule contained a pair of parasites with convoluted posterior ends (Fig. 3). The parasites had gonochoristic bodies expressing weak sexual dimorphism, with underdeveloped male and female organs in both, female and male partners, respectively (Figs 2B–C). Measurements and description of the female specimen shown in Fig 2B and 2D are as follows: 77,680 µm length, oral sucker 62 x 47 µm, pharynx 45 x 34 µm and ventral sucker 103 µm. Pharynx, oesophagus and proximal part of caeca covered by gland cells. Ovary and vitellarium simple, anterior and posterior to genital junction, respectively (Fig. 2D). Mehli's glands present, uterus with one loop. Mature eggs 15–17 x 10–11 µm. Measurements and description of the male specimen shown in Fig. 2C are as follows: 53,861 µm long, oral sucker 54 x 57 µm, pharynx 52 x 38 µm and ventral sucker 117 µm(Fig. 2C). Pharynx, oesophagus and proximal part of caeca covered by gland cells. Two parallel testes. Uterus filled with few eggs 15 x 10 µm.~~

In the transversal histological sections (Fig. 4), several body structures of parasites were identified. Parasite body wall was arranged in an external layer of compact eosinophilic material (tegument), supported by an underlying layer of cells. No spines along the ~~worms~~parasites were detected. Parasites had all organs embedded in parenchyma, which ~~was~~were more abundant in the dorsal side of the body (Fig. 4A). In female ~~worms~~specimens, the parasite anterior section had the narrowest diameter and was predominately located in the inner part of the capsule (Fig. 4A). It contained a single ovary, a lightly eosinophilic structure composed of cuboidal yellow oocytes and paired intestinal caeca, appearing as two elliptical structures delineated by a thin wall. In the median portion, the ovary was still detectable, although located peripherally to multiple ~~section~~sections of the uterus (up to 3), identifiable as large cystic structures delimited by a thin wall and containing embryonated yellow-shelled eggs ~~with a miracidium inside~~. Due to the presence of the uterus, this

segment was the largest part of the parasite body and the section which had the most extensive contact with the host connective capsule (Fig. 4B). The posterior portion, in contact with the host capsule, was characterised by the presence of two sections of the uterus and the vitellarium, a peripherally located organ ~~constituted~~formed by polygonal cells with ~~scanta~~ small deeply basophilic cytoplasm, arranged in a pseudoglandular pattern (Fig. 4C). In male ~~worms~~specimens, testes were recognizable as partially ~~cystic~~oval paired basophilic structures, detected exclusively in the smallest parasite sections in the inner portion of the cyst. Mature sperm, characterised by basophilic elongated strands, was intermingled with developing sperm, identifiable as round deeply basophilic cells (Fig. 4D).

~~**Demography of infection**~~

Statistical analysis of the parasite population

Of the 205 host specimens analysed, 92 (P%= 45%, CI~~:~~= 38%-52%), were infected with didymozoids. The smallest and the youngest fish infected were 20 cm long and 2 years old, respectively. General linear model for presence/absence data showed that the prevalence increased significantly with fish length ([Intercept] Wald= -4.91, SE= 0.7, z= -7.38, p= 1.64 e^{-13}; [TL] Wald=0.12, SE= 0.002, z=7.44, p= 1.01 e^{-13}; Fig. 5A), explaining 41% of the variation of the data. Predicted prevalence indicated a cumulative increase of the infection with fish growth. The model predicted the infection prevalence of 50% for specimens of ~~40~~42.2 cm TL and 100% for those of ~~65cm~~65 cm and above.

The abundance of capsules per infected fish ranged between 1 and 24. The mean abundance and mean intensity of parasites were 8.6 (CI: 6.7-11.0) and 13.6 (CI: 11.2-16.8), respectively. The Poisson GLM model fitted to parasite abundance indicated over-dispersion, with an estimated dispersion parameter of 4.95, thus a Poisson GLM corrected for overdispersion was applied. Fish length had a significant effect on parasite abundance explaining 24% of the variation in the data ([Intercept] Wald= -0.39, SE=0.42, z= -0.9, p= 0.36 [TL], Wald= 0.04, SE= 0.01, z= 5.0, p= 2.9 e^{-6}). The abundance of capsules increased with the increasing host length (Fig 5B).

Discussion

Sampling *Epinephelus marginatus* from the western Mediterranean ~~demonstrated high prevalence of infection by the~~Sea a large number of didymozoid *Didymodiclinus* sp., encapsulated on the gills and pseudobranchs were discovered. Didymozoid capsules of similar shape were previously reported on the gills of dusky groupers from the eastern Atlantic (Gijón-Botella & López-Román, 1987) and the Adriatic (Canestri-Trotti~~, Fioravanti, Patarnello & Restani,~~ *et al.*, 1994). Eastern Atlantic infections were attributed to a trematode species, *Didymodiclinus branchialis* (Yamaguti, 1970), while there were genera identification uncertainties between *Gonapodasmius* Ishii, 1935 and *Indoglomeritrema* Madhavi and Hanumantha, 1983 for the Adriatic infections. According to the criteria of Pozdnyakov & Gibson (2008), the parasite described herein likely ~~belonged~~belong to the genus *Didymodiclinus* Podzdnyakov, 1993. Some of the ~~parasite species'~~morphological features observed in ~~our~~the present study were similar to those described for *D. branchialis*, *Didimodiclinus epinepheli* (Abdul-Salam, Sreelatha ~~&~~and Farah, 1990), *Didymodiclinus pacificus* (Yamaguti, 1938) and *Didymodiclinus pristipomatis* (Yamaguti, 1934). The specimens examined, however, had accessory gland cells around the oesophagus, a ~~feature~~characteristic not previously observed in *D. pacificus* and *D. pristipomatis*. and Mehli's glands, which had not been reported for *D. epinepheli*. Moreover, all previously described parasite species were characterised by different hosts and location within the host, and ~~came~~are from other localities: *D. branchialis* was recorded in the nostril of *Epinephelus quernus* Seale, 1901 from Hawaii, *D. epinepheli* ~~in~~on the gills of *Epinephelus tauvina* (Forsskål, 1775) from the Arabian Gulf and *Epinephelus coioides* (Hamilton, 1822) from Philippines, *D. pacificus* ~~in~~on the gills of an epinephelid from the Pacific Ocean, *D. pristipomatis* in the mouth of *Epinephelus akaara* (Temminck ~~&~~and Schlegel, 1842) from the Japan Sea (Yamaguti~~,~~ 1971; Abdul-Salam, Sreelatha & Farah~~,~~ 1990; Cruz-Lacierda, Lester, Eusebio, Marcial & Pedrajas~~,~~ 2001). Furthermore, compared to other species, the ~~*Didimodiclinus*~~ specimens ~~of~~examined in the present study showed differences in body, suckers and pharynx dimensions, highlighting the need for further taxonomic ~~analysis investigating the~~ and phenotypic plasticity ~~and an in-depth re-examination of the genus~~analysis.

Histological findings of the three types of capsules revealed ~~the presence of a very~~that their shape and colour were closely related to growth and maturation of the parasites. White capsules

contained only early mature stage of adult parasites, yellow capsules had fully mature parasites while the brown contained only eggs, parasite debris and the necrotic cells. In the white and yellow capsules, a distinct relationship between host tissues and viable *Didymodiclinus* sp. was evidenced. The parasites were surrounded by a thick capsule of collagen layers in host lamellar lamina propria, beneath gill and pseudobranch epithelium. The increased thickness of compact collagen in the distal portion of the capsule could be explained by the chronic effect of mechanical pressure exerted by the parasite growth toward the site that offered less resistance to its expansion. On the other hand, collagen layers of brown capsules surrounding ~~degenerated~~degraded didymozoids were thinner than those detected around viable parasites, and eggs were generally found within a more loosely arranged connective tissue, in contact with areas of epithelial erosion. The reduction of the distance between eggs and the external environment can represent an advantage for their potential release in the water. The mechanism by which this happens is still ~~poorly understood.~~under researched. It is generally accepted that, at some point of maturation, eggs are released in the ~~outer~~external environment ~~by~~after capsule rupture or other traumatic mechanisms (Mladineo & Bočina 2009~~)~~), and it is believed that the inflammatory response could help the egg release (Abdul-Salam & Sreelatha~~,~~ 1992). This mechanism has also been observed in other trematodes, such as *Schistosoma* sp., where the egg-derived ribonuclease Omega-1 is thought to activate inflammation required for egg to cross through host tissues, in order to reach the external environment (Fitzsimmons ~~et al.,~~, Schramm, Jones, Chalmers, Hoffmann, Greveldingc, Wuhrerd, Hokked, Haasb, Doenhoffe & Dunne 2005; Hewitson, Grainger & Maizels~~,~~ 2009).

Generally, encapsulation is a common host response against parasites that allows isolation of the pathogen inside a capsule of fibrous tissue (Álvarez-Pellitero~~,~~ 2008; Bullard & Overstreet~~,~~ 2008). In the present study the term "capsule" simply describes thick collagen layers ~~dividing~~separating the parasite from the gill and pseudobranch epithelium, rather than a true immunological anatomical isolation against the pathogen. ~~In fact, the "proper"~~ A true capsule should provide a complete isolation of the parasite from the surrounding host tissue (Álvarez Pellitero~~,~~ 2008), not observed in our case.

~~Newly formed~~A large number of small blood vessels close to the efferent artery were seen in the connective septa dividing the coils of viable *Didymodiclinus* sp. Similar findings were also

reported for the gill lesions associated with *D. epinepheli* in *E. tauvina* and *E. coioides* (Abdul-Salam & Sreelatha, 1992; Cruz-Lacierda, Lester, Eusebio, Marcial & Pedrajas, *et al.*, 2001). There is evidence that the parasite is in fact not separated from the host tissue but, on the contrary, its body surface has an extensive contact with the capillary endothelia along theits entire worm length. It is likely that this complex capillary network, covering the parasite, develops during growth of *Didymodiclinus* sp., suggesting a constant modulatory role on host vascular development exerted by the parasite. The ability to influence vascular proliferation has also been observed in other parasite taxa. The nematode *Onchocerca volvulus*, (Leuckart, 1893), infecting the human eye, is thought to produce molecules able to stimulate angiogenesis and induce neovascularisation (Tawe, Pearlman, Unnasch & Lustigman, 2000). A similar ability can be argued for *Didymodiclinus* sp. where, by locally inducing neoangiogenesis and promoting vascular growth, fish tissues can be modified to favour the parasitic success. Another histological feature of viable *Didymodiclinus* sp. is the absence of an inflammatory response, despite the intimate contact of the parasite body surface with the blood vessels of the host, and thus exposure to the fish immune system. The lack of host response points to an evolutionary adaptation and a possible presence of immune evasion mechanisms in these parasites. Numerous parasites are known to be able to escape fish inflammatory response by the presence, for instance, of host-like molecules on their surface, which can act as a camouflage from the immune system (Roberts, Lewis, Wiegertjes & Hoole, 2005). In the present study a moderate to marked inflammatory infiltrate was observed only associated with degenerated parasites. It is possible that non-viable parasites were no longer able to maintain the mechanism of immune evasion, thus the release of the parasite contents and antigens following the cellular death could have triggered the host's general immune responsedegraded parasites, most likely because the immune response was triggered by the release of products following the parasite death. Presence of smaller brown capsules could have been the result of this mechanism but also coupled with other factors, including non-viable, developing forms of the didymozoids limited by space and nutrients. Structural differences were not observed between white and yellow capsules, suggesting pigmentation changes over the time.

Inflammatory cells have been observed in the gill capsules of several other didymozoids in variable proportions (Marino et al., Giannetto, Cavallaro, Paradiso, Bottari & De Vico 2003;

Mladineo, 2006; Justo, Kohn, Pereira & Flores-Lopes, 2013; Abdul-Salam & Sreelatha, 1992). Presence or lack of an inflammatory response and its magnitude in tissues infected bywith didymozoid species could be related to the different developmental stage of parasite (Perera, 1992), to the anatomical location (Mladineo, 2006), but also with the host-parasite co-evolution processes (Gibson, MacKenzie & Cottle, 1981).

An increase in mucous cells and epithelium hyperplasia was observed in pseudobranchs and gill filaments, in response to both, viable and degenerateddegraded parasites. Similar host responses have been reported for the didymozoid gill infections in other groupers, as well as in the Atlantic bluefin tuna, *Thunnus thynnus* (Abdul-Salam & Sreelatha, 1992; Cruz-Lacierda, Lester, Eusebio, Marcial & Pedrajas,Lacierda*et al.*, 2001; Mladineo & Bočina, 2009). The gill and pseudobranch immune responses are considered non-specific and can be caused by many aetiological agents (Bullard & Overstreet, 2008). However, in absence of inflammatory cells, the capsules of the viable parasites examined in this study only experienced a mild increase in mucous cells in the distal portion of the collagen layer.

The life cycle of the Didymozoidae is currently poorly understoodunderstood (Pozdnyakov & Gibson, 2008). They are heteroxenous parasites with teleosts as definitive hosts, and two or three possible intermediate hosts: the first assumed to be gastropod molluscs, the second crustaceans, and the third cephalopods and/or fish. The specific intermediate hosts are unknown, while the larval stages of the didymozoids of groupers remain undescribed. Present results demonstrated absence of *Didymodiclinus* sp. in juveniles of less than 20 cm TL, suggesting that these smaller groupers did not prey on the intermediate hosts. However, the observed trends of higher infection prevalence and parasite abundance, with increasing TL, indicated a likely progressive importance of the intermediate hosts as food source for the growing definitive host. Studies of stomach contents and stable isotope analyses characterised the dusky grouper as a highly opportunistic generalist, mainly associated with benthic food webs (Linde, Grau, Riera, & Massutí Pascual, *et al.*, 2004; Machado, Daros, Bertoncini, Hostim-Silva & Barreiros, 2008; Condini et al., Tanner, Reis-Santos, Albuquerque, Saint'Pierre, Vieira, Cabral, & Garcia 2016). Moreover, the dusky grouper undergoes ontogenetic diet shift, with juveniles feeding mainly on brachyuran crustaceans, while adults feed on cephalopods and fishes

(Reñones, Polunin & Goñi, *et al.*, 2002; Linde, Grau, Riera, & Massutí-Pascual, *et al.*, 2004). Thus, the relationship between the occurrence of *Didymodiclinus* sp. and the length of *E. marginatus* could be related to the change in diet and feeding on the possible intermediate hosts, cephalopods and fishes. Among the prey items of dusky groupers identified by Reñones, Polunin & Goñi (*et al.* (2002) and Linde, Grau, Riera, & Massutí-Pascual (*et al.* (2004), only the stripped mullet, *Mullus surmuletus* L. (Osteichthyes: Mullidae), has been found infected with didymozoid metacercariae in the western Mediterranean Sea (Ferrer-Castelló, 2015). In different parts of the world however, immature didymozoid stage infectionsstages have been observed forin other dusky grouper prey items;: in picarel, *Spicara smaris* (L.) (Osteichthyes: Sparidae), from the eastern Mediterranean Sea and in unidentified *Scorpaena* species (Osteichthyes: Scorpaenidae) from the western Atlantic Ocean and Gulf of Mexico (Fischthal &Thomas, 1968; Papoutsoglou, 1976, León-Regagnon *et al.* 1997, Pérez-Ponce de León & Lamothe-Argumedo1997). Unfortunately, none of these records managed to assign the parasite to a specific taxon. Records of didymozoid metacercariae infections ofin cephalopods are currently very few (Overstreet & Hochberg, 1975). Culurgioni (2014) looked for different studied the parasites in a number of several cephalopod species from the Sardinian coastsoff Sardinia (western Mediterranean Sea) and suggested that none of the species examined, on which the dusky grouper is known to prey upon, hadwas infected with larval didymozoids infections. According to Cribb, Bray, Wright & Pichelin (2002), morefurther parasitological data on the dusky grouper prey items (e.g. sympatric cephalopods, mullids, sparids and scorpaenids), coupled with the use of molecular techniques, can help shed light on the life cycle of the didymozoids of the dusky grouper and enhance the knowledge of the host ecology in the Mediterranean Sea.

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Tables

Table 1. Biological data of dusky grouper *Epinephelus marginatus* caught off Majorca Island (western Mediterranean Sea). N, number of specimens; TL, total length; TW, total weight; na, data ~~available~~unavailable. U, I, F, T and M indicate unsexed, immature female, mature female; transitional and male, respectively.

Group and sampling period	N	Sex (U/I/F/T/M)	TL range (cm)	TW range (g)	Age (years)
All samples 1998-2003	205	22/114/52/3/14	6.6-103.5	4-18,700	0-60
Subsamples 1998-1999	94	4/41/41/2/6	11.6-77.4	18-9,000	1-57
Additional samples 2014-2016	4	na	31.7-107.0	500-22,000	na

Figure legends

Figure 1. Dusky grouper *Epinephelus marginatus* sampling area. Full points indicate the sampling stations ~~in~~off Majorca Island (western Mediterranean Sea).

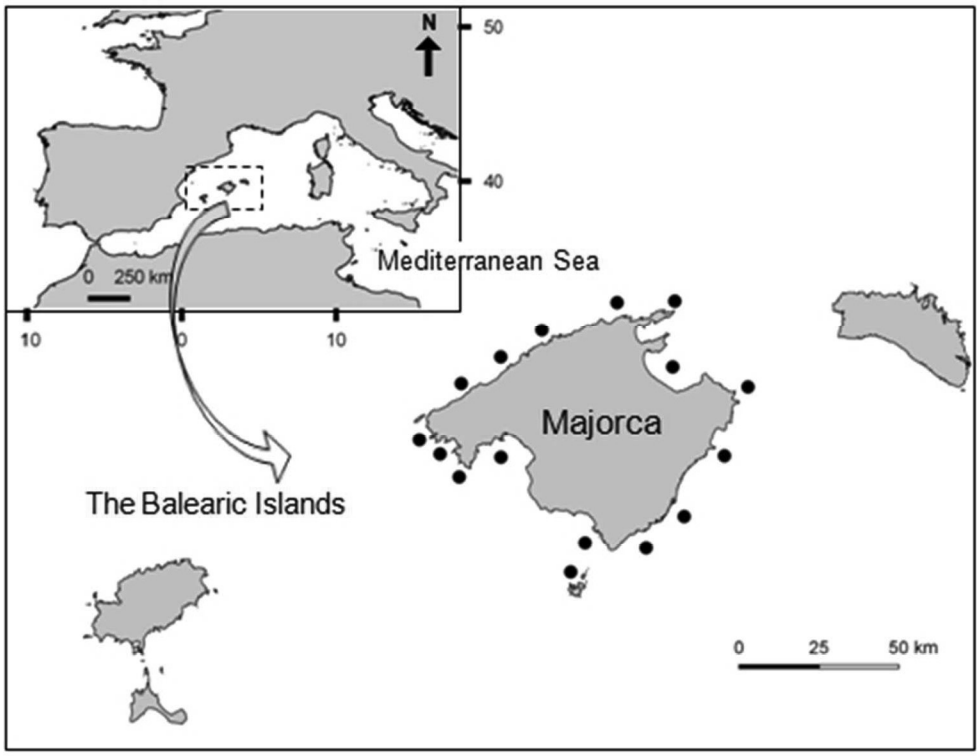
Figure 2. *Didymodiclinus* sp. ex pseudobranchs of *Epinephelus marginatus*. **A**. Parasite capsules with parasites in B-D figures. B. Female anterior end. C. Male anterior end. D. Female genital junction. Abbreviations: AU, ascendant uterus; BN, brown nodule; DU, descendant uterus; ED, ejaculatory duct; GJ, genital junction; I, intestine; MG, ~~Mehli~~Mehli's glands; OE, oesophagus; OS, oral sucker; P, pharynx; YC, WC, yellow and white capsule; V, ~~vitellogenous~~vitellarium; VS, ventral sucker; T, testis. Bars in micrometres: A, 5000 µm; B- D, 200 µm.

Figure 3. Encapsulated *Didymodiclinus* sp. on pseudobranchs and gills of *Epinephelus marginatus*. **A.** A thick connective capsule (C) enveloping two convoluted viable ~~worms~~parasites attached close to the efferent arteries (arrowheads) of pseudobranch (asterisk). HE, 2X. **B.** Connective capsule. External layer of loosely arranged collagen fibres (arrow) around an internal portion of compact collagen (double arrows). Mucous cells in the overlying epithelium (arrowhead). Masson's Trichrome, 10X. **C.** Particular of connective capsule. Fibrovascular septa (arrowhead) departing from the connective capsule (C) divide parasite coils (P). Note vascular capillaries (asterisks). Masson's Trichrome, 20X. **D.** Particular of fibrovascular septa. Parasite coils (P) in contact with capillary endothelia (asterisk) allowing the passage of single erythrocytes (arrowhead). Masson's Trichrome, 40X. **E.** ~~Degenerated~~Degraded parasites in gills. A thin connective capsule (C) surrounding ~~degenerated~~degraded parasites (P). Masson's Trichrome, 4X. **F.** ~~Degenerated~~Degraded parasites in gills. Mixed inflammatory infiltrate (asterisk) around connective capsules (C). Note necrotic material intermingled with parasite remnants (P). HE, 20X. Bars in micrometres: A, 250 µm; B, 50 µm; C, D, F, 10 µm; E, 100 µm.

Figure 4. Section of viable *Didymodiclinus* sp. encapsulated in pseudobranchs and gills of *Epinephelus marginatus*. **A.** Sections of parasite anterior segment. Ovary (O) located dorsally and

medially to paired caeca (arrows). HE, 10X. **B.** Section of parasite median segment. Multiple sections of uterus (U) in the central part of the section. Ovary displaced laterally (O). Most median segments are in contact with connective capsule (asterisk). HE, 4X. **Inset.** Magnification of Fig. 5B. Mature eggs containing ~~miracidium~~embryo show a yellow shell (arrows). HE, 40X. **C.** Section of parasite posterior segment. Peripherally located vitellaria (V) and two sections of the uterus (U) present. **D.** Section of parasite inner portion. Testis showing mature sperm (arrows) in ovular cystic structure. HE, 20X. Bars in micrometres: A 20 µm, inset of B, D, 10 µm; B-C, 50 µm.

Figure 5. Relationship between dusky grouper total length (TL in cm) and prevalence (**A**) and abundance (**B**) of *Didymodiclinus* sp. Solid lines represent the optimal GLM models describing the relationships, dashed lines are 95% confidence intervals. Analyses based on 205 (**A**) and 94 (**B**) dusky groupers.



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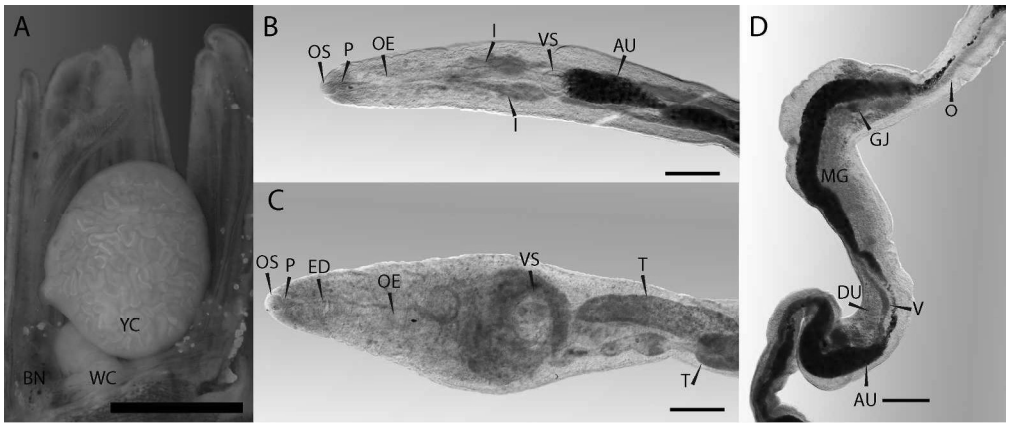

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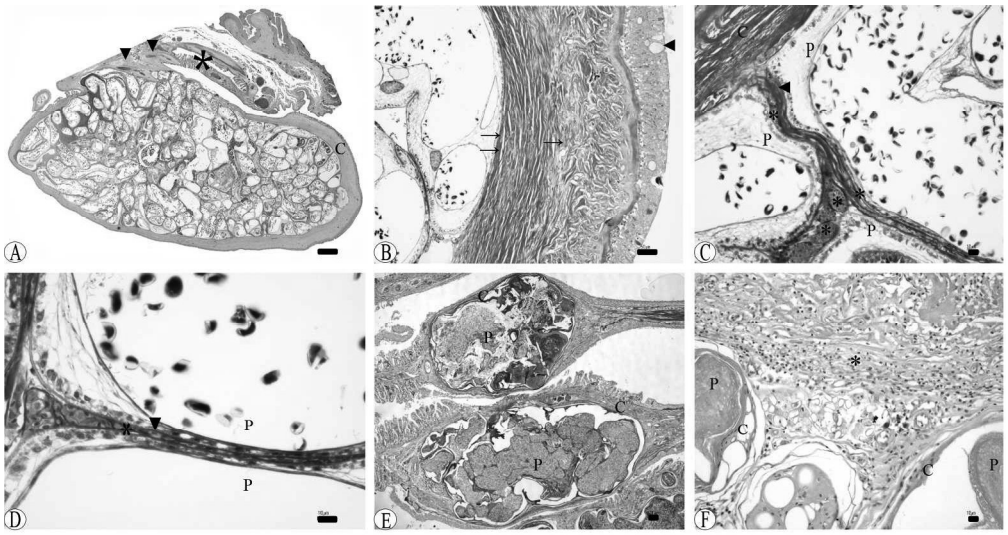

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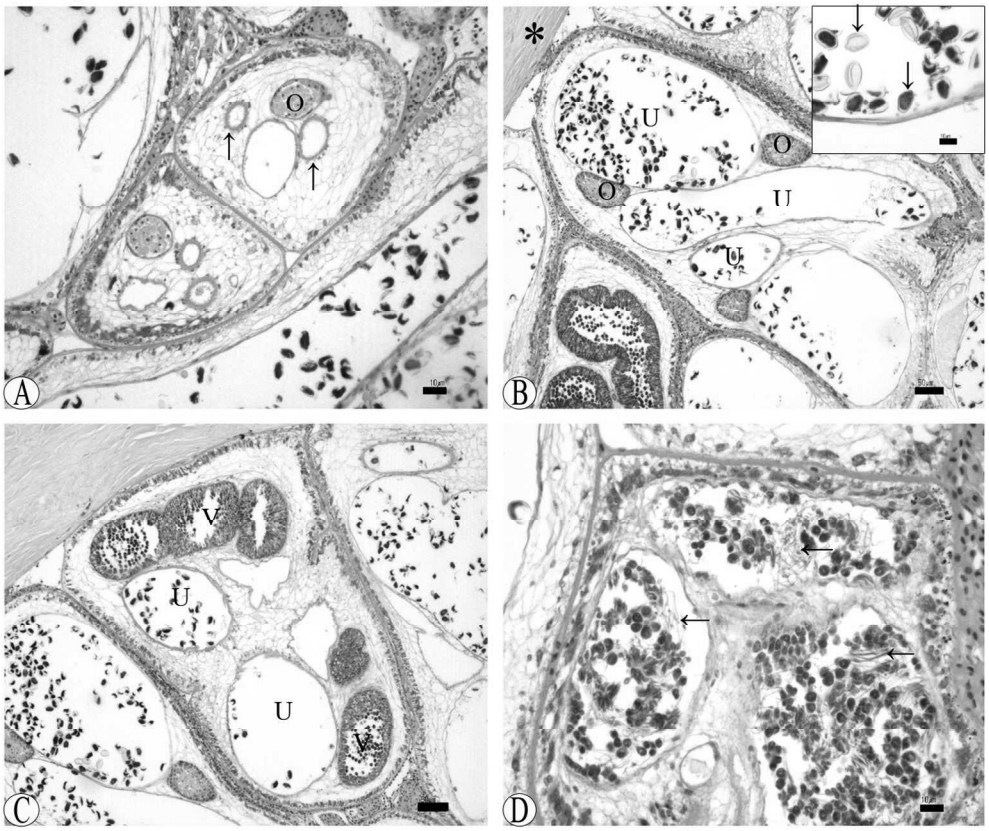

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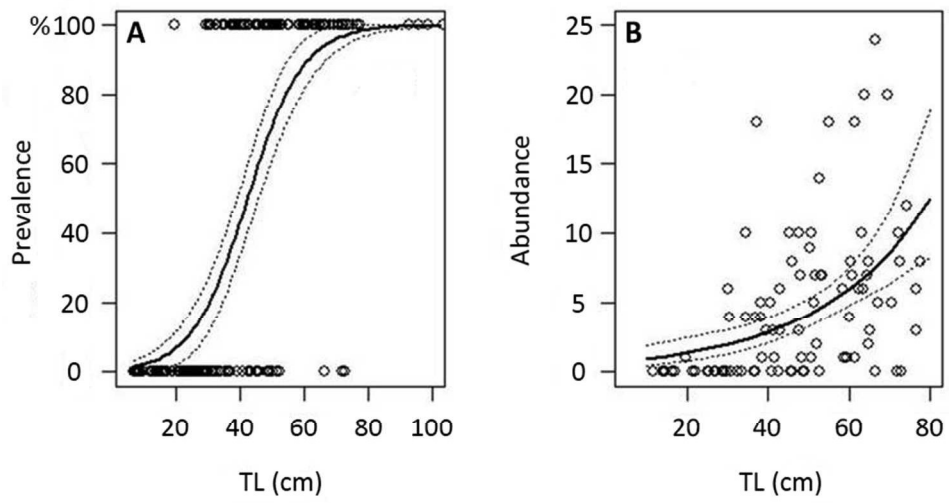

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