An investigation of the health status of wild Libyan dusky grouper, *Epinephelus marginatus* (Lowe), with characterisation of a new disease, Dusky Grouper Dermatitis (DGD)

A THESIS SUBMITTED TO THE UNIVERSITY OF STIRLING

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

by

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2016

To my parents

Declaration

This thesis has been composed in its entirety by the candidate. Except where specifically acknowledged, the work described in this thesis has been conducted independently and has not been submitted for any other degree. All images presented in this thesis are original, unless otherwise stated.

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Acknowledgements

This thesis was produced with the help, support and contributed of a network of people who deserve recognition for their role in making it possible. First and far most I would like to thank my supervisors, James Bron, Hugh Ferguson and Andy Shinn, for their advice, support and guidance. With whom I have spent a number of hours discussing my research and proposal ideas among other topics. Thanks also to the Libyan Ministry of Higher Education and Scientific Research for funding this work.

My thanks are also due to all the staff at the Institute of Aquaculture, with special thanks to Debbie Faichney, Jane, Jacquie Ireland and Linton Brown.

Thanks to all the experts of various field that I consulted along the way. My particular thanks to Dave little, Giuseppe Paladini, Tom Cribb, Matt Longshaw, Stephen Mitchell, Marion Stevenson and John Taggart

I would like to thank Alison at the university of Stirlings library for her help in getting the final copy ready to print.

I would also like to thank Mahir Kuba and Turkeya for allowing the use of the laboratory facility at the faculty of veterinary medicine in Tripoli. My thanks are also due to Abdallah Ben Abdllah for introducing me to the El Nadi El Bahri some 10 years ago, for his encouraging words and support. I also express my gratitude to Amer El Migri, former dean of the faculty of agriculture, for giving me credit and support. You are an inspiration.

My thanks are due to Abdallah Elmgawshi, Daw Hadood at the Marine biology research centre/ Tajura, and special thanks to Naser Al Kaber for urging me to study fish disease.

I wish to thank the members of the Facebook group Libya youth, with special thanks to Adam Fozi, Mohammed Sbeta, Ahmed Ali Abu Khshaim, Abdu Alangodi and Marwan Alwalid. Special thanks are due to Ahmed Gashout, Ahmed Bobakra, Mahmoud H. Khalaf, Muez Eddin-Elmari Ben Salem and El Shagmani, for sharing their experience and observations and permitting the use of the dusky grouper images. Your enthusiasm and love for the sea are exceptional.

My immense gratitude goes to all the fishermen, and salesmen at the Elnadi Elbahri in Tripoli for welcoming me in their universe. With special thanks to Salem, Hayda and Monir for their particular assistance. My sincere thanks are also due to Ganem Dernawi for his support assistance and enthusiasm.

My thanks are due to my friends, scattered around the world, Rim, Nesreen, Kadeja, Aisha, Ibtisam and Soad, Amal, Hatem, Ibrahim, Tharanghani and Lenantha, my dear Mina and Francoise. Thank you my dear Howida for making all this possible from the first day in 2008 when I first came into your office, Sophie thank you for your friendship and for helping in correcting the first draft.

I would like to thank my family, my brothers and our little ones Celini, Naila, Nelson and Scott for their constant love and support.

Abstract

The dusky grouper *Epinephelus marginatus* (Lowe 1834), is a protogynous sequential hermaphrodite and is considered to be one of the most important fish species in the Mediterranean Sea. It is a K-strategist, being slow growing and late maturing, and this, coupled with its reproductive biology and relatively sedentary behaviour, has made it extremely sensitive to overexploitation, leading it to be classified by the IUCN as an endangered fish species.

Wild dusky grouper have suffered from disease outbreaks in the past decade, leading to mass mortalities across the Mediterranean Sea, including Libyan coastal waters. These mortalities have mostly been attributed to *Nodavirus* infections. In Europe and Brazil, efforts are in place to culture this fish for commercial grow-out and stock enhancement programmes. In Libya, the dusky grouper is consumed regularly and is considered a prime-eating fish. Its importance for the Libyan internal market, as well as its potential for export, makes it an ideal candidate for future Libyan aquaculture activities. Given the scarce literature regarding the dusky grouper in Libya, this study aimed first to assess dusky grouper fisheries, spawning seasons and to identify the main threats that the fishing sector poses for wild stocks. Second this study aimed to determine the health status of wild dusky grouper offered at a local fish market in the capital Tripoli, in order to identify pathogens, pathologies or other health issues that might pose a hazard to cultured populations but also to remaining wild dusky grouper stocks.

To achieve these aims, twelve field surveys spanning the period of 2013-2015 were conducted. From these surveys, it was established that the dusky grouper is captured throughout the year, including the spawning season. Fish sizes offered for sale ranged

between 20-92 cm total length (TL), with the fish being sold from local fishing grounds around Tripoli, but also from as far as Benghazi, 1300 km to the east of Tripoli. The dusky grouper is principally caught in artisanal fisheries and by spearfishing, with approximately 300 spear-fishermen serving one particular fish market in Tripoli that was a focus in this study, and with dusky grouper being one of their main targets.

Over the period of the survey, 267 landed dusky grouper were inspected for visible lesions prior to sampling. A total of 50 dusky grouper with sizes ranging from 27- 66 cm TL including the gonads from a further five fish measuring 66-92 cm TL that were sampled separately and examined to assess the stage of sexual maturity and to look for the presence of parasitic infections mainly affecting the gills, skin and gonads.

The spawning season was found to extend from May to early September, with females ranging between 39-68 cm TL, males measuring 57-92 cm TL, and transient fish measuring 58-68 cm TL. From otolith readings of 8 fish, the youngest fish was a 3 year old juvenile of 28 cm TL and the oldest was an 8-9 year old 56 cm TL female.

Whilst the highest prevalence of parasitic infection was found to be monogenean infection of the gills, with 100% prevalence, followed by gnathiid isopods infecting the oral cavity with 92% prevalence, it was the nematode *Philometra* sp. infecting post-spawning ovaries at 52% prevalence, that gave the highest apparent pathological impact. Necrosis potentially attributed to *Philometra* sp. in one particular ovary, was at a level likely to have caused complete parasitic castration, while others showed varying levels of probable functional reduction. The pathologies described need further investigation, especially in relation to possible synergies between *Philometra* sp. and bacteria in causing the necrosis.

From the 267 inspected dusky grouper, 55 fish ranging in size from 42-92 cm TL were observed to be affected by external skin lesions of unknown aetiology. Twenty-six of these fish were sampled, having lesions at various stages of severity, and 5 further unaffected fish were used for histological assessment of the skin as negative controls. Histopathologically, the lesions comprised a multifocal, unilateral or bilateral dermatitis, involving the epidermis, superficial dermis and scale pockets, and sometimes, in severe cases, the hypodermis. Severe lesions had marked epidermal spongiosis progressing to ulceration. Healing was observed in some fish. Bacteria and fungi could be isolated from severe lesions, although they were not seen histopathologically in early-stage lesions. By contrast, metazoan parasite eggs were observed in the dermis and epidermis of some fish with mild and moderate dermatitis. Unidentified gravid digenean trematodes, carrying similar eggs, were also seen within the blood vessels of the deep and superficial dermis. The newly described condition was termed dusky grouper dermatitis (DGD).

DGD's geographical distribution along the Libyan coastline was investigated using a novel application of the social media network Facebook. Using Facebook, it was possible to document skin lesions of dusky grouper in Libyan waters from images attached to the entries of spear-fishermen. Thirty two Facebook accounts and 8 Facebook groups posting from 23 Libyan coastal cities provided a retrospective observational dataset comprising a total of 382 images of dusky grouper caught by spearfishing from December 2011-December 2015. Skin lesions were observable on 57 / 362 fish, for which images were of sufficient quality for analysis, giving a minimal prevalence for lesions of 15.75%. Only dusky grouper exceeding an estimated 40 cm total length exhibited lesions. The ability to collect useful data about the occurrence and geographical distribution of pathological conditions affecting wild fish using

social media networks, demonstrates their potential utility as a tool to support epidemiological studies and monitor the health of populations of aquatic animals.

The gravid digenean trematode described from mild lesions of five fish was identified using reconstruction through histological sectioning as belonging to the Family Aporocotylidae Odhner, 1912. This is the first description of a blood fluke from the dusky grouper, as well as from dermal blood vessels. The parasite was relatively long; the longest section of the parasite that could be measured was 1500 µm and 20-80 µm in width, while the total length of the parasite was estimated at 1500-2000 µm. Minute tegumental spines, possibly covering only a few parts of the parasite, were seen from some cross-sections. The parasite had one post-testicular ovary, which might overlap the testis, a pre-ovarian ascending uterus, and a post-ovarian descending uterus. It also possessed an oesophagus surrounded by oesophageal glandular cells and a pre-ovarian and pre-testicular extension of the vitelline cells, mostly at the level of the ascending uterus. The parasite was observed to be intra-vascular, the uterine lumen varies in size to accommodate between 1-7 eggs. The uterine eggs were embryonated and observed to span several stages of maturation. Eggs were also found in the dermal blood vessels, in the dermis, and in the epidermis, with the latter appearing to provide a potential route of egress of eggs into the environment. The extra-uterine eggs were 23.5 to 37.52 µm long and contained a ciliated miracidium. The eggs seemed to elicit a mixed inflammatory reaction, with degranulation of eosinophilic granular cells attached to the external surface of some of the eggs within the blood vessels but also the dermis. From observations made in the current study, this parasite appears to be a new species, most closely allied to none of the currently described Aporocotylidae genera.

In summary, the present study has demonstrated that the dusky grouper is extensively fished in Libya without discrimination to sizes and season, by both artisanal and spearfishing, with the latter as one of the main fishing methods, posing treats to the spawning potential and conservation of dusky grouper in Libya.

The philometrid infecting the ovaries has a potential to reduce fecundity or to result in parasitic castration of wild broodstock. Gill-infecting monogeneans might represent a hazard for all stages of dusky grouper production. Dusky grouper dermatitis is a skin lesion, although there are no indications that infections may result in mortalities. Under culture conditions, however, this might change due to increase bacterial loads, which might lead to secondary bacterial infection. The presence of skin lesions would undoubtedly reduce the market value of whole fish. These findings are important for existing wild stocks, and for future plans regarding the aquaculture of dusky grouper. Future studies need to focus on the pathology of DGD, describing the disease process and aetiology using laboratory techniques such as TEM and virology as well as using morphology and molecular-based tools to describe the blood fluke and to determine their potential role in the initiation the disease. The novel approach to disease surveillance using social media Facebook posts could be further expanded by attracting citizen scientists, for future research assessing disease in wild fish, for sightings of mortality events and/or the appearance of disease outbreaks, or, for mapping marine mammal stranding's and/or turtle nesting activity.

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Chapter 1

1.1 General Introduction

Groupers (class Actinopterygii, order Perciformes, family Serranidae, and sub-family *Epinephelinae*) are mostly monandric, protogynous hermaphrodites (Smith 1965; Shapiro 1987) and are classified into 14 genera at the sub-family level Epinephelinae (Tuker 1999). They have an extensive distribution, being found in tropical and subtropical waters of all oceans (Heemstra & Randall 1993). Grouper are long lived and slow growing (Heemstra & Randall 1993), with some species capable of living for many decades *e.g.* 40 years in female brown-marbled grouper *Epinephelus fuscoguttatus* (Forsskål, 1775) (Pears *et al.* 2006) and 60 years in dusky grouper.

Grouper sizes vary from one species to the next (Heemstra & Randall 1993) with the Atlantic goliath grouper *Epinephelus itajara* (Lichtenstein, 1822), being, in the western Atlantic, the largest reef fish (Sadovy & Eklund 1999). The oldest recorded specimen, described by Bullock *et al.* (1991) from the eastern Gulf of Mexico, was 37 years old (yr) and the longest recorded was 2.5 m in length (Heemstra & Randall 1993).

Most grouper are solitary fish, only forming spawning aggregations during the spawning season (Heemstra & Randall 1993; Whaylen *et al.* 2004). they exhibit variation in spawning behaviour, with some spawning in pairs *e.g.* Hong Kong grouper *Epinephelus akaara* (Temminck *et* Schlegel, 1842) and brown-marbled grouper (Heemstra & Randall 1993)*;* and dusky grouper *Epinehelus marginatus* (Lowe, 1834) (Zabala *et al.* 1997a), while others spawn in large aggregations of several hundred *e.g.* Nassau grouper *Epinephelus striatus* (Bloch, 1792) (Whaylen *et al.* 2004; Sadovy de Mitcheson *et al.* 2012). While some, *e.g*. Nassau

grouper, migrate hundreds of km to spawning sites (Bernard *et al.* 2016), others, *e.g.* dusky grouper, show vertical migrations of only a few metres (Zabala *et al.* 1997a), information on grouper spawning grounds and migration is relatively sparse, therefore greater knowledge needs to be gathered to allow understanding of behaviour and ecology, which may help in assessments of population vulnerability and protection.

Spawning aggregations can make grouper very vulnerable to overfishing, to the point of almost complete depletion as is the case for the Nassau grouper in the Cayman Islands (Whaylen *et al.* 2004). The combination of fisheries targeting spawning aggregations (Whaylen *et al.* 2004; Bijoux *et al.* 2013), loss of habitat and overfishing of several grouper species *e.g.* Atlantic goliath grouper (McClenachan 2009), leads to some species being listed by the IUCN Red List of Threatened Species as *e.g*. Critically Endangered A2d ver 3.1 *e.g.* Atlantic goliath grouper (Craig 2011), Endangered *e.g*. dusky grouper (Cornish & Harmelin-Vivien 2004), or Near Threatened *e.g.* camouflage grouper *Epinephelus polyphekadionand* (Bleeker, 1849) (Russell *et al.* 2006) and brown-marbled grouper (Cornish 2004).

Measures to reduce fishing pressure on grouper populations include protection of grouper spawning aggregations (Bijoux *et al.* 2013), and the introduction of minimum and maximum size limits for captured fish. Such measures were implemented in Queensland for the brown-marbled grouper, with maximum and minimum fish sizes allowed to be caught as well as closed fishing during the spawning season (Sadovay 2013). Similarly, a minimum size for dusky grouper in Libya (30 cm TL) (Reynolds *et al.* 1995) and Brazil (47cm) (de Almeida Rodrigues Filho *et al.* 2009).

In some instances a total fishing ban has been implemented *e.g.* Atlantic goliath grouper in the USA (Porch *et al.* 2006). Culturing programmes for food (Pierre *et al.* 2008) and restocking programmes are also used *e.g*. restocking programmes of dusky grouper in the Mediterranean Sea (Spedicato *et al.* 1995; La Mesa *et al.* 2008) and Brazil (de Almeida Rodrigues Filho *et al.* 2009) .

1.2 Grouper culture in Asia

Almost 200K tonnes of the annual global wild fisheries catch comprise wild groupers (Epinephelinae) (Tupper & Sheriff 2008), these achieving high market values in tropical and sub-tropical areas especially in Southeast Asia. For this reason, grouper aquaculture started in the region in the 1980's (Pierre *et al*. 2008). Since that point they have been subject to widespread successful culture (Pierre *et al.* 2008), with FAO (2010) estimates for groper production in 2008 to have increased from 22000 in 2002 to 78000 tonnes. Taiwan is one of the leading countries producing grouper in Southeast Asia, with the most commonly produced species being *Epinephelus lanceolatus* (Bloch, 1790), *Epinephelus coioides* (Hamilton, 1822)*, Epinephelus malabaricus* (Bloch & Schneider, 1801) *and E. fuscoguttatus*, but also other countries *e.g.* China, Malaysia, Indonesia and Thailand (FAO). In Thailand the most important cultured species are *E. coioides and E. malabaricus* (Pomeroy *et al.* 2002), which are cultured to supply the ever growing market for live grouper (De Silva & Phillips 2007). Whilst there has been an increase in the supply of full-cycle cultured grouper (hatched and hatchery-reared fingerlings), 20% of cultured grouper are from broodstock (Pomeroy *et al.* 2002), with most being capture-based (Leong 1998; Pierre, Gaillard & Pre 2008) and comprising wild-collected fingerlings and alevins (70%-80 % of total) (Leong 1998; Tupper 2008; De Silva & Phillips 2007). As well as culturing for the live food-fish market, Thailand is also the largest wild-caught grouper seed supplier (Pomeroy *et al.* 2002).

Most grouper culture relies on feeding grouper with low value/trash fish (Hasan *et al.* 2009), which according to Funge-Smith *et al.* (2005) includes fish with *e.g.* low commercial values either due to their low quality, or small size. The implication of feeding low value/ trash fish to cultured grouper is in the introduction of pathogens including parasites, bacteria and viruses to the culture system (Sim *et al*. 2005).

Grouper culture is affected by several pathological problems, which can lead to mortalities and loss of production including viral (Chua *et al.* 1994; Chi *et al.* 1997; Gibson-Kueh *et al.* 2003; Lio-Po & de la Peña 2004 & Huang *et al.* 2015), bacterial (Lee 1995; Yii *et al.* 1997; Lee *et al.* 2002; Cruz-Lacierda & Erazo-Pagador 2004 &Tendencia & Lavilla-Pitogo 2004); and parasitic infections (Abdul-Salam *et al.* 1990; Sreelatha & Farah 1990; Supamattaya *et al.* 1991; Cruz-Lacierda *et al.* 2000; Cruz-Lacierda *et al.* 2001; Cruz-Lacierda & Erazo-Pagador 2004; Jithendran *et al.* 2005 & Wang *et al.* 2015) see **Table. 1.1.**

The health problems affecting cultured grouper (*Epinephelus* spp).create a significant constraint to culture (Somga *et al* 2002). Some of these pathogens have caused economic loss to cultured groupers as seen during iridovirus infections, the causative agent of Sleepy Grouper Disease' (SGD), affecting net cage cultured *Epinephelus tauvina*. The grow-out stage and marketable size fish were those mostly affected, with mortality in infected fish reaching up to 50% (Chua *et al.* 1994). Bacterial infections such as *Vibrio* also cost the grouper industry (Yeh *et al.* 2008) and in some cases, high loss of production has been reported for pond reared grouper in the Philippines (Somga *et al* 2002). In 1989, Thailand's losses due to disease in marine cage-cultured seabass and grouper were about US$ 1.9 M, while in Malaysia losses in 1990, due to vibriosis in sea-caged farms, totalled more than US$.5 million. Finally, 75% of rural small-scale farmers culturing grouper in the Philippines,

experienced reduced gain due to fish health and disease problems (Bondad-Reantaso *et al*.2002).

1.3 Dusky grouper

The dusky grouper is a monandric (males being the terminal sex), protogynous (female-male), hermaphrodite (Bouain 1980; Bouain & Siau 1983; Zabala *et al.* 1997a, b; Hereu *et al.* 2006). *E. marginatus* also has the synonyms *Epinephelus guaza* and *Epinephelus gigas* (often being confused with *Epinephelus haifensis* (Ben-Tuvia, 1953) which shares the synonym *E. gigas* (Brünnich, 1768)) (Randall & Heemstra 1993). The dusky grouper has an extensive geographical distribution and is endemic to the East Atlantic Ocean, the British Isles and South Africa, and is widely distributed in the Mediterranean Sea, but not in the Black sea. It is also found along the coast of southern Brazil (Heemstra & Randall 1993; Figueiredo & Menezes 1980) southern Oman (Randall 1995) and south of Madagascar (Randall & Heemstra 1991) (**Fig. 1.1**).

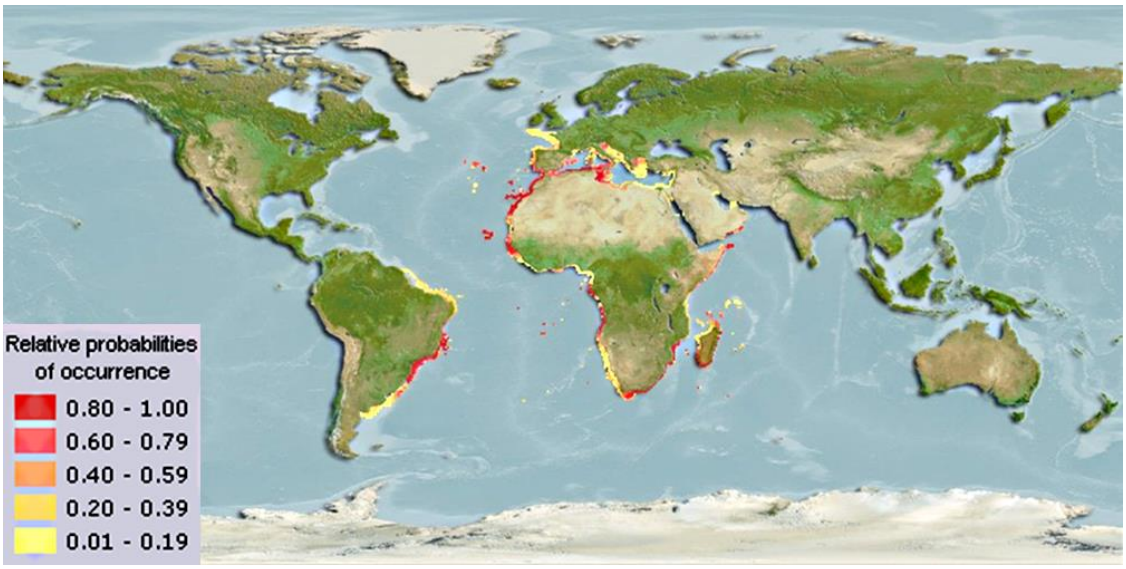

Figure 1.1. Geographical distribution and probabilities of occurrence for dusky grouper, which is found in the Mediterranean Sea, west coast of Africa, Brazil and Oman, as well as south of Madagascar. (Map from FishBase, accessed 31 May 2016)

For many years *E. marginatus* has been considered to be an over exploited fish species in most of the Mediterranean Sea (Bruslé & Brusel 1976; Chauvet 1991; Zabalaet *et al.* 1997a; Relini 1999), due to its late maturation, long life, slow growth and spawning aggregation behaviour, all factors which make it susceptible to being captured at commercially unsustainable levels (Chauvet 1991; Zabala *et al.* 1997a, b; Marino *et al.* 2001; Coll *et al.* 2004; Sadovy de Mitcheson *et al.* 2013). In 2003 the dusky grouper was placed on the IUCN list of endangered fish A2d ver 3.1 (IUCN) (Marino *et al.* 2003; Cornish & Harmelin-Vivien 2004; GFCM 2013).

The colour of the dusky grouper, according to Heemstra & Randall (1993), is reddish brown to greyish dorsally, and yellowish gold ventrally, with a vertically arranged series of irregular white, pale greenish yellow or silvery grey blotches usually visible on the head and body, and a distinct black maxillary streak and dark brown median fins. A narrow white band can often be seen on the distal edge of the anal fin as well as on the pectoral fin. Whilst the pelvic fins are distally blackish, the pectoral fins are dark reddish brown or grey, and the margins of the spinous dorsal fins and basal part of paired fins are golden yellow (**Fig. 1.2**) for further description see Heemstra & Randall (1993).

Figure 1.2. Juvenile female dusky grouper showing typical patterning and colouration *e.g.* brown head and body and blotchy skin with a yellowish pectoral region.

1.3.1 Gross morphology

Although there seems to be no difference between males and females in terms of skin colour and pattern, during the spawning season the skin colour of spawning males often changes into darker and light variations (**Fig.1.3**) and is adorned by silver streaks (Zabala *et al.* 1997b). This 'silvering' of the skin of fish is considered by Han *et al.* (2003) to be a measure of gonadal development.

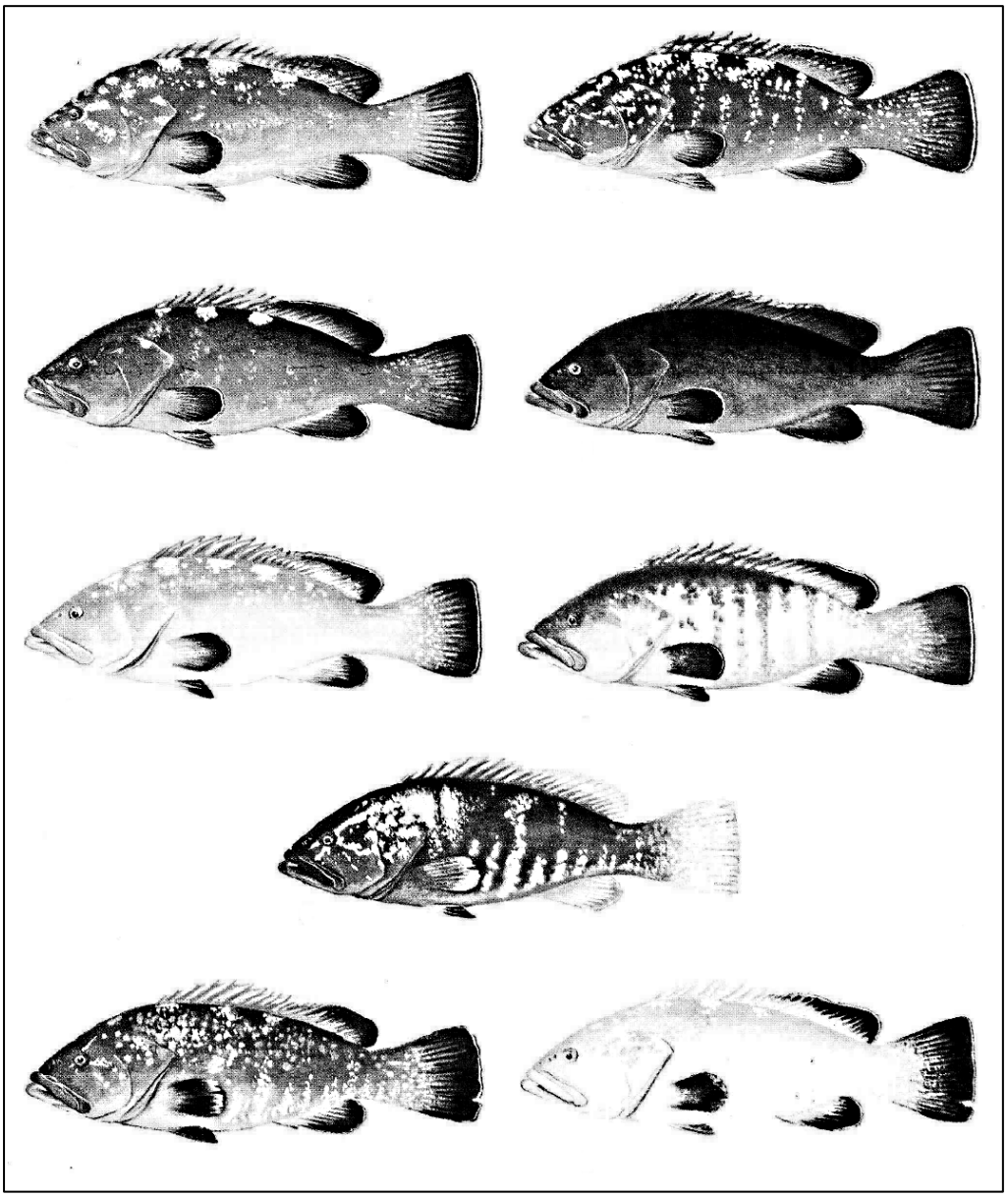

Figure 1.3. Diagram showing changing skin pattern and colouration (not evident from this figure) of male grouper during the spawning season (taken from Zabala *et al.* b (1997))**.**

1.3.2 Habitat and feeding habit

A year-round resident (Koeck *et al.* 2014) the dusky grouper is believed to be a solitary, territorial fish and lives most of its life in rocky shelf environments, alternating between two to three lairs and hunting fish within a specific territory (Koeck *et al.* 2014). The bathymetric range is positively correlated with their size (Harmelin & Harmelin-Vivien 1999), with larger fish found at depths ranging from 6-35 m (Kara & Derbal 1999) to up to 200 m (Tortonese 1986).

Azevedo *et al.* (1995) found juvenile < 13 cm TL, in tide pools. Although mostly they can be found in shallow waters (upper infralittoral) and in small cavities which provide cryptic shelter (Harmelin & Harmelin-Vivien 1999). While dusky grouper are not limited to a certain depth range they have been found mostly in depths of 12-20 m (Harmelin & Harmelin-Vivien 1999), while young dusky grouper of sizes ranging from 13 to 40 cm TL, were mostly found in depths less than 15 m (Coll *et al.* 1995; Derbal & Kara 1995).

The main prey collected from the stomach contents of dusky grouper of sizes > 90 cm TL were the cephalopod molluscs *Octopus vulgaris*, while for dusky grouper < 90 cm TL, prey largely comprised crustaceans and fish *e.g. Centrolabrus trutta, Scyllarus arctus* and *Ophioblennius atlanticus atlanticus* (Barreiros *et al.* 1998). Harmelin & Harmelin-Vivien (1999) reported that the stomach content of dusky grouper having sizes ranging from 4-7 cm TL, indicated that they fed mostly on amphipods, isopods, cumaceans, shrimps and crabs, while 13-25 cm TL individuals mostly used crabs as their predominant food, and to a lesser extent fish. 30-60 cm TL fish largely consumed fish, crabs, which were mostly brachyuran genera (*Scyllarides, Parthenope, Maj and Cancer*) and molluscs in equal quantities.

1.3.3 Age, sexual maturity and reproduction

The dusky grouper is a K-strategist and can live up to 60 years (Reñones *et al.* 2007). It is a slow growing and late maturing top predator (Tupper& Sheriff 2008). Early studies of Bruslè (1985) suggested fish weighing between 1-3 kg to be juveniles, while maturity in females was reported as occurring on average at 5 years, at a total length (TL) of 40-50 cm (Chauvet 1988). In the south-western Atlantic fish were observed to be 49 cm TL at first maturity (Condini *et al.* 2014). Larger individuals are often males (Zabala *et al.* 1997a) ranging in size between 70-120 cm LT (Zabala *et al.* 1997a; Kara & Derbal 1999).

Females reach sexual maturity at about 5 to 6 years in the western Mediterranean (Chauvet 1988; Fennessy 2006) and from 2-5 years in Tunisia (Bouain & Siau 1983) with the size ranging from 47 cm to 62 cm TL. Sex change occurs (becoming male) at 80-90 cm TL (Bruslé & Bruslé, 1976; Bruslé 1985; Chauvet 1991) when aged between 9-16 years old (Chauvet 1988). The maximum TL recorded to date is 120 cm, with a maximum body weight of 60 kg (Rocha & Costa 1999), whilst the smallest recorded mature female to date is 38.6 cm TL (Reñones *et al.* 2010).

Spawning season extends from May until October (Marino *et al.* 2001; Tsikliras *et al.* 2010), depending on the specific habitat zones within the Mediterranean Sea (Zabala *et al.* 1997a; Tsikliras *et al.* 2010). The longest spawning season was recorded by Marino *et al.* (2001) for dusky grouper spawning in the Pelagie islands (Italy), which extends from May to October. Studies by Kara & Derbal (1999) on the other hand described the spawning season along the coast of Algeria to extend from July to August. The variation in spawning times and the extent of the spawning season are thought to be attributable to temperature variation (Tsikliras *et al.* 2010).

1.3.3.1 Spawning

It has been almost 20 years since Zabala *et al.* (1997b) reported upon the first dusky grouper spawning aggregations in Medes Islands Marine Reserve in Catalonia in the Mediterranean Sea. During aggregation behaviour, Zabala *et al.* (1997a) noted vertical migrations of 4-8 m.

These spawning aggregations consist of one male and several females, with the sex ratio of sexually mature males to females in one spawning aggregation being approximately 1:7. Actual spawning occurs between a single male and a single female at a time, with dusky grouper spawning in pairs (Zabala *et al.* 1997 a).

Zabala *et al.* (1997a) suggested the possibility of female spawning migrations due to the appearance of juvenile females not previously observed within marine protected areas (MPA's) prior to the spawning season. Further evidence for spatial (lateral) migrations of dusky grouper during times of spawning has also been provided by Koeck *et al.* (2013).

1.4 Dusky grouper culture programmes in the Mediterranean

Dusky grouper culture programmes in the Mediterranean aim to offer an alternative source of this species for the market as commercially reared food-fish, and also to assist in future dusky grouper restocking programmes, with artificial propagation regarded as an important method to improve wild stocks (Glamuzina *et al.* 2000, La Mesa *et al.* 2008, Cunha *et al.* 2013). In the Mediterranean, dusky grouper culture is in its infancy (Pierre *et al.* 2008), and is faced with many problems (Glamuzina *et al.* 1998). One of the main problems encountered, as for all cultured grouper species, is in the rearing of larval stages, during which there are high mortalities in first feeders due to the small mouth gape (opening diameter ranging from 250-300 µm) (Glamuzina *et al.* 1998)., which accounts for high losses (Spedicato *et al.* 2000).

However, this has been resolved by Cunha *et al*. (2013) through the use of small prey, feeding enriched rotifers (*Brachionus* sp.) initially, and later newly hatched and enriched *Artemia* sp. and dry feed to rear dusky grouper hatchlings to juvenile stages (Cunha *et al.* 2009).

There is a scarcity of available early life stages of dusky grouper (Cunha *et al.* 2013), and pilot studies relied on wild-caught broodstock (Bruzón 2007). Following fertilisation, eggs hatch in about 30-40 hours (Cunha *et al.* 2013; Barnabé 1974) up to 48 hours (Bruzón 2007). Size estimates for newly hatched larval lengths for laboratory-reared dusky grouper, varied from 1.52 mm (Glamuzina *et al.* 1998) to 2.3 mm (Cunha *et al.* 2013).

The yolk-sac larva, (the stage of development lasting from hatching until the complete absorption of the yolk sac and oil globule), begins yolk absorption at about 1 day post-hatching (dph) (mean temperature 24·3°C, salinity 36·5, dissolved oxygen 6·4mg L^{-1} and pH 8·2. (Cunha *et al.* 2013) to 4 dph (at 23°C) (Glamuzina *et al.* 1998) post hatching. This period is followed by a pre-flexion stage extending from 4-10 dph, where the lipid droplet is completely absorbed and morphological changes characteristic of *Epinephelinae* start to show *e.g.* emergence of posterior pre-opercular angle spines. This stage is followed by the flexion stage, extending from 12-14 dph, post-flexion stage to 16 dph, transforming stage to 20 dph, pelagic juvenile stage to 25 dph, settling juvenile stage to 30 dph, and demersal juvenile stage to 35 dph (**Fig.1.4**). Dusky grouper larvae are characterised by their "kite-shaped" body (Cunha *et al.* 2013) a shared feature for all grouper species larvae (Heemstra & Randall 1993). A detailed description of the main ontogenetic and morphological characters of larvae and juveniles was provided by Cunha *et al.* (2013).

Table 1.1 Illustrating some of disease affecting cultured grouper

Causative agent	Name	*e.g.* Grouper sp. affected	Symptoms/ affected organ	Stage	Ref
Viral Iridovirus	Sleepy grouper disease	*Epinephelus tauvina*	Lethargy & 50 % mortality	Juveniles & broodstocks	Chua *et al.* (1994); Lio-Po & de la Peña (2004); Gibson-Kueh *et al.* (2003)
Iridovirus	Fish Lymphocystis Disease virus (FLDV)	*Epinephelus malabaricus, Epinephelus fuscoguttatus, Epinephelus coioides*	Small, pearl-like (single or in clusters) nodules on body surface & fins	Fingerlings, juveniles & adults	Lio-Po & de la Peňa (2004); Huang *et al.* (2015)
Betanodavirus	Viral nervous necrosis (VNN)	*Epinephelus* spp	Corkscrew darting swimming, anorexia, hyper inflated swim bladders, , corneal opacity & mortalities, up to 100%	All growth stages	Chi *et al.* (1997); Chi, Shieh & Lin (2003); Lio-Po & de la Peňa (2004)
Bacterial *Vibrio alginolyticus* *V. parahaemolyticus* *V. vulnificus* *V. carchariae*	Vibriosis ''Haemorrhagic septicaemia''	*E. malabaricus, E. tauvina, E. coioides*	Anorexia, gastroenteritis syndrome, skin ulcers, fin rot & up to 50 % mortalities	Fry, fingerlings juveniles, adults & broodstock	Lee (1995); Tendencia & Lavilla-Pitogo (2004); Yii *et al.* (1997); Lee *et al.* (2002)
Pseudomonas sp.	Pseudomonad haemorrhagic septicaemia	*E. tauvina*	Extensive haemorrhagic erosions of the body, exophthalmia and corneal opacity 60% mortalities	All growth stages	Tendencia & Lavilla-Pitogo (2004)
Parasitic **Protozoa** Trypanosomes	Trypanosomiasis	*E. fuscoguttatus*	Listlessness, anorexia & mortalities	Net-cages	Wang *et al.* (2015)
Cryptocaryon irritans	White spot disease	*E. coioides, E. malabaricus, E. tauvina*	Whitish or greyish spots on the skin and gills, inappetence, lethargy, abnormal, swimming behaviour, darkened body, skin haemorrhages, exophthalmia	Intensive culture systems, Fish of all sizes	Cruz-Lacierda & Erazo-Pagador (2004)

Causative agent	Name	*e.g.* Grouper sp. affected	Symptoms/ affected organ	Stage	Ref
Myxozoans *Sphaerospora epinepheli*	Renal sphaerosporosis	*E. malabaricus, E. coioides*	Kidney. Disorientation, haemorrhage, & mortalities	Wild & cultured	Supamattaya *et al.* (1991)
Isopod *Rhexanella* sp.		*E. coioides, E. malabaricus*	External. loss of appetite, parasite attaches on the body surface, mouth nasal cavity and opercular cavity	Nursery, grow -out, & broodstock	Cruz-Lacierda & Erazo-Pagador (2004)
Trematodes Digenea G*onapodasmius epinepheli*	Didymozoid digeneans	*Epinephelus sp, E. coioides, E. tauvina*	Capsules or cysts on the gills	Nursery and grow-out, wild grouper	Cruz-Lacierda & Erazo-Pagador (2004); Cruz-Lacierda *et al.* (2001); Abdul-Salam Sreelatha & Farah (1990)
Monogenea *Pseudorhabdosynochus* spp, *P. lantauensis*	Gill monogeneans	*E. coioides; E. malabaricus*	Gills. Swimming near the surface, loss of appetite & increased mucus, production of the gills	Nursery, grow-out, & broodstock	Cruz-Lacierda & Erazo-Pagador (2004); Abdul-Salam , Sreelatha & Farah (1990)
Benedenia sp.		*E. tauvina*	Gill, fin& skin. Erratic swimming behaviour and restlessness small focal haemorrhages on the body	Wild; cultured broodstock	Jithendran *et al. (*2005)

Causative agent	Name	*e.g.* Grouper sp. affected	Symptoms/ affected organ	Stage	Ref
Leech	Marine leech,	*E. coioides*	Parasites attached to the pectoral, ventral, anal, and caudal fins, skin folds behind the lower jaw, under the operculum, oral cavity. Frayed fins, haemorrhages and swollen, areas on the parasite's attachment and feeding sites.	Nursery, grow -out, & broodstock	Cruz-Lacierda *et al.* (2000), Cruz-Lacierda & Erazo-Pagador (2004)

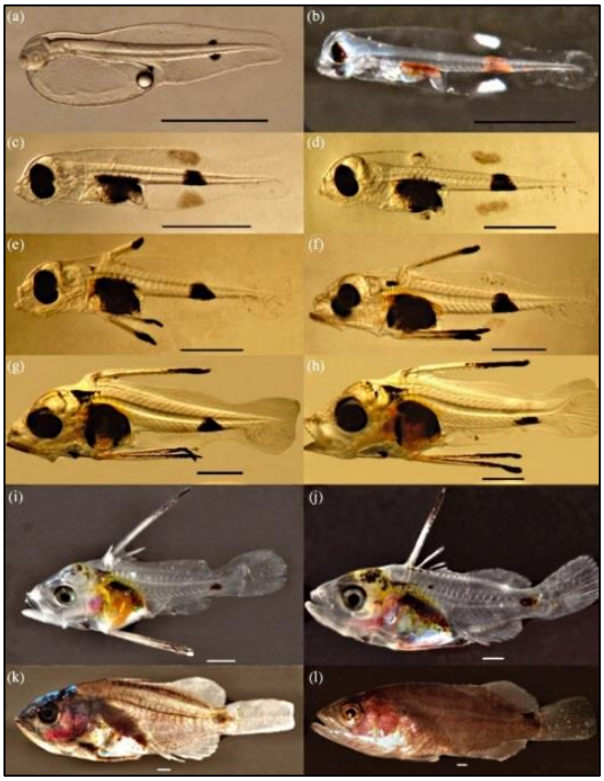

Figure 1.4. Different larval stages of *Epinephelus marginatus*. Yolk sac: (a) newly hatched, 1 day post hatching (dph) (b) 2 dph. Pre-flexion: (c) 4 dph (d) 6 dph (e) 8 dph and (f) 10 dph. Flexion: (g) 12 dph (h) 14 dph. Post-flexion: (i) 16 dph. Transforming: (j) 20 dph. Juveniles: (k) settling 30 dph and (l) demersal 35 dph *Scale bars* = 1 mm (Taken from Cunha *et al.* (2013)).

During the planktonic phase of dusky grouper, which starts at 25 days (Cunha *et al.* 2013) and under laboratory conditions lasts up to 2 months (Barnabé 1974) following yolk resorption, larvae are fed a mixed diet of *Brachionus plicatilis* and *Artemia* sp in mesocosms (Cunha *et al.* 2009; Cunha *et al.* 2013). Most juveniles start to settle at 20.1 mm and most were benthic once they had reached a mean TL of 26.8 mm (Cunha *et al.* 2013). La Mesa *et al.* (2008) reported the successful release of batches of juvenile dusky grouper at an MPA on the south-western Sicilian coast (central Mediterranean) as part of a pilot study examining prospects for restocking efforts.

1.5 Infectious disease affecting grouper spp. and dusky grouper

Wild dusky grouper are recognised to be affected by an array of pathologies caused by parasitic (Roumbedakis *et al.* 2013), bacterial (Eissa 2011) and viral infections (Marino 2001; Vendramin *et al.* 2013; Kara *et al.* 2014). In the Mediterranean episodes of mass mortalities affecting wild grouper are known to be caused by bacterial (Marzouk *et al.* 2009; Eissa *et al.* 2011), and viral infections (Marino & Azzuro 2001; Vendramin *et al.* 2013; Kara *et al.* 2014). In Libya it was suspected, but not confirmed, that a viral agent was associated with wild grouper mortality (Al-Attar *et al.* 2013; Attar *et al.* 2009; OIE 2016).

Cultured dusky grouper may share similar parasite infections to wild fish of the same species (Roumbedakis *et al.* 2013), with most parasites found affecting wild fish also being found to affect cultured fish of the same species (Scholz 1999). As an example, grouper species are frequently found to be affected by monogeneans belonging to the genus *Pseudorhabdosynochus*, Leong & Wong (1988) described *Pseudorhabdosynochus epinepheli* (Yamaguti, 1938) affecting greasy grouper *Epinephelus malabaricus* (Bloch & Schneider, 1801) to show a higher intensity in cultured fish when compared to wild. Under culture conditions, infection of grouper *e.g.* orange-spotted grouper *Epinephelus coioides* (Hamilton, 1822), with monogeneans *e.g. Pseudorhabdosynochus lantauensis*, can lead to mortalities or loss of production during heavy infections (Nagasawa & Cruz-Lacierda 2004). Roumbedakis *et al.* (2013) reported that for both, wild and cultured dusky grouper, *P. beverleyburtonae* had 100% prevalence during all seasons, giving no indication for intensity.

A commonly reported parasite associated with dusky grouper belongs to the family *Philometra*, which are nematode parasites infecting a wide range of fish species (Moravec & De Buron 2013). In the dusky grouper they are frequently found to infect the ovaries,

causing major damage to the post spawning gonads, and they also have the potential to cause parasitic castration (Moravec *et al.* 2003).

The implications of infectious disease for the success of future aquaculture projects and conservation schemes (*e.g.* restocking programmes) have not been fully assessed; however, it is evident from the initial data provided that impacts are likely to be considerable without the instigation of effective management programmes. For aquaculture it is also clear that the use of integrated pest management (IPM) strategies is likely to be necessary to minimise the development of drug resistance in pathogens (Akinbowale *et al.* 2006; Salama & Rabe 2013).

The understanding of disease conditions in wild stocks is vital for monitoring purposes (Lloret *et al.* 2012) and given that most parasitic infection are expected to occur in cultured fish of the same species (Scholz 1999), understanding the factors that affect the health of wild fish is vital for future culture plans.

1.6 Libya country profile

Artisanal fishing in Libya is one of the principal modes of fishing practised (Lamboeuf *et al* 2002; (Lamboeuf *et al.* 2007; Khalfallah, Belhabib & Zeller 2015). In the countries bordering the Mediterranean Sea artisanal fisheries provide an important source of food and employment. Total catch from Mediterranean marine capture fisheries rose from 420,000 tonnes in 1950 to close to 1,000,000 tonnes in the 1980s. In 1995 it reached a maximum peak of 1,093,000 tonnes. Since that point, Mediterranean capture fisheries have been in decline (Sauzade & Rousset 2013). The decline of artisanal fisheries, accompanied by low levels of recruitment and decreasing incomes, poses a risk to their future wellbeing (Maynou

et al. 2013). A contributory factor to this decline might be the low income experienced by participants, and in countries like Lebanon, fishermen can typically earn 25% less than the minimum wage of the country (Pinello & Dimech 2013).

However, fishing activities form a core part of the social fabric and cultural identity of several Mediterranean coastal regions, providing 100,000 direct jobs in the European Union (EU) alone (Maynou *et al*. 2013) and with 319,000 individuals employed in the fisheries sector in the south Mediterranean region (Sauzade &Rousset 2013).

Libya has an estimated population of 6.3 million population as of 2016 (http://countrymeters.info/en/Libya). Fishing in Libya is mostly artisanal (Lamboeuf 2000), with the fisheries fleet constituting a range of boats, mainly batah, flouka, mator and lampara (**Fig. 1.5**) and (**Table.1.2**).

Figure 1.5. Fishing vessels used along the Libyan coast: (A) Mator, (B) Flouka.

Along the 1,700 km long Libyan coastline, 300 harbours and fish-landing sites were recognised according to a survey by the Marine Biology Research Centre (MBRC) in 2003 (FAO 2005), while Shakman & Kinzelbach (2007) counted 76 active landing sites. Lamboeuf (2000) reported 1266 boats. Fishing largely employs long lines, trawls and trammel nets, the

latter being most frequently used in depths from 1-50 m, as well as gill nets (Shakman & Kinzelbach 2007).

In the study conducted by Shakman & Kinzelbach (2007), of the 1511 of the boats recorded in 2006 along the Libyan coastline, 64.26% were “Flouka”, 24.09% were “Mator”, 6.88% were “Lampara” and 4.77% were “Batah” making the local boats called ‘’Flouka’’ the most commonly used for fishing, these most frequently using gill nets and long lines as fishing gear. Other vessels like the larger “Mator”, are owned by a Libyan fishermen, and operated by foreign crews *e.g.* Egyptian.

The species of fish targeted by the local ‘’Flouka’’ boats is seasonally-dependent and includes several fish species, of which the dusky grouper constitutes 0.98% of total catch (Shakman & Kinzelbach 2007).

Table 1.2. Main description of the fishing vessels used in Libya, from Lamboeuf (2000)

Fishing vessel	Description
Batah	7-8 m flat-bottomed boat using gillnets and pots (octopus) in shallow lagoon waters; propelled by outboard engine.
Flouka:	Small fishing craft of varied sizes ranging from 2 to 7 m; shapes are diverse but generally with a flat transom and no deck; powered by outboard engines.
Mator	Generally greater than 5-6 m in length running up to 18 m or more, with deck and roof for the smallest units, wheel house, fish hold, and net hauler for the largest; shape and design similar to units found in Tunisia, Greece and Egypt.
Lampara fishing unit	
Lampara	Usually 12-13 m with deck, inboard engine, a small roof and a purse seine winch; associated with one to three Dghaissas carrying kerosene or butane gas lights to catch small pelagic fish using light attraction at night some units may convert to net and/or line fishing during the off-season; only present in the western part of Libya
Dghaissa	7-8 m, without deck and engine; serves as light boat in association with the lampara

Fishing operators are obliged to hold a fishing permit issued by the Libyan Fisheries Society (Reynolds *et al.* 1995), and despite the presence of pre-printed forms for landed fish (Reynolds *et al.* 1995) most landings remain unrecorded, a trend commonly observed in small-scale, multi-species fisheries (Sadovy de Mitcheson *et al.* 2013).

Data from a scientific campaign in 1993-94 conducted under the LIBFISH project concluded that demersal fish stocks between the Tunisian border and Misurat are nearing full exploitation, urging not to increase fishing effort (FAO 2005). Updated fisheries data from Libya is lacking and most data listed from Libya are out of date and need further assessment (FAO 2006).

For the coastal population, fisheries provide one of the most important economic activities (Hamza *et al.* 2011). Sauzade & Rousset (2013) estimated that in Libya the number employed in the fisheries sector to be 7,657 individuals, which in 2005 was estimated at 1,500 individuals (FAO 2005). While there seem to be a possible change in trend / growth, no differences were drawn between fisheries type. Furthermore, there were no data on the number of individuals using spearfishing either part time or full time. As there has been no explicit reference to this type of fishing, it is unclear if they were included in these data.

In the Mediteranea, Pita (2014) estimated approximately 6000 spearfishers operate in Galcia, and these are responsible for 16% of the shared species landing with commercial fisheries. Godoy *et al.* (2016) has shown that the revenue derived from spearfishing was 2-3 times the monthly wage in Chile, which has the potential to become an incentive for the expansion of this unregulated fishing practice in Chile.

The lack in spearfishing data, being an uncontrolled fishing method, might be the reason for it drawing little attention from research (Pawson *et al.* 2008; Zeller *et al*. 2008). On the other hand data from spearfishing competition may give information for an otherwise lack of data

(Coll *et al.* 2004; Richardson *et al*. 2006).). Pita & Freire (2014) provided estimates from records of recreational spearfishing competition (1953–2007), observing up to 76% decline The lack in spearfishing data, being an uncontrolled fishing method, might be the reason for it drawing little attention from research (Pawson *et al.* 2008; Zeller *et al.* 2008). On the other hand data from spearfishing competition may give information for an otherwise lack of data (Coll *et al.* 2004; Richardson *et al.* 2006).). Pita & Freire (2014) provided estimates from records of recreational spearfishing competition (1953–2007), observing up to 76% decline in coastal rocky reef fishes in Galicia (NW Spain). Worldwide fish stocks are in decline (FAO 2012), and the role artisanal and spearfishing on fishing stocks needs investigation. In a study on spearfishing catch in Cape Creus Marine Protected Area (MPA), Lloret(2008) estimated that 40% of the annual biomass extracted from this area was by spearfishing. The selective nature of spearfishing in targeting large individual fish, has the potential to exert changes in both the trophic structure and the intrinsic vulnerability of taxa in the catch, and requires regulation. With the absence of current scientific surveys and statistics on commercial catches there is need for studies of the socio economical values on artisanal fisheries in Libya (FAO 2006), as well as to clarify the numbers of spear fishermen to be able to further estimate their impact on coastal fisheries.

1.6.1 Dusky grouper fisheries & regulations

The total production from Libya's capture fisheries in 2013 was 36,004 tonnes (FAO FishStatJ 2013) valued at an estimated US$ 100 million. Of that landed, approximately 33,700 mt was represented by 30 finfish species, from which approximately 660 mt were of dusky grouper. With a minimum size for capture of dusky grouper being set at 30cm (Reynolds *et al.* 1995), no maximum size is defined. Fishing is prohibited during the spawning season, which, for the dusky grouper, is June-July in Libya (Reynolds *et al.*1995). In **Table 1.3** the minimum capture

size of several commercial fish and the months in which fishing is banned, which coincides with the spawning season of these fish, are shown as reported by Reynolds *et al.* (1995).

In the Mediterranean more widely and in Libya more specifically, the dusky grouper is an economically important fish species in terms of artisanal and spearfishing activities (Heemstra & Randall 1993; Marino *et al.* 2001; Reñones *et al.* 2007, Kasem *et al.* 2009).

Dusky grouper are caught throughout the year in depths ranging from 20 to 65 m, often by artisanal fishermen using bottom longlines (Mallo & Goňi 2008) and by recreational spear-fishermen from 0 to 40 m depth (Coll *et al.* 2004). In Libya grouper in addition to bottom set longline (locally called: bringali dechi), is often caught by spearfishing, which is conducted in combination with scuba / compressor divers who fish in depths of 20-50 metres from March to November, by reaching diving site with either a Fluka with 1-2 divers or Mators with 3-4 divers (Lamboeuf 2002). Captures reach their maximum from summer to autumn, coinciding with maxima in feeding and reproductive activities (Reñones *et al.* 2007).

Table 1.3. The minimum capture size allowed in Libya, and closed seasons for key target commercial fish and other marine organisms (Reynolds *et al.* 1995)

Species	Min allowed size cm	Spawning season
Dusky grouper (*Epinephelus marginatus*)	30	Jun–July
White grouper (*Epinephelus aeneus*)	35	June–July
Common Dentex (*Dentex dentex)*	30	May–Aug
Red mullet (*Mullus surmuletus*)	15	Jun–July
Horse mackerel (*Trachurus trachurus*)	15	July
Sea bass (*Dicentrarchus labrax)*	25	Feb–Mar
Gilthead bream (*Sparus aurata*)	20	Oct–Jan
Grey mullet (*Mugil cephalus*)	25	July–Oct
Golden mullet (*Liza aurata*)	20	Jun–Aug

The adverse impact of artisanal fisheries is mostly observed for fish adopting sex reversal strategies, in particular protogynous fish *e.g*. dusky grouper (Lloret *et al.* 2008; Lloret *et al.* 2012; Rodríguez-Rodríguez 2014). Capture of larger individuals, which are either males or larger females will tend to deplete super-spawners in natural stocks, and the male population more generally (Whaylen *et al.* 2004; Lloret et al. 2008; Bijoux *et al.* 2013). In a study along the Natural Park of Cap de Creus Catalonia (Spain), Lloret *et al.* (2012) concluded that the artisanal fishery catch comprises a wide range of target fish, nominally reducing the pressure on one species. In comparison, spearfishing targets large individuals and the selective nature of this fishing practice means that some species *e.g*. dusky grouper (Harmelin & Robert 2001) are at higher risk. This makes this fishing practice highly detrimental to the grouper population at large and leads to it being considered as one of the main causes of mortality (MEPA 2011).

Given the artisanal and recreational nature of capture (Heemstra & Randall 1993), coupled with current political tensions in the region, it is extremely difficult to accurately assess and control landings. As a whole, artisanal fishery statistics are lacking, given the dispersed nature of capture operations, the universal existence of illegal and unreported fishing activities as well as the basic technical problem of fish/ product counting and measuring (*e.g.* gutted or whole, salted or not) making it difficult to collect complete catch information (Sauzade & Rousset 2013). Libyan artisanal fisheries need further evaluation, which includes training of fishermen. Also, greater artisanal fisheries research by the Marine Biology Research Center in Libya (MBRC) is urgently required in order to further ensure sustainable management of marine fisheries. This would support national economies and ultimately protect the livelihoods of the individuals involved in the fisheries sector (FAO 2006). A similar approach had been made by some other Mediterranean countries (Pinello & Dimech 2013).

1.7 Dusky grouper status and marine protected areas (MPA)

The dusky grouper is characterised as a year-round resident (Koeck *et al.* 2014) and shows site fidelity (Lembo *et al.* 1999). Owing to these biological characteristics, e.g. site fidelity (Lembo *et al.* 1999), it is considered a flag fish in the establishment of marine protected area (MPA) (Maggio *et al.* 2006). The Mediterranean sea has several sites designated as marine protected areas (MPAs) (Andrello *et al.* 2013) (**Fig. 1.6**), and although most of the Mediterranean dusky grouper stocks are described as depleted or over exploited (Abdul Malak *et al.* 2011;Tsikliras *et al.* 2013), the species continues to be caught despite its status as a threatened fish and position on the IUCN red list, In some countries, *e.g*. in South Africa, rules limiting a bag limit to five fish per day exist (Cornish & Harmelin-Vivien 2004).

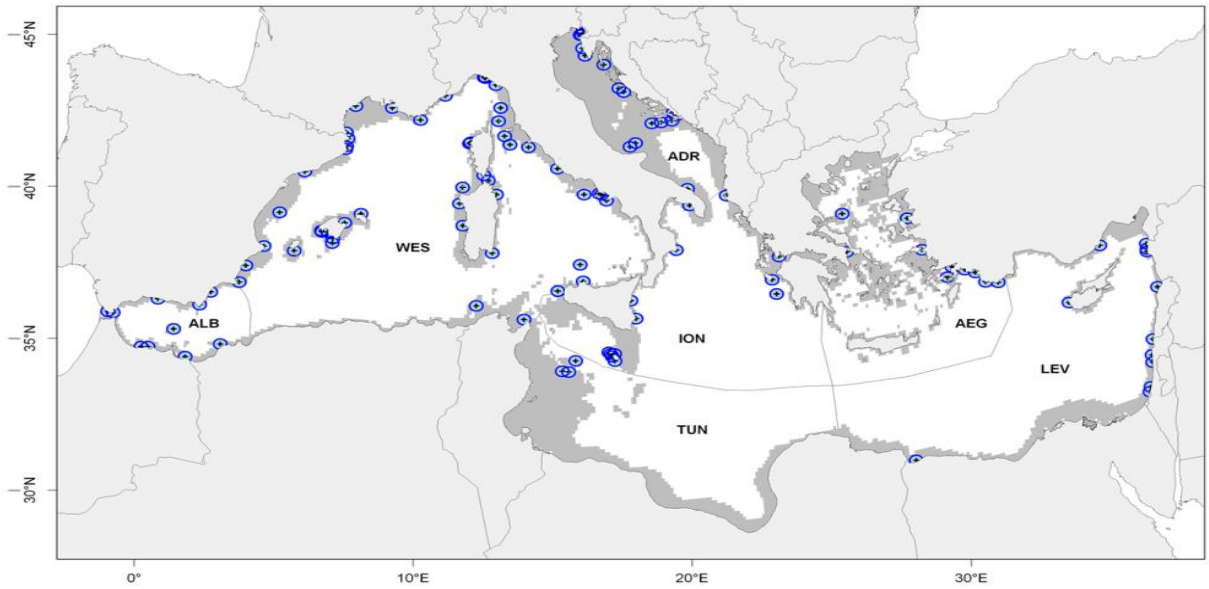

Figure 1.6. The existing marine protected areas (MPA's) as reported by Andrello *et al.* (2013). Abbreviations: ALB, Alboran Sea; WES, Western Mediterranean; TUN, Tunisian Plateau and Gulf of Sidra; ION, Ionian Sea; ADR, Adriatic Sea; AEG, Aegean Sea; LEV, Levantine Sea. (Image taken from Andrello *et al.* (2013))

In general dusky grouper are only protected in MPAs, where a spearfishing ban is implemented in addition to a bag limit (Harmelin & Robert 2001; Echwikhi, Jribi, Saidi & Bradai 2014) and capture size limit (Harmelin *et al.* 2010).

As described above, dusky grouper is considered a flag fish, with efforts being made to ensure its protection in various parts of the Mediterranean Sea by instituting MPAs with constrained fishing zones and limited bag sizes for spearfishing (Pawson *et al.* 2008) and with formal restocking programmes (La Mesa *et al.* 2008).

1.7.1 Marine protected areas in Libya

There are three established marine protected areas along the Libyan coastline. Two are located in the North east of Libya: El Kouf National Park on the north-west flank of the Jabel Al Akhdar, near the town of El Beidha and Aïn Ghazalah MPA and Hisha nature reserve, and Farwa MPA in the north-west about 180 km west of Tripoli (Haddoud & Rawag 2007; Hamza *et al.* 2011). (Hamza *et al.* 2011) proposed 24 more sites for consideration as part of a future network of Libyan MPAs (**Fig.1.7**).

1.8 Aquaculture in Libya

Libya's aquaculture industry is relatively small, with aquaculture being introduced in the early 70's (FAO 2013). The first formal entry for culture of freshwater cyprinids found in FAO's FishStatJ database was in 1987 at 30 tonnes and although there was moderate growth over the next few years, production appeared to stop in 1992 when there was 80 tonnes produced. From 1993 onwards, there was a switch to Nile tilapia, *Oreochromis niloticus* (L.), although the annual reported output has remained unchanged at a constant 10 tonnes per annum. In 1995 European seabass, *Dicentrarchus labrax* (L.) and gilthead seabream, *Sparus*

aurata (L.) culture were introduced, with both industries recording annual productions of 15 tonnes. Both industries appeared to remain fairly constant for the next few years but then rose sharply from 2003 onwards. The production of these latter two marine species then stopped abruptly in 2008 and thereafter only data for tilapia are recorded.

The recent war and political instability within the area has impacted aquaculture activities in the region, most notably with the destruction of one of the two main culture systems and hatcheries, *i.e.* the governmentally owned Ras Al-Helala sea bass and sea bream fish farms (pers. comm. Abdallah Elmgawshi)

Figure 1.7. The location of the nearest cities to current (highlighted with yellow dots) and proposed (numbers) MPAs in Libya. Current MPAs: a = Farwa lagoon and island, b = Kouf National Park, c = Aïn Ghazalah, d = Hisha nature reserve. Proposed MPA's: 1 = Wadi Maseed; 2 = Wadi Turghat; 3 = Ain Wadi Kaam; 4 = Sebkhet Qaser Ahmed-Taourgha Complex; 5 = Ain Taourgha; 6 = Sandy beaches and waters of Al Araar-Bouerat lahsoun; 7 = Al-Thalateen Beach; 8 = Sandy beaches of Bishr, Ajdabiya and Zwuitina; 9 = Garah Island; 10 = Shat Elbadine; 11 = Al-Mtefla Beach. 12 = Sebkhet Jeliana-Benghazi; 13 = Ain Zayanah; 14 = Tolmitah-Ugla rocky coast; 15 = Kouf Beaches; 16 = Sebkhet Ain Azzarga; 17 = Sebkhet Ain Shakika (Ain Eshgaiga); 18 = Wadi Khalij; 19 = Wadi Hamassah; 20 = Gulf of Bumba; 21 = Abulfrais Beach; 22 = North beaches of Ain Al Ghazalah; 23 = Beaches of Gurdaba; 24 = Gulf of Burdiya (Bardiyah). Image adapted from Hamza *et al.* (2011).

1.9 Aims and objectives

The work presented in this thesis had the overarching aim of assessing the health condition of wild dusky grouper in Libyan waters. This was achieved by research that targeted the following principal objectives:

Objective 1. Assessment of principal landed sizes, seasonal fishing activity and adherence to closed season and minimum size regulations.

Objective 2. Assessing evidence for the presence and impact of infectious disease in wild dusky grouper in terms of presence of pathogens, disease pathologies and effects on specific tissues and fish growth parameters

Objective 3. Investigation of the potential for use of social media networks *e.g.* Facebook and Google as alternative method for an epidemiological tool for surveying the presence of pathologies in dusky grouper captured along the Libyan coastline

Objective 4. To characterise the potential impact of identified health issues for wild and cultured dusky grouper populations, and to identify future research priorities

The relationship of these objectives to the structure of the thesis was as follows:

Chapter 2 A Field survey investigation of the general health condition of landed dusky grouper from a local fish market in Libya, This chapter provides the results of a field survey aimed at monitoring the health of wild dusky grouper offered for sale at a local fish market in Libya. It attempts to identify the key health issues that might affect wild and cultured dusky grouper populations and in addition provides an assessment of parameters related to fishing activities, landing sizes and fish ages across various seasons. This chapter serves to provide information which

might be beneficial for monitoring wild dusky grouper fish stocks, as well as highlighting disease issues that might pose concern in the event of future aquaculture programmes.

Chapter 3 A novel use of social media to evaluate the occurrence of skin lesions affecting wild dusky grouper, *Epinephelus marginatus* (Lowe, 1834), in Libyan coastal waters, this chapter addresses the epidemiology and distribution of skin lesions showing an apparent pathological continuum and identified in Chapter 2, this work uses a novel approach that employs use of social media networks to examine relevant parameters. This chapter serves to improve understanding of the prevalence of these lesions in fish captured along the Libyan coastline and neighboring countries, as well as highlighting the extent of spearfishing practice in Libya.

Chapter 4 Dusky grouper dermatitis (DGD), this chapter addresses the histological and gross morphological description of the skin lesions. This chapter serves to give a morphological and histological description of these skin lesions and the effect these lesions have on dusky grouper is also further investigated. As well as assessing the age group of affected fish, seasonality of infection and prevalence of the occurrence of these skin lesions on landed dusky grouper during the period of the study.

Chapter 5 Metazoan parasite morphological description, pathogenesis, this chapter describes the parasite seen affecting the fish with these skin lesions using histological serial skin sections, and the parasite with known blood fluke genera affecting fish. Furthermore, the possible role this parasite has in initiating this pathology is highlighted to further understand the parasite role in the DGD pathology.

Chapter 6 General discussion and conclusions, this chapter summaries the findings of the described research and attempts to assess the impact of these findings on wild and cultured dusky grouper populations and for grouper more widely, as well describe future research.

Chapter 2

A field survey investigation of the health of landed dusky grouper from a local fish market in Libya

2.1 Introduction

In the Mediterranean, the dusky grouper is listed as an endangered fish species (IUCN, 2015). Efforts have been made across this area to culture dusky grouper for commercial purposes (Glamuzina *et al.* 1998; Glamuzina *et al* 2000; Spedicato *et al.* 2000; Marino *et al*. 2003) and for stock enhancement programmes (La Mesa *et al.* 2008). In Libya, dusky grouper is considered a prime-eating fish species, which therefore achieves high market value (Reynolds *et al.* 1995; Kasem *et al.* 2009). With no culture programme currently in place, its high marketability and importance nationally and internationally makes culture of this species a priority endeavour that should be seriously considered in a Libyan context.

In this respect, assessment of the health condition of wild populations is vital (Lloret *et al.* 2012). Given that wild stocks carry many infectious agents, it is possible that these could potentially be introduced into aquaculture farms via several routes, including feeding of unprocessed trash fish and use of infected broodstock (Lafferty *et al.* 2015). Therefore understanding health conditions of wild dusky grouper population would offer valuable information for future culturing programmes. Currently, save for a number of reports and case studies on mass mortalities of wild grouper (Southgate 1985) recorded as resulting from a range of bacterial and suspected viral infections over several years (Al-Attar *et al.*2009; Soliman *et al.* 2011; Al-Attar *et al.* 2013), little is known with respect to wild dusky grouper health in Libyan waters.

This study aims to investigate the health of wild dusky grouper caught off the Libyan coastline over a study period extending from 2013-2015, through the use of two principal approaches. First, in order to assess fishing pressure, landing sizes and weights and other fisheries parameters were investigated. Second, a range of health parameters were assessed, including those established through laboratory investigation *e.g.* histological description of pathological changes to tissues induced by parasitic infestation and assessment of gonadal pathologies, which might affect capacity for stock recruitment. The approach used in this study starts with the individual fish, a bottom-up approach that can be key to the assessment of disease in a wild populations (Feist & Longshaw 2008) and can help to identify disease / pathologies that might be of relevance to both the future of and rational management of wild and cultured fish populations.

2.1.1 Grouper fishing methods and landings

Dusky grouper is caught mainly through a combination of artisanal methods and by spearfishing (Shakman & Kinzelbach 2007; Kasem *et a.l* 2009). In general, Kronen *et al.* (2005) estimated that 20% of total *Serranidae* are caught by spearfishing and Gillett & Moy (2006) estimated that grouper comprised up to 6%-14% of captures from scuba spearfishing. The detrimental effect of the combination of scuba and spearfishing on selected fish populations, by allowing access to deeper water spawning aggregations, diminishes the positive effects of deep water acting as a sanctuary for fish (Gillett & Moy 2006) and leads to the eventual reduction in fish populations.

In Libya the legal minimum size for dusky grouper fishing is 30 cm TL (measured from the tip of the head to the end of longest tail lobe) (**Fig.2.3**). Furthermore, fishing is nominally

prohibited during the spawning season in Libya, which for the dusky grouper, is June-July (Reynolds *et al.* 1995). No maximum size or bag limit (for spearfishing) is defined.

Earlier work (see **Chapter 3.**). has provided evidence for scuba spearfishing becoming an increasingly popular fishing method in Libya, being the most frequent recreational fishing method in Libya and being one of the most frequent recreational activities in North-West Mediterranean coastal zones more generally (Rocklin *et al.* 2011) Spearfishing is also a fishing method, which is used commercially using scuba in Libya (Lamboeuf 2000).

The general lack of statistics for grouper landings is consistent across the Mediterranean, mainly due to the generally artisanal nature of fishing for grouper (Sauzade & Rousset 2013). When available, most statistics give the total weight of captured grouper (FAO stat 2013) without specifying the size of fish, and in Libya, landed fish are not generally recording on the appropriate pre-printed fishing forms, which exist for coastal fishing (Reynolds *et al.* 1995).

The highly selective nature of spearfishing targets larger individuals in depths extending to 20-50 m where larger individuals usually live. It is well established that larger / older individuals are the most fecund (Green 2008) and it is this population which might, if depleted, result in the decline of fish recruitment (Green 2008). Knowledge of landed grouper sizes can 1) give an estimate of target size, 2) provide a reflection of the structure of the remaining population and 3) allow assessment of potential for impact of fishing activities.

2.1.2 Survey site

Tripoli has 12 principal fish landing sites (Reynolds *et al.* 1995), but most fish is sold at Bab Elbahar and Enadi Elbahri, the latter being the second largest fish market in Tripoli the capital of Libya (**Fig.2.1**).

Figure 2.1 Map showing the location of Tripoli, where Enadi Elbahri, the fish market and fish landing site used for the current survey, is situated. (Both images download from the internet, anonymous)

Enadi Elbahri (32°55' N 13°14' E) is a former naval installation protected by artificially made jetties, and serves as a permanent landing site for fishermen (Reynolds *et al*. 1995). It is located about 5 km west of ‘’Bab Elbahar’’ (32°54' N 13°11' E) the main port for commercial ships and comprises a harbour mainly used by medium size gillnetters and small size trawlers. Enadi Elbahri provides whole fish sale, a fish market and a fish inspection point. Dusky grouper landings from this fish market were unknown at the inception of this work, and no landing records could be found for dusky grouper at this specific harbour. At Enadi Elbahri, the fish offered are mostly caught locally and are sold whole and later gutted after purchase by the customer. Fish species include a range of Perciformes, molluscs including cephalopods and bivalves, and crustaceans and sharks are also occasionally sold. Cultured

fish imported from Malta, Greece, Turkey and Tunisia *e.g.* chilled sea bass *Dicentrarchus labrax* L. and sea bream *Sparus aurata* L., are also offered for sale.

2.1.3 Age determination

The principal method for assessing the age of fish in seasonally variable waters is by counting the annular rings of otoliths (Wright 1993). Reñones *et al*. (2007) used sagittal otoliths to estimate the age of 358 specimens of dusky grouper ranging in TL from 6.6 to 105.6 cm. By counting the annular rings they concluded that the otoliths grow symmetrically for all fish sizes, with a visible pattern alternating between translucent and opaque bands. While it was possible to easily estimate fish age from whole otoliths up to 10 years of age, beyond that it was increasingly difficult, as the rings are close packed and more difficult to distinguish, and thus the otoliths require sectioning. However, small otoliths can be used directly by immersing them in 70% ethanol to clear them, and subsequently viewing them under a dissecting microscope (pers. comm. Aisha Ambu Ali).

2.1.4 General description of dusky grouper gonads and maturation

Being a sequential protogynous hermaphrodite (Heemstra & Randall 1993) all dusky groupers are born females, and some females transform into males after reaching sexual maturity and spawning. Under cultured conditions, however, male inversion and sexual maturation have been accelerated, with Sarter *et al.* (2006) succeeding in inducing sex change in 1-year-old pre-pubertal dusky grouper juveniles within 12 weeks during their second year of life, using slow-releasing implants of 17α-methyltestosterone (MT). These irreversible males produced milt in 2 consecutive seasons. This capability could prove important for the success of dusky grouper culture, since males previously captured from

the wild displayed high rates of mortalities, and hence success in artificially induced sex inversion would eliminate or reduce the need for wild males (Seckendorff 2009).

As in most bony fish (Uribe *et al.* 2015), the dusky grouper has paired gonads located in the abdominal cavity (Marino *et al.* 2001). Both the ovaries and testis are connected to the ovipore, located posterior to the anus and anterior to the genital papilla with an opening in the genital pore (Marino *et al.* 2001). The gonads of dusky grouper are of the ovotestis type with groups of sexual cells being dispersed through the organs (Tortonese 1975; Bruslè 1985). While the testicular tissue is found in all stages of sexual maturity, it is most abundant during the transitional and male stages. The stage of gonadal development has been histologically assessed, with Reñones *et al.* (2010) and Marino *et al. (*2001) dividing the microscopic description of the developmental gonad stages into juvenile, female, transient and male (in order of development), with each stage having further sub-divisions. In the current study, Marino *et al.*'s (2001) approach will be used as the key reference for the description of gonadal development.

2.1.5 Females

Marino *et al.* (2001) described the ovaries to comprise two uneven lobes, connecting to the ovipore by a short oviduct which is enlarged in spawning females. The size of the ovaries varies, depending on age, stage of maturation and season. Islands of testicular tissue can be found in the ovaries (Marino *et al.* 2001). The ovaries according to Marino *et al*. (2001) are divided into juvenile, F1-resting, F2-developing, F3-maturing, F4-mature, F5-partially running and F6-spent. According to Marino *et al.* (2001), functional females range in size from 36.7 to 97.0cm SL (standard length) where standard length refers to the length of a fish measured from the tip of the snout to the posterior end of the last vertebra / excluding the tail (**F.2.3**).

Reñones *et al.* (2010) reported that the size of mature females ranged between 38.6 cm TL for a 5 year old fish and 100.3 cm TL for a 52 year old specimen, while they noted that the largest and oldest immature female was 54 cm TL and 7 years old. In a separate study, Marino *et al.* (2001) reported that first sexual maturity was reached at 36·7 cm somatic length (*LS*) (*n.b*. not total length, which might add 5cm) and 5 years of age in females.

2.1.6 Transient

Transient is the stage during which the ovary transforms into a testis. Sex inversion occur when dusky grouper are between 9 to 16 years old and 70 - 90cm in total length (6 - 10kg) (Chauvet 1988). Smaller sizes have been recorded by Reñones *et al.* (2010) who reported transient males to occur at sizes ranging from 52.1 to 76.9 cm and 7 to 17 years old.

2.1.7 Males

Dusky grouper testes are of an unrestricted spermatogonial testis type (Grier 1981; Marino *et al.* 2001). Testes comprise uneven lobes situated in the posterior part of the body cavity. The size of males ranged from 58.4 to 105.6 cm TL, and 7 to 60 years old (Reñones *et al.* 2010), while Marino *et al.* (2001) measured 68·5 (12 years) to 105·0 cm LS.

2.1.8 Spawning season

The dusky grouper spawning season in the Mediterranean Sea extends from May to October. Mostly lasting 4 months, the exact start- and end-points vary from one region to another *e.g.* Balieric islands (Spain) extending from May-Septemeber, while along the Algerian coast (Algeria) it extends from May to August (Tsikliras *et al.* 2010). Differences in season and extent of spawning seasons have been attributed to temperature variation (Tsikliras *et al.* 2010).

With oocytes maturing in several batches, eggs are released in waves during spawning, which can take place over a period of two months (Bouain & Siau 1983). The dusky grouper reproductive cycle is asynchronous at the population level (Bouain & Siau 1983; Reñones *et al.* 2010) this being indicated by the presence of a few regressing ovaries from July until early November (Marino *et al.* 2001).

2.1.9 Parasites infecting wild and cultured dusky grouper

Parasites can have a detrimental effect on the survival and reproductive capacity of fish, since parasites affecting fish in the wild are often found to affect cultured fish of the same species (Scholz 1999). Several parasites have been observed to affect both wild and cultured dusky grouper including gnathiid isopod juveniles (Genc 2007), Monogenea (Roumbedakis *et al.* 2013), and nematodes *e.g. Philometra* (Moravec *et al.* 2003). Didymozoid trematodes and Myxozoa have also reported to infect the gall bladder (Gunter *et al.* 2009).

2.1.10 Gnathiid isopod infection

Gnathia spp. (Isopoda: Gnathiidae) are ectoparasitic arthropods, mostly showing a low host specificity (Grutter 1994), and thus affecting a wide range of fish, including teleosts. The larval stage called praniza can be found infecting the buccal cavity of wild dusky grouper throughout the year (Genc 2007).

2.1.10.1 Life cycle

Having a direct life cycle, with no need for an intermediate host, adults are associated with the benthos, often living inside benthic invertebrates such as sponges but not feeding (**Fig.2.2**). The larval parasitic stage is termed a “praniza”, a haematophagous stage temporarily associated with fish hosts for feeding (Marino *et al.* 2004). *Gnathia maxillaris* (Montagu, 1804), as an example, affects a wide range of fish with a large geographical

distribution (Smit and Davies 2004). Genc (2007) found that infected wild dusky grouper showed no indication of mortalities, however, heavy infestations of fish have been associated with mortalities in several aquaria (Marino *et al.* 2004; Hispano *et al.* 2013) .

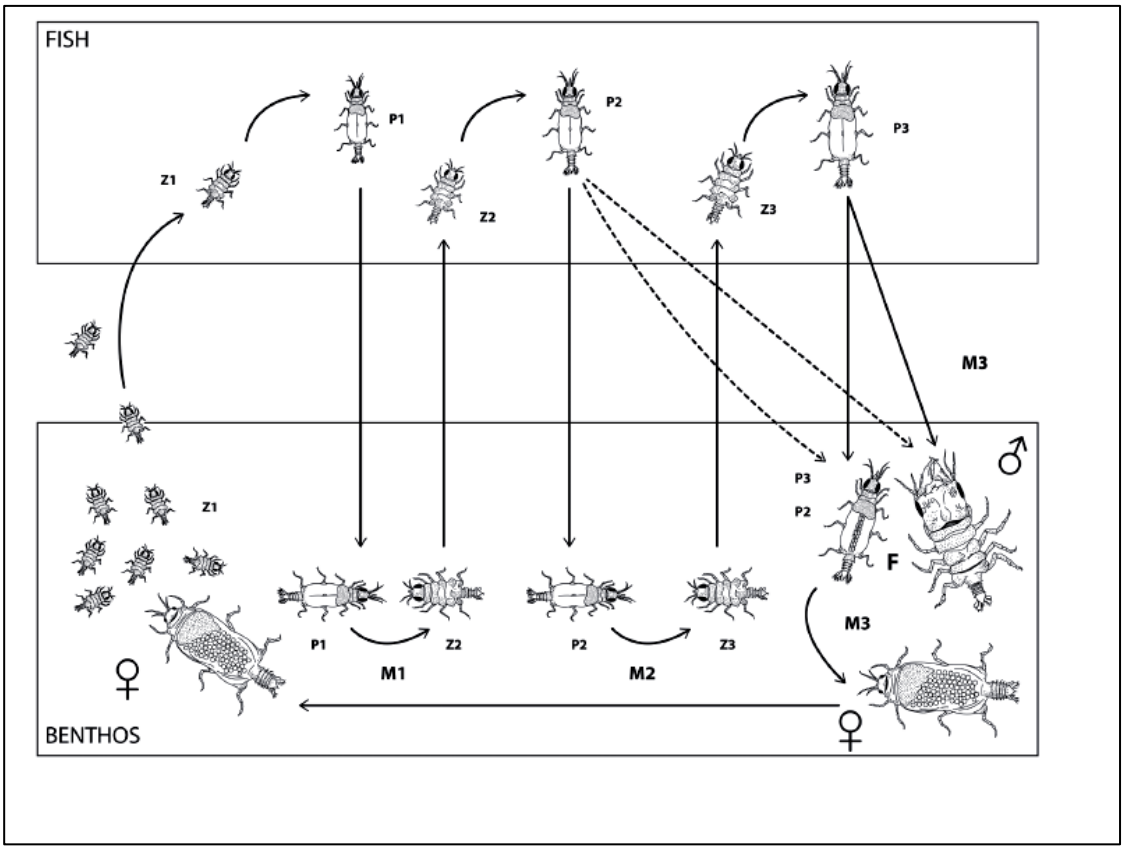

Figure 2.2 Diagram representing the life cycle of a gnathiid isopod, *Gnathia maxillaris* (Montagu, 1804) (image taken from Hispano *et al.* (2014) Pg. 281 Fig .2)

2.1.11 Monogenean infection

Monogenea are a group of largely ectoparasitic worms, normally dorso-ventrally flattened, which are associated with aquatic vertebrates, principally fish. Grouper are susceptible to a broad range of monogenean infections, and in the past 10 years alone, there have been several reports of Monogenea affecting grouper spp. With a direct life cycle, monogenean parasites have been associated with skin lesions and ulceration, and are believed to account for some of the mortalities observed in juvenile grouper including *Epinephelus coioides*

(Hamilton, 1822) and *Epinephelus fuscoguttatus* (Forsskål, 1775) in Asian aquaculture (Nagasawa and Cruz-Lacierda 2004). In India cultured greasy grouper *Epinephelus tauvina* (Forsskål 1775), infected with capsalid monogenean *Benedenia epinepheli* ([Capsalidea Order] Benedenia Diesing, 1858) showed skin lesions, erratic swimming and restlessness (Jithendran *et al.* 2005) (**see Table. 1.1 Chapter 1**). Similarly *Neobenedenia melleni* (MacCallum, 1927), when infecting cultured juvenile dusky grouper, can give rise to haemorrhages in several areas of the body, and can cause reduced growth and corneal opacities and death (Sanches 2008). In a comparative study conducted by Roumbedakis *et al.* (2013) monogenean parasites while infecting both wild and cultued dusky grouper, the Diplectanidae *Pseudorhabdosynochus beverleyburtonae* Oliver, 1984 showed a 100% prevalence found to infect the gills of both wild and cultured dusky grouper, no significant differences were seen in term of intensity of infection. While (Jithendran *et al.* 2005) has observed that the intensity varied between cultured and wild *E. tauvina*, with the cultured fish showing a higher intensity of *B. epinepheli* infection.

This parasite intensity and life cycle is greatly water temperature dependent with direct correlation between the rise in water temperature and increased monogenean infestation (Ravi & Yahaya 2016).

2.1.12 Nematodes

A large number of nematodes (Phylum Nematoda) have been reported from wild grouper. Justine *et al.* (2010) investigated the parasite on several grouper species in New Caledonia, including *E. tauvina, Epinephelus malabaricus* (Bloch & Schneider, 1801), which both seem to host a large number of parasites including nematodes. Cultured grouper are often infected by nematodes, especially when fed on unprocessed trash fish Rückert *et al.* (2009).

One group that has has been found to affect grouper are nematodes belonging to the genus *Philometra* Costa, 1845 (Nematoda, Philometridae). Dusky grouper have, for instance, been reported to be infected by the gonadal philometrid *Philometra jordanoi* (López-Neyra, 1951) (Moravec *et al.* 2016). The Philometridae comprise a group of parasitic nematodes infecting the internal organs of fresh, marine and brackish water fish worldwide. Having a wide distribution, they infect fish of the tropical and subtropical regions of the Atlantic, Indian and Pacific Oceans (Moravec 2006), and to a lesser extent the Mediterranean Sea (Moravec *et al.* 2003). Most pathologies are associated with gonad infecting *Philometra* spp., which can infect both ovaries and testis (Moravec & De Buron 2013). During the host's spawning season, the haematophagous parasite occupies large portions of the ovarian space, giving the parasite the best conditions to feed, due to the presence of abundant red blood cells (RBCs) (Moravec 2006). The adult females grossly assume a dark, pink or red colour, supporting the suggestion that they feed on blood (Bakenhaster *et al.* 2014; Moravec 2006; Moravec & De Buron 2013). Female gonadal *Philometra* spp. often synchronise their maturation to the host's ovarian maturation cycle (Perez *et al.* 2009). This synchronous development of gonadal philometrids with their hosts has been observed for several *Philometra* species (Hesp *et al.* 2002; Moravec & De Buron 2013). However, there are contradicting reports as to the effect of *Philometra* spp. infection upon ovarian fecundity. Hesp *et al.* (2002) report that the ovaries of the Australian dhufish *Glaucosoma hebraicum* Richardson, 1845 show no tissue response to live adult *Philometra* of the species *P. lateolabracis* during its spawning season. Bakenhaster *et al.* (2014) observed that after spawning in red drum *Sciaenops ocellatus,* a severe necrotic reaction occurs in the ovaries, which is possibly elicited by the parasite. This reaction is characterised by an influx of inflammatory cells, mainly eosinophilic granular cells, giant cell formation and the formation

of a fibrous tissue capsule surrounding both dead and live nematodes. These authors concluded that despite the severe reaction, there were no evidence to support a reduction in host fecundity. In direct contrast to these findings, both Moravec *et al.* (2003) and Reñones *et al*. (2010) suggest that infection may have adverse effects.

Adult females, while alive, usually cause minimal tissue reaction, this being elicited by feeding on host tissues. In general most known pathological changes caused by *philometra* spp. infections follow the death of the parasite (Bakenhaster *et al.* 2014). Severe necrotic reactions can, however, lead to parasitic castration, as they do in the speckled trout *Cynoscion nebulosus* (Perez *et al.* 2009). The long term effects of ovarian philometrid infections on dusky grouper fecundity have not been investigated.

2.1.12.1 Life cycle

With one exception (Rasheed 1963), all philometrids are ovoviviparous. As their vulva, vagina and anus atrophy after fertilization, the female, in order to release the first stage (L1) larvae, bursts following contact with water, this suggested in freshwater to follow swelling induced by osmotic pressure (Moravec 2006; Moravec & De Buron Buron 2013). In the sea, however, it might be hypothesised that the bursting of females might be due to the mechanical pressure induced by growing of the larvae or alternatively proteolytic digestion form larval secretions. In *Philometra obturans* (Prenant, 1886) infecting northern pike, *Esox lucius* L. 1758, L1 larvae can survive for up to 26 days at 5°C, however, the survival rate is temperature dependent and decreases to only one week at 20-22 °C (Moravec 1978).

Once ingested by the intermediate host, usually copepods, the larvae (in the haemocoel) moult twice into the second (L2) then third (L3) larval stages (Moravec 1978; Moravec 2006).

At this point a copepod carrying an L3 larva can pass on the infection if consumed by the final host. Moravec (2006), however, suggested that a paratenic host may also be involved in the life cycle.

After consumption of the intermediate invertebrate host (or another infected fish) by the final host, the L3 larva is released by digestion and, possibly triggered by gut pH, penetrates the gut wall and migrates to target tissues. The L3 then moults successively through juvenile to adult stages. In the final host female philometrids mature at which point copulation occurs, while gravid females then continue to grow, being filled with large numbers of first stage larvae (L1) (Moravec 2006), the males cease growing after reaching 3.1-3.3 mm (Rasheed 1963), and 2–4 mm (Moravec & Buron 2013). These very small males are consequently frequently overlooked during parasitological investigations due to the size disparity, *e.g.* 20-300 mm between males and adult females respectively (Rasheed 1963). Thus there are far more full descriptions of females in the literature than males, resulting in the lack of a full description of both sexes for many species and genera (Moravec & De Buron 2013).

2.1.13 Trematodes

Trematodes have been well documented in grouper, particularly as part of parasite checklists, with 62 species and 9 genera known to infect grouper spp. (Cribb *et al.* 2002). Leong & Wong (1988) for instance, observed that in Indonesia *Epinephelus malabaricus* were infested by the trematode *Prosorhynthus pacificus* Manter 1940, with the prevalencebeing 97.2% in cultured and 77.1% in wild fish. In Vietnam, pond -and cage-cultured *Epinephelus coioides* and *Epinephelus bleekeri* (Vaillant, 1878), with sizes ranging from 85-500 mm (mean=286.8±96.4 mm) were infected with three intestinal digeneans (*Prosorhynchus*

epinepheli, P. pacificus, and *Helicometra fasciata*), one from the stomach (*Erilepturus hamati*), and one from the skin (*Transversotrema patialense*). One group of trematodes that can be associated with mortality of infected fish is that comprising the blood dwelling trematodes, also known as blood flukes, which belong to the Family Aporocotylidae Odhner, 1912 (Platyhelminthes: Trematoda) [syn: Sanguinicolidae von Graff, 1907]. Affected fish are found dead with open mouths and flared operculi (Leong & Colorini 2002). Fish can also be found gasping for air at the water surface, with fusion of gill lamellae and hyperplasia (Seng, Tan & Enright 2006). Blood fluke pathologies are further described in **Chapter 5**.

One of the best documented digenetic trematodes causing pathologies in both cultured and wild grouper species are Didymozoidae Monticelli, 1888 [Trematoda, Digenea] parasites. Didymozoidae are trematodes parasitising fish usually live in pairs (Yamaguti 1970). They are found in capsules of connective tissue in various tissues, including, but not only, the skin (Eiras & Rego 1987; Ohiekezie *et al*. 1992), gills (Abdul-Salam *et al*. 1990; Soliman *et al*. 2011; Mladineo & Bočina 2009), oral cavity, kidneys (Yamaguti 1958, 1970; Mladineo & Bočina 2009), and muscles (Lester 1980). Both wild (Justine *et al.* 2010) and cultured grouper (Nagasawa & Cruz-Lacierda 2004) are found infected with didymozoidae parasite.One of the well documented digenetic trematodes causing pathologies in both cultured and wild grouper species are Didymozoidae Monticelli, 1888 [Trematoda, Digenea] parasites. Didymozoidae are trematodes parasitising fish usually live in pairs (Yamaguti 1970). They are found in capsules of connective tissue in various tissues, including, but not only, the skin (Eiras & Rego 1987; Ohiekezie *et al*. 1992), gills (Abdul-Salam *et al*. 1990; Soliman *et al.* 2011; Mladineo & Bočina 2009), oral cavity, kidneys (Yamaguti 1958, 1970; Mladineo & Bočina 2009), and muscles (Lester 1980). Both wild (Justine *et al.* 2010) and cultured grouper (Nagasawa & Cruz-Lacierda 2004) are found infected with didymozoidae parasite.

Wild Libyan dusky grouper have been observed to be affected by didymozoid parasites (Soliman *et al.* 2011), forming yellow capsule-like cysts situated between the basement membrane of the epithelium and the efferent artery of the primary gill filament. This was also observed in cultured *Epinephelus coioides* (Hamilton, 1822) by Nagasawa and Cruz-Lacierda (2004), were the gills in pond-reared *E. coioides* described by Cruz-Lacierda *et al.* (2001) were seen afected by *Gonapodasmius epinepheli* **see Chapter 1 Table.1.1**. Grossly the yellow cysts cause gill filament distortion, and histologically it can be seen to cause focal epithelial cell hyperplasia of the gill lamellae with an increase in mucous cell number (Cruz-Lacierda *et al.* 2001; Nagasawa and Cruz-Lacierda 2004).

Didymozoids are also reported from commercial fisheries. For instance, an unknown didymozoid was seen to infect the skin of white grouper *Epinephelus aeneus* (Geoffroy Saint-Hilaire, 1817), forming light to dark brown labyrinth-like ducts beneath the skin which were visible following the filleting of the fish, and occurring at a prevalence of 43.8% (Eiras & Rego 1987). Although there seem to be no severe health effects reported for grouper affected by didymozoids, it has been reported that infection of white grouper by didymozoids can reduce their market value (Eiras & Rego 1987).

2.1.14 Myxozoa

Myxozoans belong to the Phylum Myxozoa (Grassé, 1970) (Dyková & Lom 2007). They comprise a group of primitive metazoan parasites capable of causing major histopathological changes in fish hosts, and can be found in diverse tissues (Dyková & Lom 2007).

Several pathogenic species belonging to the genus Sphearospora Thélohan, 1892 are coelozoic parasites (infecting the in the urinary system) of fish (Dyková & Lom 2007;

U-taynapun *et al.* 2012). Wild and cultured *Epinephelus malabaricus* (Bloch & Schneider, 1801) are reported to be affected by *Sphaerospora epinepheli*, leading to loss of equilibrium with fish observed to be floating or turning upside down whilst some fish had haemorrhages on the mouth and skin (Supamattaya *et al.* 1991), *Sphaerospora epinepheli* has also been reported to cause mass mortalities in cage reared *E. coioides* (Xu *et al.* 2014).

The genus Ceratomyxa (Myxozoa: Myxosporea: Bivalvulida) is frequently observed in the gall bladder of several marine fish (Dyková & Lom 2007; Gunter *et al.* 2009), recognisable by the shape of the elongated spores, containing shell valves exceeding in length the axial width of the spore (Dyková & Lom 2006).Grouper spp. are also among the fish infected by Ceratomyxa (Thélohan, 1892), *Ceratomyxa hamour* have been described by Mansour *et al.* (2015) from the gallbladder of the orange-spotted grouper *E. coioides.* Although most infections are benign, Katharios *et al.* (2007) observed 100% mortality in Greek cultured sea bream *Sparus aurata*, although this might have been associated with the combination of hormonal treatment previously administrated to the fish to induce sex inversion with and consequent reduced immunity leading to a heavy infection of *Ceratomyxa diplodae*. Often the intra-hepatic bile duct of fish is found to be infected by myxosporean plasmodial stages belonging to *Zschokkella* sp. (Auerbach, 1910) (Ferguson 2006; Lom & Dyková 2006; Dyková & Lom 2007). The gall bladder of wild fish such as the Mediterranean parrotfish *Sparisoma cretense* (Linnaeus, 1758) is also noted to be infected by *Zschokkella* sp. (Kalatzis *et al.* 2015). These infections might lead to cholestasis and bile duct breakdown should they lead to invasion of the liver parenchyma, as described for dusky spinefoot (rabbitfish) *Siganus luridus* (Rüppell, 1829) infected with *Zschokkella icterica* (Diamant & Paperna 1992). Since the 1970s, dusky spinefoot has been recorded off the Libyan coast, and is considered as one of the species having invaded from the Red Sea (Stirn 1970, Lamboeuf 2000).

2.1.14.1 Life cycle

The life cycle of myxosporeans involves fish as final hosts and invertebrates as intermediates (**Fig.2.3**), these latter potentially including annelids or bryozoans according to species (see Dyková & Lom (2006) for a further detailed description of myxozoan life-cycles).

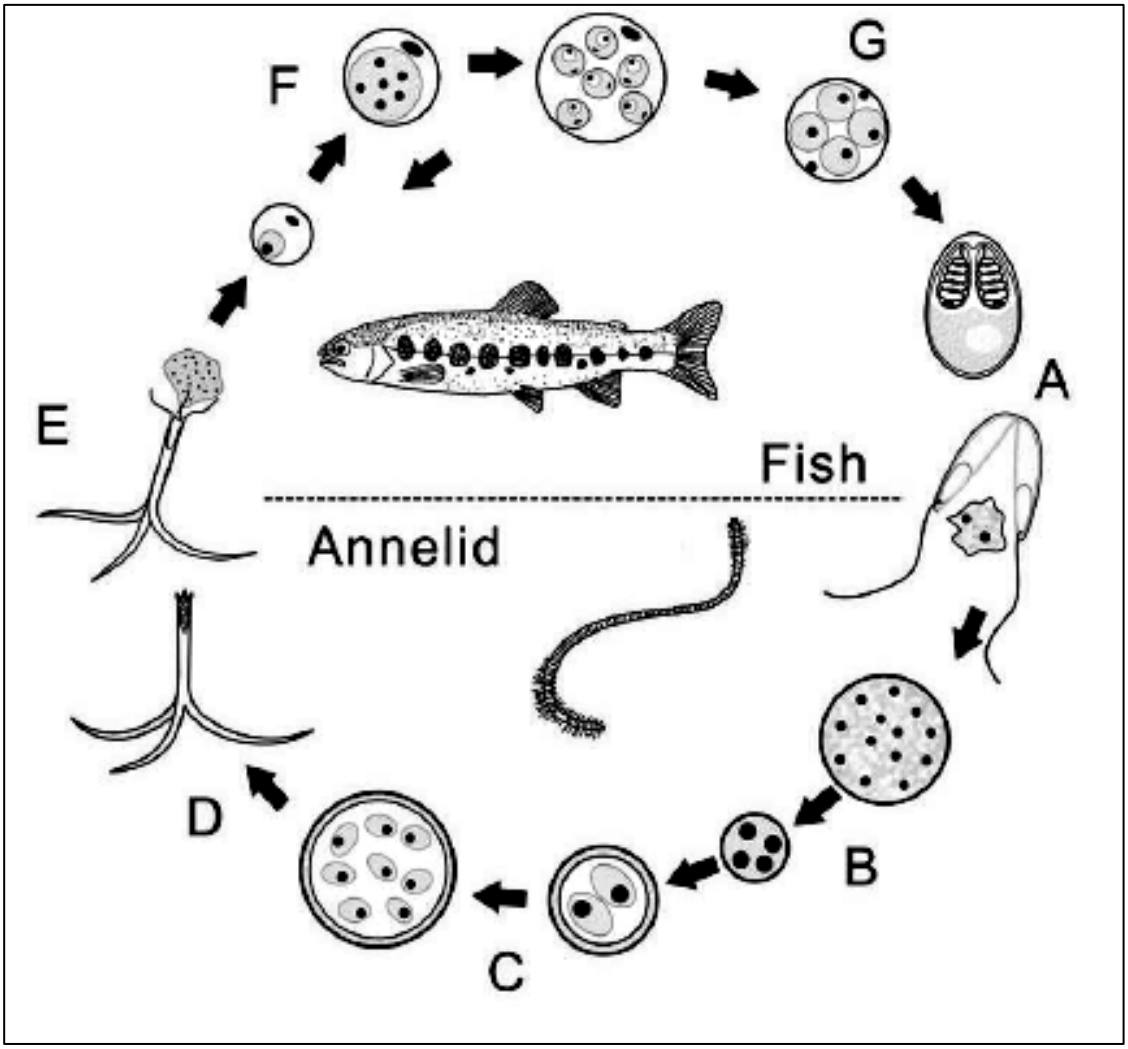

Figure 2.3. Diagram illustrating one of the few established life cycles for myxosporeans involving fish as final hosts and invertebrates as intermediate host. *Abbreviations*: A= polar filaments; B= gametogony; C= Sporogony of actinosporean phase; D: Mature actinospore stages; E: actinospores, polar filaments extrude to anchor the spore; F: Presporogonic multiplication in a cell-in-cell state; G: Sporogony of myxosporean phase. (Image taken from Yokoyama *et al.* (2012) Pg11, Fig. 4)

2.1.15 Protists

Cryptocaryon irritans and *Trichodina* spp. are ciliates often found to infect cultured fish in the Mediterranean (Rodgers & Furones 1998) with *C. irritans* recognised to cause serious pathologies in intensively cultured grouper (Nagasawa and Cruz-Lacierda 2004) **see Chapter 1 Table.1.1.** During a *C. irritans* disease outbreak affecting juvenile brown-spotted grouper (*Epinephelus chlorostigma*) (Valenciennes, 1828) in Kuwait, mortalities of up to 50% were observed (Rasheed 1989).

From the above it is clear that grouper are affected by a wide range of parasitic diseases, showing various levels of pathogenicity, these affecting both wild and cultured fish. Despite numbers of reports for grouper in general, reports on diseases affecting dusky grouper are scarce, particularly given that dusky grouper are being considered both for commercial culture and for restocking programmes in the Mediterranean (Zabala *e al*. 1997a; Spedicato *et al.* 2000; Cunha *et al.* 2009). Knowledge of diseases affecting wild population are increasingly important in terms of restocking and disease surveillance programmes. Assessing the disease status of wild dusky grouper in Libya, including provision of information on parasitic infections, is important to help predict both their impact on future culture programmes and their possible impact on wild stocks. The health condition of wild dusky grouper from which broodstock often derive makes the investigation for the health condition of dusky grouper in Libya essential for future culture and disease surveillance.

2.2 Aims and Objectives

The aims of the research presented in this chapter, were to examine locally caught Libyan dusky grouper, to describe the principal members of their parasite fauna and other aspects

of disease status, to note pathologies associated with parasitic pathogens and from these observations provide some estimates of the potential for impact of conditions described.

The main objectives of the work were:

1) To survey a local fish market and assess the relationships between size, age and sexual maturity of wild dusky grouper as well as examine landed fish sizes and key fishing methods.
2) To inspect landed grouper for health issues and examine the seasonal prevalence of parasites and pathologies that might threaten population conservation and have impacts on fish health and welfare, following the introduction of dusky grouper to intensive aquaculture systems.

2.3 Materials and Methods

2.3.1 Field survey

2.3.2 A survey of the health of wild-caught dusky grouper

Dusky grouper offered at a local fish market were periodically inspected for external signs of disease in 12 sampling trips from 2013-2015 see **Table. 2.1.** No sampling was carried out during the months of February and December. Fish samples were obtained from a local harbour / fish market, Al Nadi Al Bahri, located in Tripoli, the capital of Libya. Visual inspection included examination for malformations, skin discolouration and skin lesions, abdominal distension, protrusion of parasites from the cloacal opening, gill colouration (*e.g.* haemorrhagic, pale), and external parasites (*e.g.* isopods). These changes were recorded and pictures were taken as mentioned below. Measurements were taken of total length (TL) as described by Heemstra & Randall (1993) **See Fig. 2.4**. Fish were weighed (gram= g) whenever possible. Pictures were taken using an Olympus "Tough" camera (TG-810).

Table 2.1 Field sampling timing from March 2013-January 2015

Year	Months									
2013			**Mar**	**Apr**	**May**	**Jun**	**Jul- Aug**	**Aug-Sept**	**Oct-Nov**	**Dec**
			x	x	*nos*	*nos*	6-4	30-10	20- 23	12-29
2014	**Jan**	**Feb**	**Mar**	**Apr-May**		**Jun**	**Jul- Aug***	**Aug-Sept**	**Oct**	**Dec**
	nos	*nos*	*nos*	12-10		11-19	16-01	30-10	12-30	24-
2015	**Jan**									
	18									

nos = no sampling trips; x= preliminary trip in preparation for sampling trips; *= disrupted sampling due to political unrest.

For laboratory investigation a total of fifty fish (50) alive or freshly dead were sampled over the period of the study with sizes ranging from 27-68 cm TL. Gonads from an additional five dusky grouper sold at the fish market were examined, from individuals with sizes ranging from 68-92 cm TL, these gonads being sampled for histological and parasitological

investigation. From the fifty fish sampled, twenty six fish showed visible skin lesions. Five of the remaining fish, which showed no external skin lesions, were used for a description of normal skin histology (**Chapter 4**).

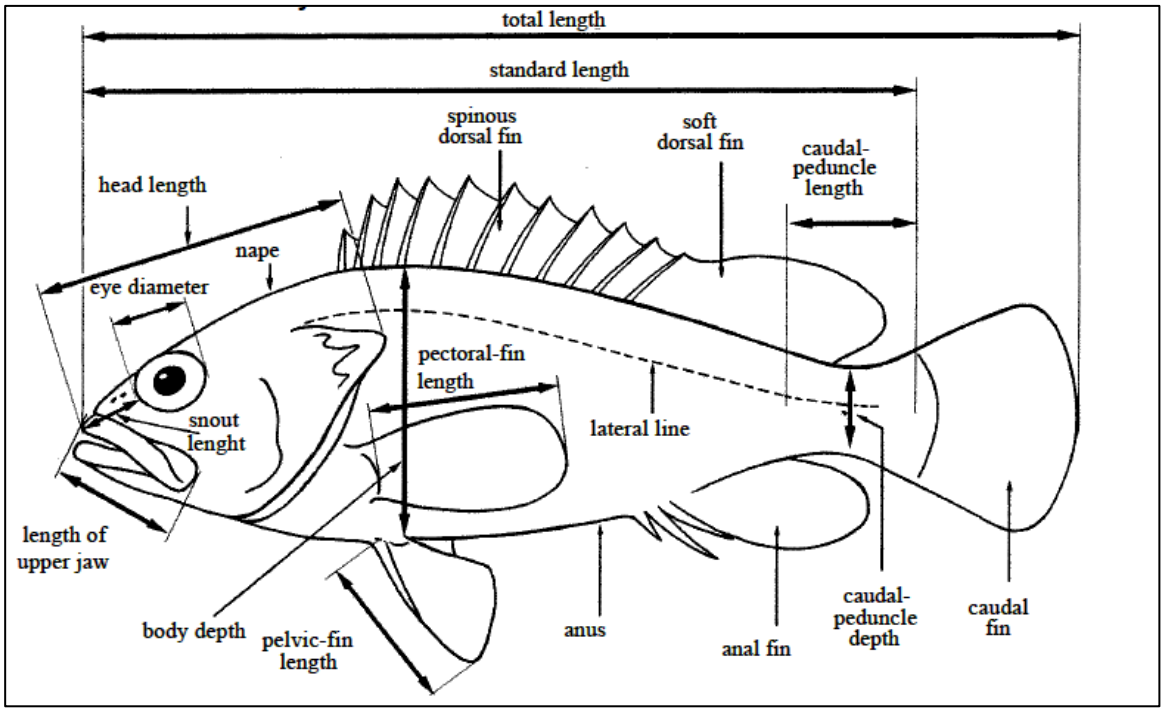

Figure 2.4. External morphology and measurements used throughout this study are as described by Heemstra & Randall (1993). Taken from Heemstra & Randall (1993)

During the sampling period, the number of fishermen, the fishing methods employed, the fishing fleet, as well as shops offering dusky grouper were recorded. Following fish inspection, the capture method was established through direct enquiry and by inspecting the fish for hooks or hook damage in the oral cavity and injuries to the body typically caused by spearfishing. Data were recorded at the time of sampling and entered into Microsoft Excel (2010) spreadsheets. Graphing was also conducted using the Excel package. Valid dusky grouper species names and authorities followed validated terminologies provided by Heemstra & Randall (1993) and FishBase (www.fishbase.org).

2.3.3 Euthanasia

Fish was either sampled at the fish market, or transported in aerated water to a nearby laboratory and killed by schedule one methods.

2.3.4 Age determination

For age determination, the sagittal otoliths of ten (10) dusky grouper having sizes ranging from 28 -56 cm TL were used for age determination. It was not possible to obtain the otoliths from 8 of the sampled fish, and unfortunately the remaining 36 otoliths could not be retrieved from storage in Tripoli due to a worsening political situation.

2.3.4.1 Otolith preparation

Sagittal otoliths were removed from fish, rinsed with local tap water and left to dry in a fresh Petri dish. Once dry they were stored dry for further inspection (see Reñones *et al.* (2007)). To facilitate the viewing of the annual rings, the otoliths were placed in 70% ethanol, and while immersed in the 70% ethanol, the otoliths were viewed at 10x under a dissecting microscope. Following the method described by Reñones *et al.* (2007), the age of the fish was determined by counting the number of annual rings. For each otolith, the annual rings were counted blind by two people, pictures were taken and results were compared

2.3.5 Histology

Samples were taken from the skin (head, flank close to the lateral line, cloaca), eyes, gills and internal organs (brain, heart, pyloric caeca with attached pancreas, liver, spleen, gonads, head and tail kidney) from all 50 fish sampled. All tissues were placed in 10% neutral buffered formalin (NBF) and were kept for a minimum of 24 hours to a maximum of 7 months before being embedded in paraffin.

For compound light microscope observations, samples were routinely processed following an overnight cycle using chloroform as the clearing agent (Shandon Citadel 2000 tissue processor, Thermo Fisher Scientific Inc.). The tissues were embedded in paraffin wax, and trimmed with a Leica microtome using disposable microtome blades (MX35 Premier Microtome blades 34°/80mm/ Thermo Fisher Scientific Inc.) at 20µm. All organs except skin and eyes were soaked in distilled water for 5-10 min at room temperature, excess water dried off and placed for 1-2 min on a cold plate. Five µm thick multiple serial sections were subsequently cut. Skin and eye tissue blocks were pre-treated and decalcified in a rapid decalcifier (RDC™ and RDF™ cellpath) for 1 to 2 hrs. Tissue blocks were then rinsed with running tap water, dried and processed as above.

All tissue sections on slides were placed on a hot plate (Raymond Lamb) at 40°C, allowed to dry and then arranged in a staining rack and placed in an incubator (Windsor incubator). Slides were incubated at 66°C for 1 hr (internal organs) and at least 2 hrs to overnight (skin and eyes). Incubation of skin and eyes for less time would cause them to be lost during staining, especially for skin with lesions.

Dewaxed sections were stained with haematoxylin-eosin stain (H&E) (Drury & Wallington 1980). Tissue slides were viewed under 10x, 20x, 40x and 100x objectives using a compound light microscope (Olympus BX51TF, Olympus Optical Co., Ltd) and pictures were taken using an attached camera (Zeiss AxioCam MR colour, r 1.1) and software package (Carl Zeiss Vision,AxioVision Viewer v4.8). Pictures were processed using the free software package paint.net (http://www.getpaint.net/index.html).

For sex determination and examination of maturity and phase of the reproductive cycle, a mid-section of the gonad for both ovaries and testis was sampled. Each section was placed in 10% NBF, and processed for histology as described above. Histological criteria were used to establish stage of sexual maturity and phase of the reproductive cycle. The terminologies used to describe these stages of maturation followed the guidelines of Marino *et al.* (2001). These divide dusky grouper maturation into the following recognisable stages: juvenile females; maturing females: F1=resting, F2=developing, F3=maturing, F4=mature, F5=partially running, F6=spent; males: M1-maturing, M2-mature and M3-running ripe testes; and transitional: T1-early transitional, T2-bisexual, T3-late transitional.

2.3.6 Parasitological investigation

Sampled dusky grouper were subject to an external visual inspection followed by investigated of the oral cavity, eye and orbit, gills, fins, abdominal cavity and gonads. Skin scrapes were taken using the back edge of a scalpel blade along transects running along dorsal and abdominal surfaces of the fish. Skin scrapes were placed on a microscope slide, diluted with a small quantity of water, covered by a coverslip and viewed directly under stereo and compound microscopes.

2.3.6.1 Preparation of tissue for parasitological examination

2.3.6.1.1 Gonads

Whole gonads were dissected from 50 sampled fish 27-68 cm TL, and an additional 5 gonads were obtained from the guts of dusky grouper that had been sold at market, ranging from 68-100 cm total length (TL). The gonads were placed individually in plastic bags and transported in a cooled container to be dissected at a nearby laboratory. Although care was taken to immediately screen the gonads for parasites, this was not always possible due to

the frequent power cuts which extended from several hours to over 13 hours. During such periods, gonads were kept in a fridge and processed as soon as possible after sampling.

Depending on the size of the gonad, the gonad was placed whole or in parts in a clean Petri dish with a little PBS added. If whole, then an incision was then made in the external connective tissue. It was observed that by placing the opened gonads in a covered Petri dish for few minutes *Philometra* sp. would be induced to migrate from the ovary into the Petri dish, thus facilitating the removal of the tiny 2-3 mm long *Philometra* using a fine paintbrush.

The parasites were viewed alive using both a dissecting (Nikon) at 50x magnification and a compound light microscope (Nikon) under 5-40x objectives. Pictures were taken for recording purposes using a camera (Olympus/Tough). Isolated parasites were placed in either 80% ethanol, or 10% NBF.

To assess the survival and longevity of *Philometra* larvae, ~1000 (estimated) larvae were sampled from ruptured mothers within the ovaries of a single dusky grouper sampled on July 2014. The larvae were placed in aerated oxygenated water (2 L) for seven days and during short power cuts the air pump with attached air stone was run on battery. Twice a day a sample from the water with the parasites was taken using a plastic pipette, placed on a microscope slide and the movement and activity of larvae were observed using a stereo microscope. Unfortunately, due to deterioration in the political situation in Tripoli between July-August 2014, the laboratory work had to be suspended before the completion of the experiment.

2.3.6.1.2 Gills

Gill arches were collected from 50 fish. Where possible, gills were viewed directly by dissecting out gill filaments and mounting them on a microscope slide with a drop of seawater. Filaments were covered with a coverslip and viewed under a compound light microscope. Photos were taken of live parasites as described above and any parasites observed were placed in 80% ethanol and 10% NBF for transport and further identification. If such examination was not possible, the entire gill arches were placed in 80% ethanol or 10% NBF for later viewing.

2.3.6.1.3 Abdominal cavity

The abdominal cavity was grossly screened for parasites, encysted parasites, abnormalities and visible pathologies or anomalies.

2.3.6.1.4 Gallbladder

The gallbladder was dissected from its attachments and its contents were emptied into a glass receptacle. A drop of the retrieved contents was placed on a clean glass slide, covered with a coverslip and viewed under a compound light microscope (Nikon) under a 40X objective. Pictures were taken as described above. For photos taken using the Olympus camera, exact magnifications are not provided.

2.3.7 Scanning electron microscope

Preparation of parasites for scanning electron microscopy was conducted with the help of Mr. Linton Brown. In brief, parasite samples were fixed in 4% glutaraldehyde buffered with 2.5% cacodylate buffer, post-fixed in osmium tetroxide, dehydrated through an ethanol series, critical-point dried in liquid CO2 using a Bio Rad critical point dryer, mounted on

aluminium stubs using double-sided tape and sputter coated with gold-palladium in a Polaron Edwards 150 B sputter coater. Specimens were then viewed on a Jeol JSM 6460LV scanning electron microscope at an accelerating voltage of 7-10KeV. Parasites fixed in 10% neutral buffed formalin (NBF) were similarly processed.

2.4 Results

2.4.1 Field survey

2.4.2 Harbour and fishing method survey

2.4.2.1 Fish market:

Elnadi Elbahri was converted into a fish landing site and recreational boat anchoring harbour in 1986 and since then has continued to support these activities. The harbour is enclosed by two large walls, East and West, and one large wall in the North built from concrete, with an opening to the Large Tripolitania natural harbour. Twenty-five shops sell the landed fish directly to domestic consumers, restaurants, hotels and small scale distributers (**Fig. 2.5**). It also provides space for drying boats, and barracks for workers. Nationalities working at the fish market include Libyan, Tunisian, Egyptian, Algerian, Sudanese and Nigerian nationals.

This site has its own ice-distributing facility, a coffee shop and a mosque, with some of the shops and boats being owned by families of fisherman across two generations. A restaurant was newly built during the time of this study at the main entrance to the fish market. An estimation based on personal observations suggests that ~100 fishermen work in fisheries at this harbour on a regular basis, with a total of 350 small and large boats anchored in the harbour, and an estimated 300 spear-fishermen associated with the facility (**Tab.2.2**).

Table 2.2. Elnadi Elbahri daily and seasonal fishing capacity and number of fishermen

Equipment	Small boat (Flouka)	Large boat	Divers
Fishing method	Longline & net	Longline & net	Spearfishing
No. fishermen	1-2	5-7	1-?
Fishing Depths.	<100 gama*	<300 gama	2-40m
Fish sp.	Seasonal	Seasonal	Grouper sp. seasonal
Total fishermen	~50 daily;~100 occasional; in season ~400	10 -12 daily and ~150 summer	~300

*A gama is the distance between the fingertips and the centre of the chest with arm outstretched: 1 Gama = ~90 cm

Fish from other surrounding fish landing sites were also sold directly at the fish market located in this harbour.

When the sea was rough and fish landed from around Tripoli were scarce, fish were observed to be transported from as far as Benghazi (1050 km east of Tripoli) and sold at this market. Fish were normally transported by road in cooled vehicles, however, this practice was seen to decrease over the period of the study due to the political situation and the dangers of the coastal road connecting the east to the west.

The fish species offered for sale were seasonally dependant and included but were not limited to Carangidae (*Seriola dumerili*); Sciaenidae (*Umbrina cirrosa*); Scombridae (Tuna); Serranidae (dusky grouper *Epinephelus marginatus;* gold blotch grouper *Epinephelus costae,* white grouper *Epinephelus aeneus,* Dogtooth grouper *Epinephelus caninus* (Valencienne)); Sparidae (bogue *Boops boops*, sand steenbras *Lithognathus mormyrus* (L.); salema porgy *Sarpa salpa*, gilt-head sea bream *Sparus aurata*, black seabream *Spondyliosoma cantharus*, Saddled seabream *Oblada melanura*, common pandora *Pagellus erythrinus*); Siganidae (dusky spinefoot *Siganus luridus*); Merlucciidae (European hake *Merluccius merluccius* (L.)); Mogilidae; Mullidae (red mullet *Mullus barbatus, striped red mullet Mullus surmuletus*); Zeidae (John Dory *Zeus faber* (L.)); Balistidae (grey triggerfish *Balistes carolinensis*), Lobotidae (tripletail *Lobotes surinamensis*) (seen only twice) and occasionally sharks. While crustacean (shrimps) and molluscs (octopus) were sold seasonally, bivalves were seldom offered (only seen once). Farmed fish were also offered along with landed fish, these comprising mainly sea bass and sea bream, cultured in Turkey and Malta and transported by air or sea (**Fig. 2.5**). Frozen fillets of marine or fresh water fish were not observed to be offered at this fish market during the sampling period.

2.4.2.2 Fish sale

Chilled fish are offered whole, arranged on ice in wooden or plastic boxes and placed on display each morning until early evening (**Fig. 2.5**).

Fish were only gutted and prepared for consumption after they were sold to the consumer. The gut and gills were usually thrown back into the water of the harbour, the facility lacking a formal waste management system. A large number of fry and fingerling from different fish species were seen swimming in groups in the harbour.

2.4.2.3 Dusky grouper landings

Dusky grouper are offered almost daily at the fish market. Most dusky grouper comes from fishing grounds around Tripoli, extending to Zwara to the west and Misratah in the east. On one occasion, due to bad weather, fish were lacking, and dusky grouper caught in the vicinity of Benghazi were transported across to the market (more than 1000 km from the west), with these fish showing clear skin lesions (**Fig 2.5C**).

2.4.2.3.1 Fishing method and fish size

Fish were mostly caught using artisanal methods, and by spearfishing. Injuries caused by spearfishing were often seen on the fish, and occasionally hooks were embedded in the stomach. A typical bag catch for a spear fisherman would comprise 1-4 grouper a day (weather permitting). During the spawning season, females are particularly sought after by customers due to their ovaries. Fish over 70 cm TL are sold to restaurants, while fish ranging from 30-60 cm TL were most sought after by domestic consumers. The size of fish offered at the fish market ranged from 20-92 cm TL (**Table.2.3**). It should be noted although from direct inquiries it was not always possible to define the fishing method, fishing ground and depths.

Lesions induced by harpoons were observed on 20% of the dusky grouper offered. The remaining grouper fishing methods involved baited hooks, including longlining and also possibly included capture by blast fishing, although this was not confirmed but suspected based on inquiries.

Figure 2.5. Images of the harbour / fish market at Nadi Bahri, Tripoli. (**A**) Harbour basin with fishing boats and nets (July 2014) (**B**) Large fishing boats taken out to dry (October 2014) (**C**) Fish laid out chilled on ice to be sold, note a dusky grouper with a skin lesion at the top left corner of the image (white circle) (April 2014). (**D**) Sea bream (1) caught from the wild and (2) cultured (probably brought in from Malta (not confirmed)) (January 2015)

Out of the 267 captured dusky grouper observed, most fish offered at the local fish market ranged in size from 30-39 cm LT, such fish comprising 70 out of the 267 fish, giving 26% of the total. Sizes from 50-59 TL came second (62, 23.23% of total), followed by 40-49 cm TL (47 fish, 17.6% of total), followed by 60-69 cm TL (26 fish, 9.7% of total), then 70-79 and 80-

89 cm TL (both by 9 fish, 3.3% of total) and finally fish sizes ranging from 90-99 cm TL (2fish, 0.7% of total) (**Fig. 2.6 & Table. 2.3**). The relationship of length to weight in dusky grouper measured in this study is shown in (**Fig. 2.7**).

Table 2.3. Size range and percentages of total capture for a total of 267 dusky grouper offered at a local fish market in Tripoli. Landings were recorded in intervals including July- December 2013, , April-May 2014, October 2014, December 2014 and January 2015 (**see Table 2.1 for details**).

Size cm	20-29	30-39	40-49	50-59	60-69	70-79	80-89	90-99	Total No.
Number	42	70	47	62	26	9	9	2	267
Total %	15.7	26.2	17.6	23.2	9.7	3.3	3.3	0.7	99.70%

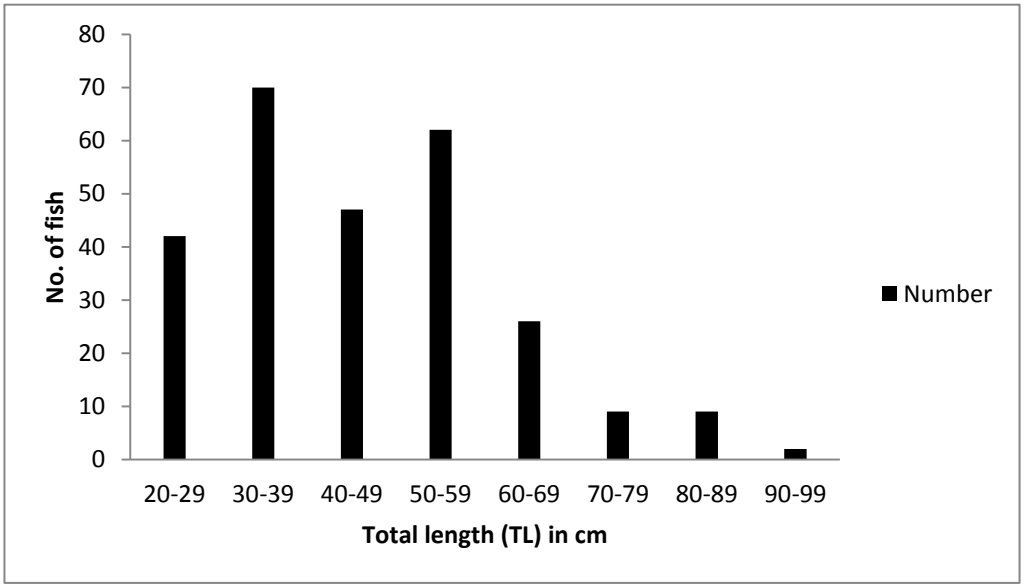

Figure 2.6. Graph showing captured dusky grouper sizes for multiple surveys conducted between April 2014 and Jan 2015

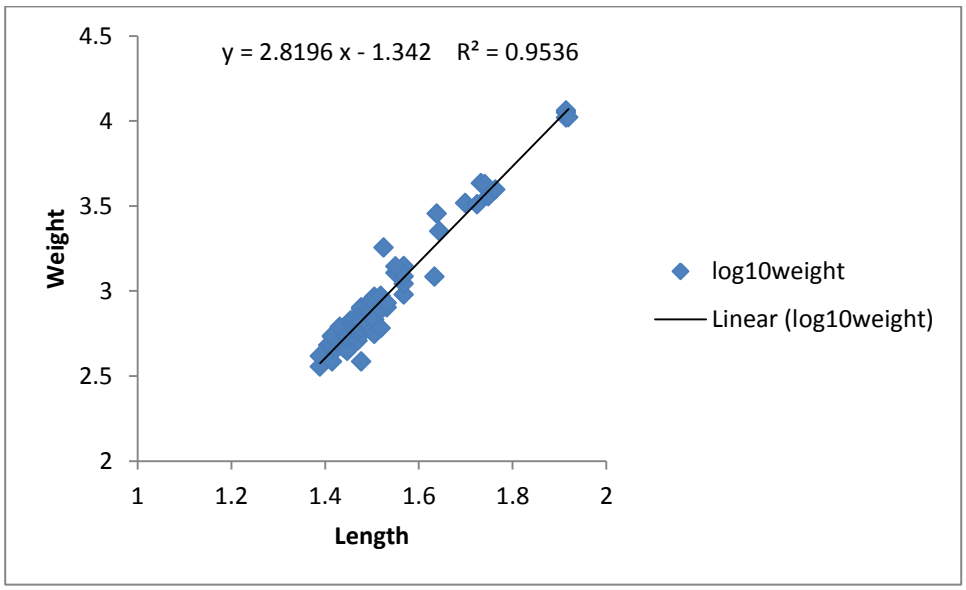

Figure 2.7. Linear regression graph showing the positive correlation between the length and the waight of dusky grouper captured and offered at a local fish market (Tripoli) April 2014

2.4.3 Age determination using otoliths

It was possible to assess the annual rings (**Fig.2.8**) from 8 fish ranging in size between 28 -59 cm TL. All fish assessed were females with ovarian maturation stages of: juvenile, F2 and F6. Ages ranged between 3 years and 8/9 years (**Table. 2.4**). No male or transient individuals were among the assessed otoliths.

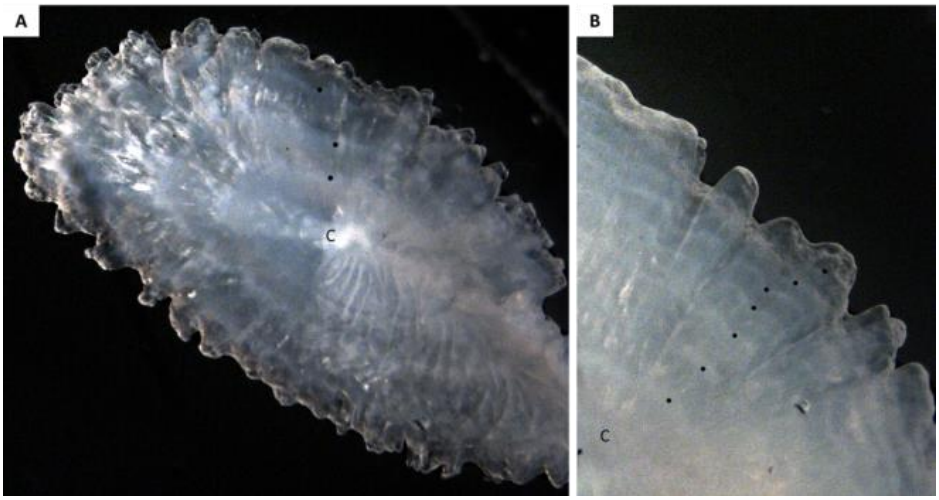

Figure 2.8. The sagittal otoliths of two dusky grouper, (**A**) 3 year old dusky grouper with visibly detectable white annual rings (points). (**B**) The sagittal otolith of a 7 year old dusky grouper with

visible annual rings (points) becoming harder to resolve at the margins of the otolith. c = centre of otolith

Table 2.4. Readings of 8 dusky grouper otolith rings sampled Octoebr 2013 to November 2014

Fish size cm TL/g	Approximate age	Sexual maturity	Sampling month
28/560	3	Juvenile	Nov
27/440	3	Juvenile	Nov
34/590	4	Juvenile	Oct
40/880	4	F6	Oct
41/1250	8	F1	Oct
52/2400	7	F6	Nov
56/3425	9	F6	Nov
59/*no*	6	F2	June

2.4.4 Maturation and spawning season

In general, the gonads of the dusky grouper observed varied in size relative to the size of fish and season of the year, with ripe ovaries being best represented among the sampled ovaries. The 55 sampled gonads came from females sized 27-68 cm TL (of which juvenile females were 27-35.5 cm TL), males sized 64-92 cm TL and transient state dusky grouper sized 58-68 cm TL (**Table 2.5 & 2.6**).

The ovaries often contained islands of testicular tissue at the borders of the lamella, and only in one fish was it intermingled among the oocytes. Eosinophilic granular cells (EGC's) are frequently seen in the connective tissue surrounding the gonads of both male and adult females, but more frequently in female adults. Some degranulating EGC's were seen in resting (F1) and early transitional (T1) gonads. It was not always possible to define stages transitioning to the next.

Table 2.5. Various stages in dusky grouper development and numbers of fish sampled and sizes. F1-resting, F2-developing, F3-maturing, F4-mature, F5-partially running, F6-spent; T1-early transitional, T2-bisexual, T3-late transitional; M1-maturing, M2-mature and M3-running ripe testes. (*) only the gonads were sampled

Stage	Size cm TL/No. fish
Juvenile	27-35.5 / 13
F1	40-44 / 4
F2	39-59 / 3
F3	56*-65 / 4
F4	36-65 / 3
F5	40*-61 / 3
F6	41-68 / 10
T1	*None*
T2	58-66* / 4
T3	65-68 / 2
M1	64/ 1
M2	57-92*/ 6
M3	63-72*/ 2

2.4.4.1 Spawning period

Vitellogenesis in maturing ovaries was first observed in grouper females sampled in May (**Fig. 2.11**). These fish showed yolked oocytes (**Fig.2.12 & 2.13**) and a partially spawned ovary, characterised by the presence of pre-vitellogenic and vitellogenic follicles. Running ovaries (F5), were seen from July to September (**Fig. 2.14**) and spent ovaries (F6) were seen in October (**Fig. 2.15**, **Table. 2.6**). During this study period ovaries with hydrated oocytes were not sampled. Juvenile Females (**Fig. 2.9**) were seen all year round (**Table. 2.6**).

Males at various stages of development were seen from May to October (**Figures 2.18,2.19, 2.20 & 2.21**). Ripe males (**Fig. 2.21**) were seen in May, while transient males, at the early and late stages (**Fig. 2.16 & 2.17**) were seen January, October and November, with almost all fish sampled in October being transient, male, or spent and resting females (**Fig.2.10**) (**Table. 2.6**). All ovaries sampled in October were post-spawning with signs of atresia and necrotic changes to the ovaries (**Fig. 2.26 & 2.28**).

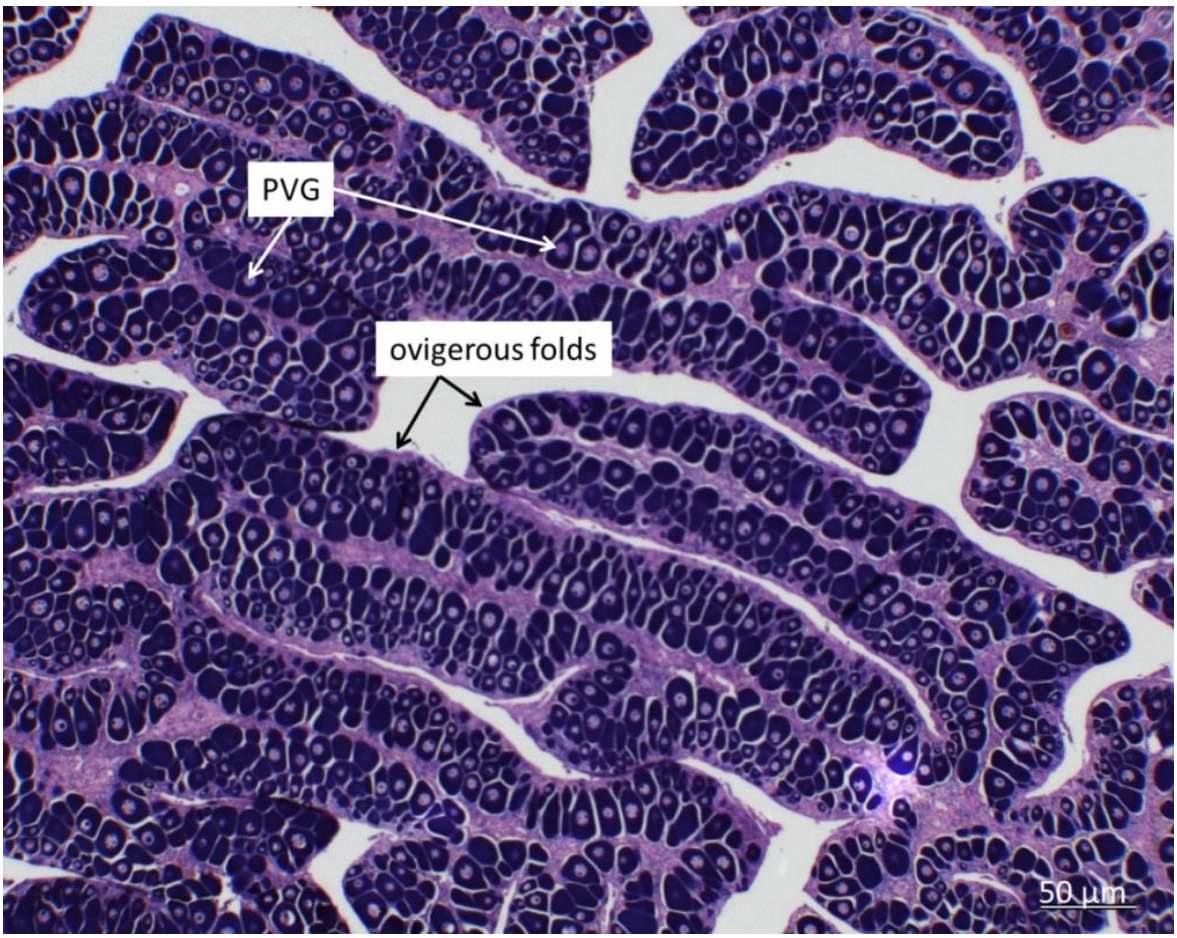

Figure 2.9. Juvenile: Ovary of a juvenile female dusky grouper, showing the ovarian lamella with predominantly pre-vitellogenic ovarian follicles (POF) and closely packed ovigerous folds. (H&E)

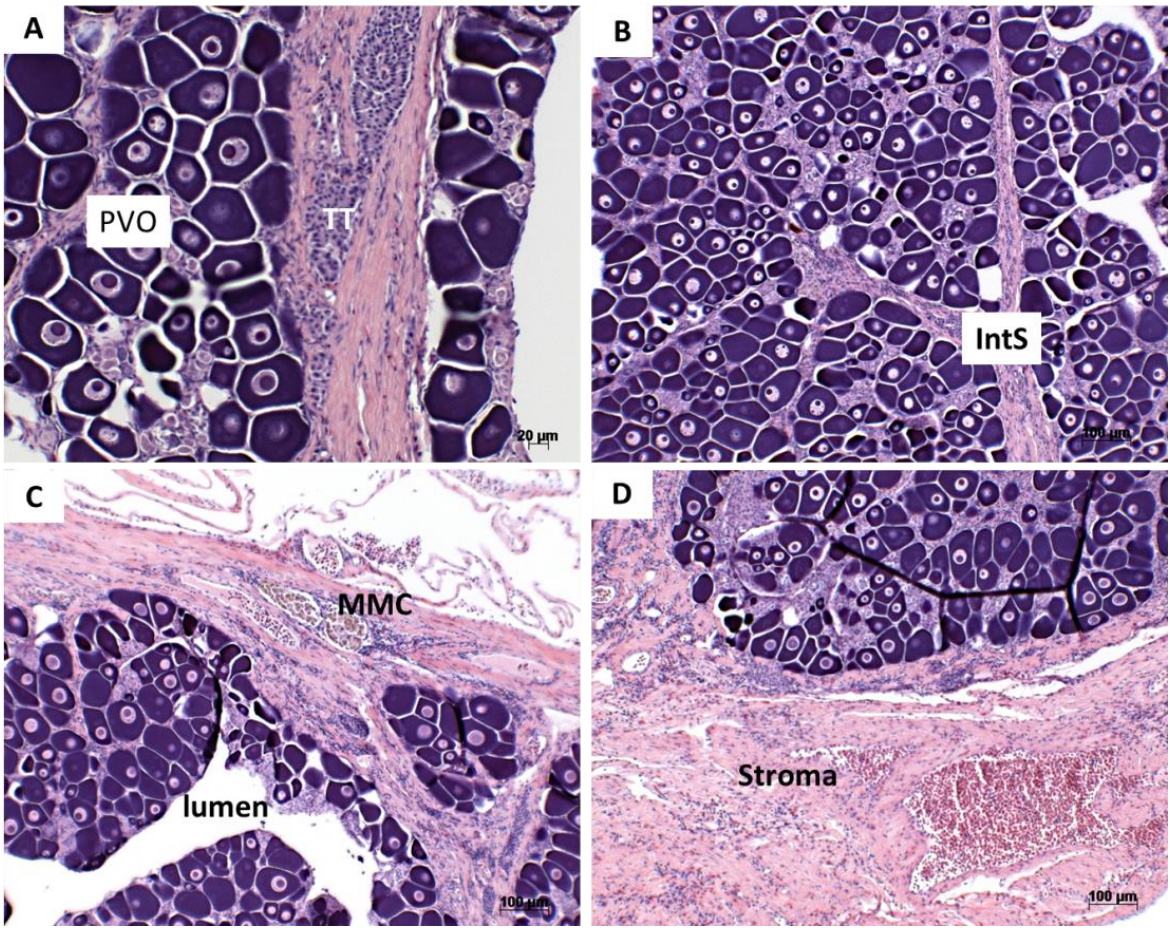

Figure 2.10. Ovary sampled from a female (58cm/3105 g) October 2014 classified as resting mature inactive female (F1). (**A**) With predominantly pre-vitellogenic oocytes (PVO), with scattered clusters of testicular tissue (TT) and (**B**) intra-lamellar stromal strands (IntS). (**C**) Melanomacrophage centre (MMC), and (D) thick stroma. (H&E)

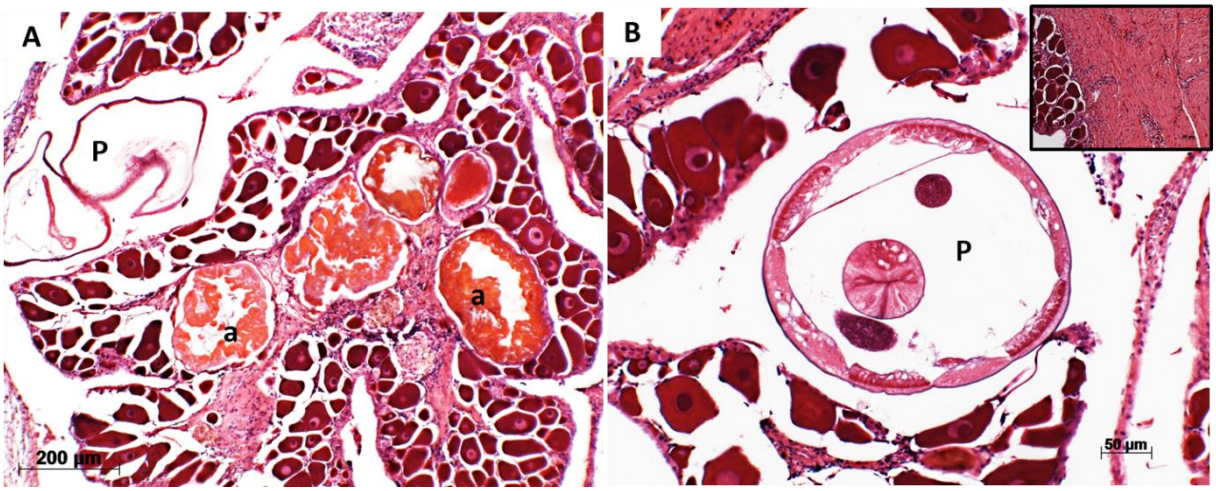

Figure 2.11. Ovary of a dusky grouper collected May 2014 at (F2) stage. **(A)** Atretic bodies can be seen (a) in cross section, and the majority of follicles are in the pre-vitellogenic and lipid vesicle stage. (**B)** Cross section of adult gravid *Philometra* (P), within ovarian tissue. The ovary has a thick stroma (insert). *Abbreviations*: P=adult gravid Philometra. (H&E)

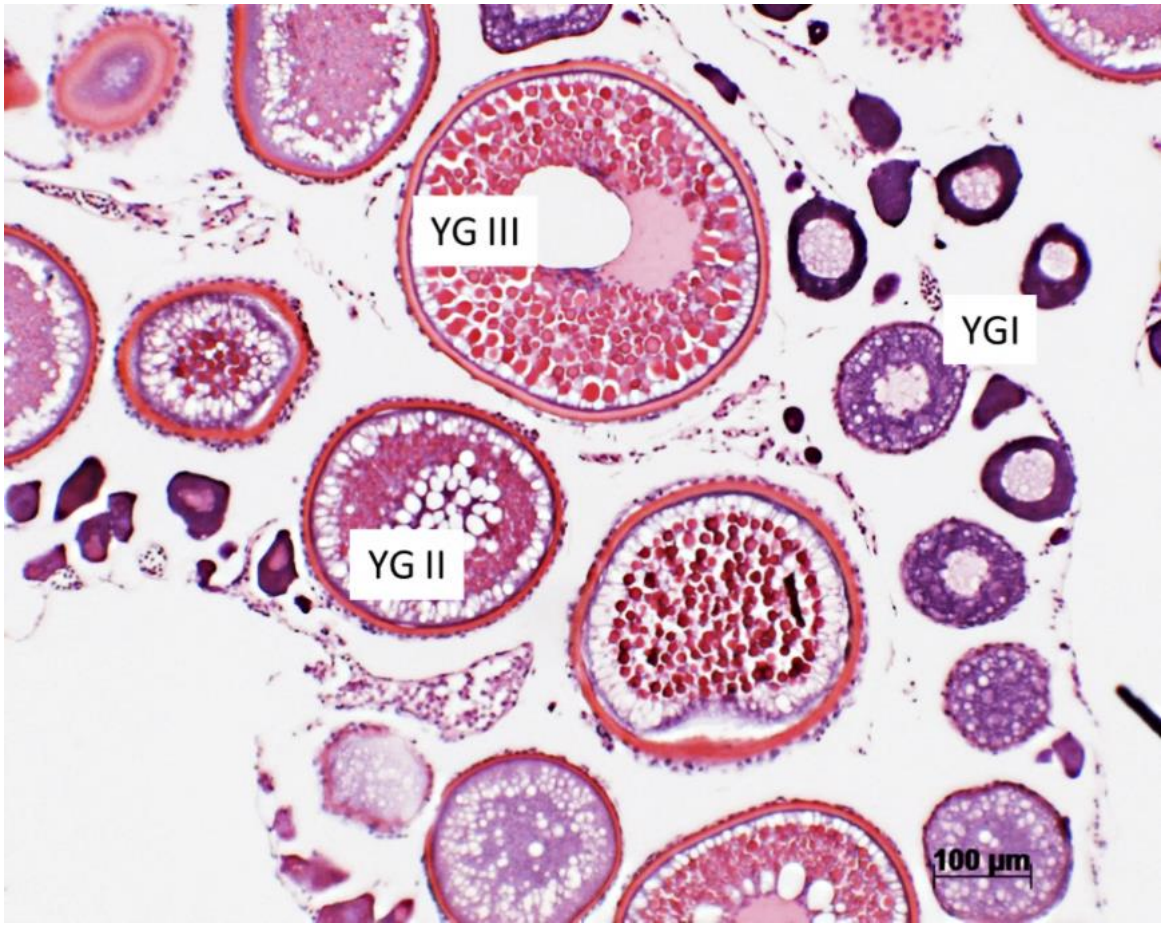

Figure 2.12. Maturing ovary from a dusky grouper sampled in late April (F3), with vitellogenic follicles (yolk granule (YG) stages I, II, and III). No hydrated oocytes are seen or any post-ovulatory follicles. *Abbreviations*: YGI=Yolk granule stage I; YG II=Yolk granule stage II; YGIII=Yolk granule stage III. (H&E)

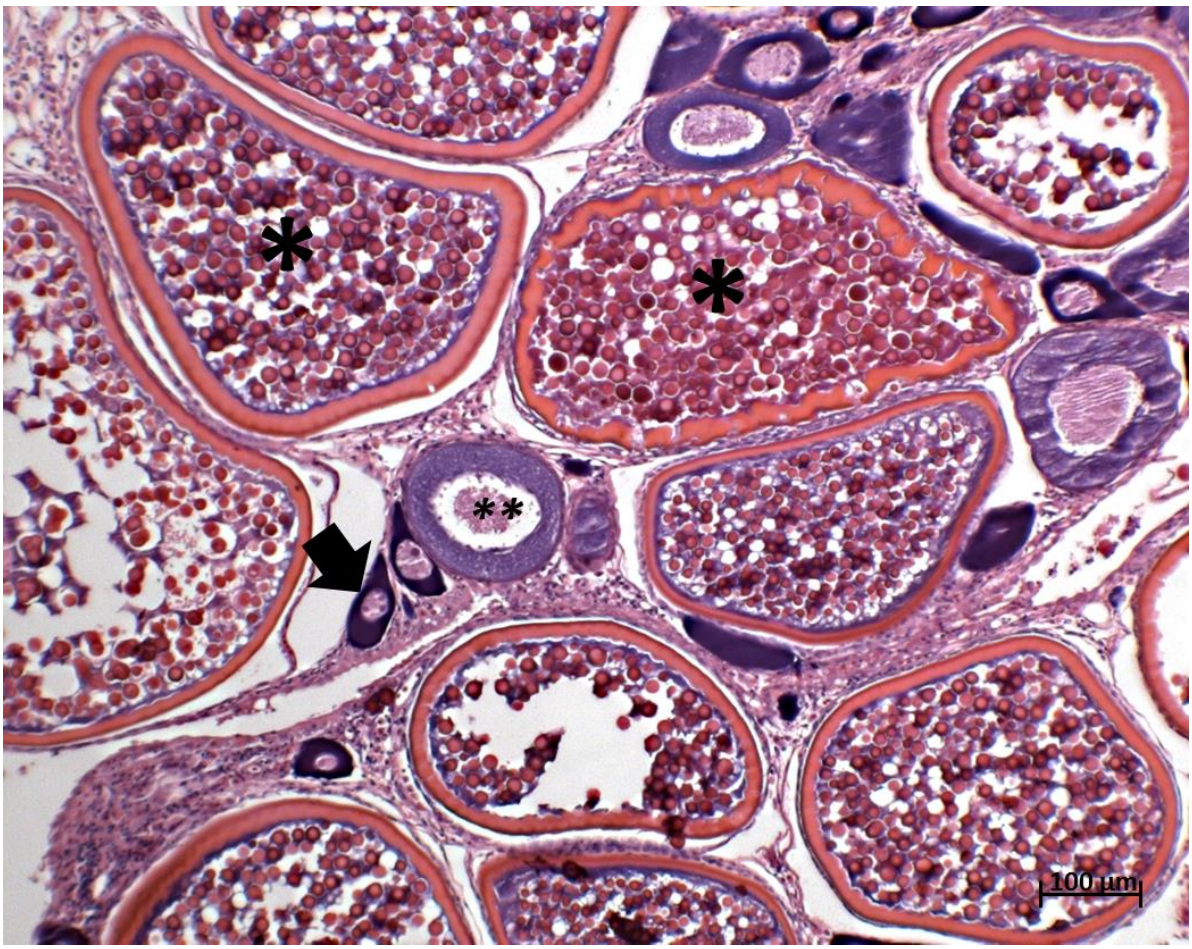

Figure 2.13. Section taken from the ovary of a dusky grouper showing late maturing ovary (F4), with a predominance of ovarian follicles filled with yolk granules (*), vitellogenic follicles (**) and few pre-vitellogenic ovarian follicles (thick back arrow). (H&E)

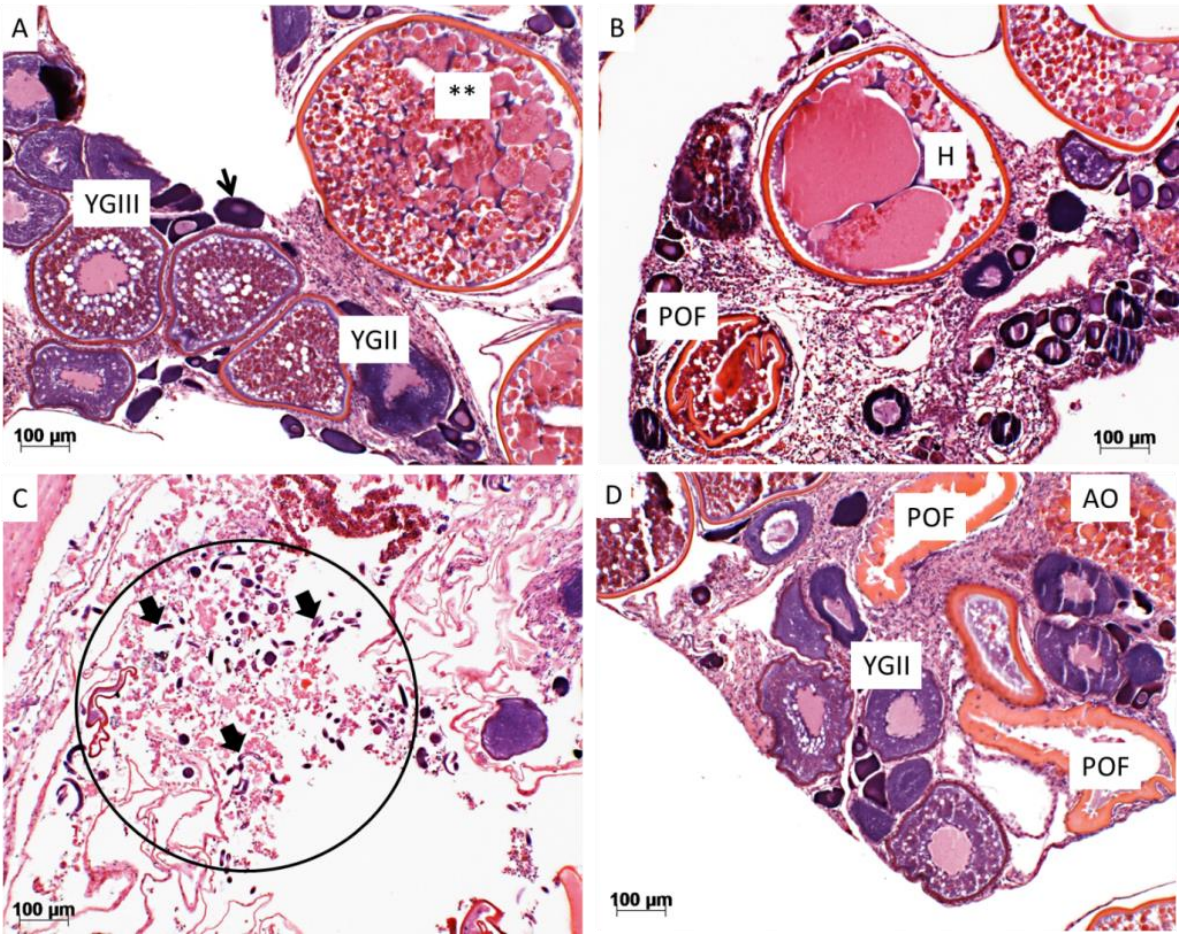

Figure 2.14. Partially running ovary (F5) sampled from a female dusky grouper indicated by (**A**) groups of vitellogenic oocytes at different developmental stages with follicles undergoing hyalinisation (**), and (**B**) the presence of oocytes with fused yolk mass (H). (**C**) Note the presence of ruptured *Philometra* sp. and the release of larvae (arrow) at various stages of development (circle). (**D**) A few atretic vitellogenic oocytes (AO) can be seen, vitellogenic oocytes (yolk granule (YG) stage II) and post-ovulatory follicles (POF). *Abbreviations*: AO=Atretic vitellogenic oocytes; H=Fused yolk mass; MMC=Melanomacrophage centre; POF=Post-ovulatory follicles; YGII=Yolk granule stage II; YGIII=Yolk granule stage III. (H&E)

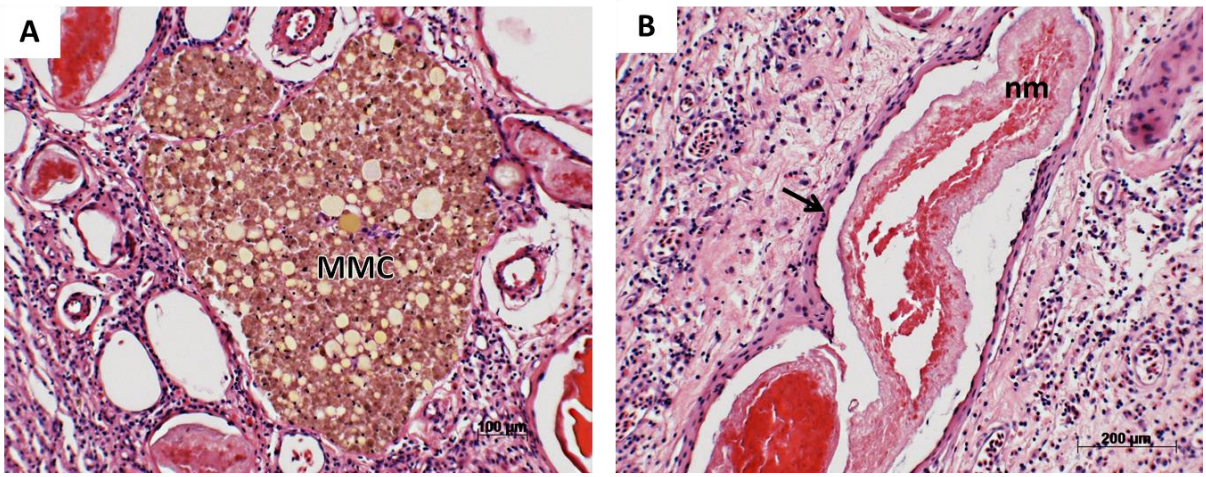

Figure 2.15. Spent ovary (F6) (**A**) showing numerous melanomacrophage centres (MMC), and (**B**) thick stroma, and necrotic material (nm) surrounded by connective tissue (black arrow). *Abbreviations*: MMC= Melanomacrophage centre. (H&E)

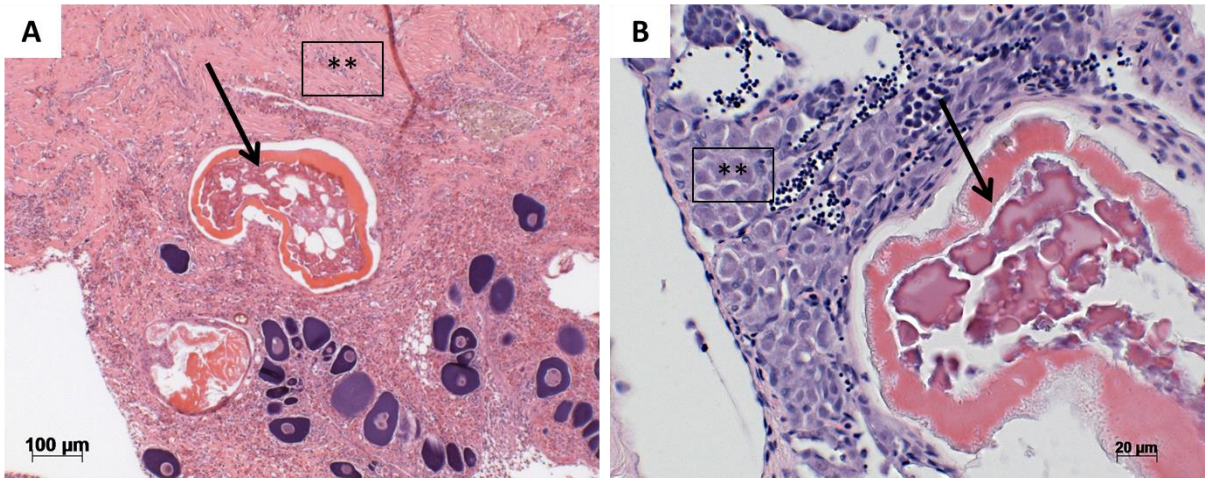

Figure 2.16. The gonads of a dusky grouper at the bisexual stage (T2) (**A**) atretic follicles can be seen (arrowed) and remaining pre-vitellogenic oocytes within a thick stroma (**). (**B**) Cysts of spermatogonia (**) and atresia (arrow). (H&E)

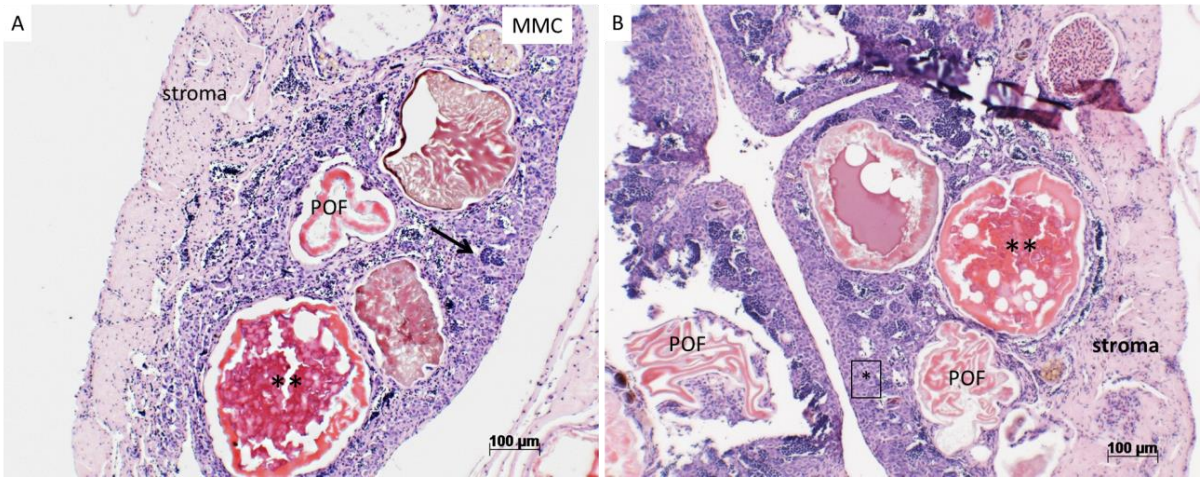

Figure 2.17. Gonads of a dusky grouper at the late transitional stage (T3) characterised by (**A & B**) a few scattered post-ovulatory follicles (POF) of the residual ovarian tissue (**) and the presence of MMC as well as spermatozoa (arrow) and spermatogenic cysts with cells at different stages of development. *Abbreviations*: POF=Post-ovulatory follicles; MMC=melanomacrophage centre. (H&E)

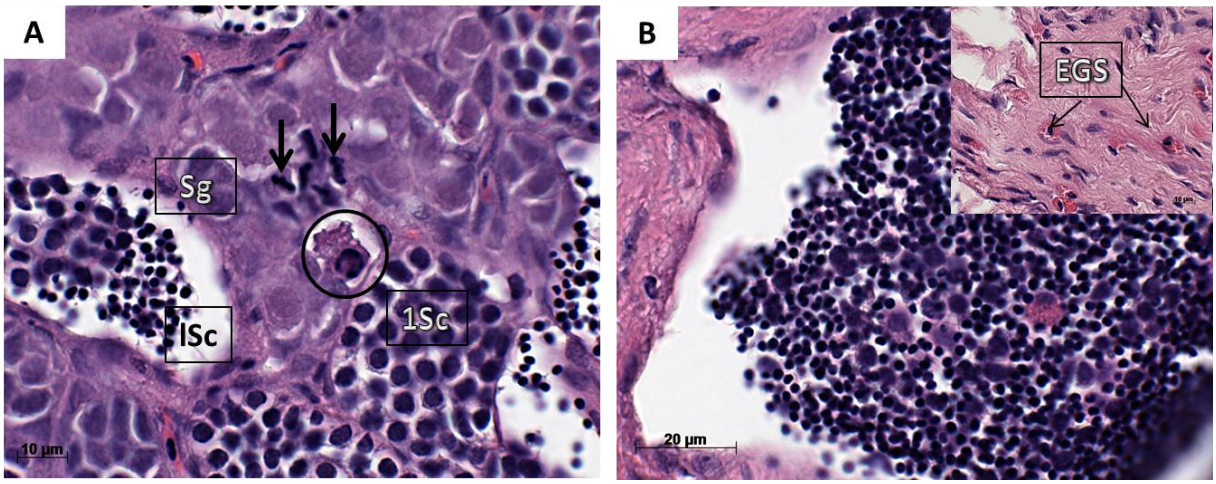

Figure 2.18. Section of the testis of a dusky grouper sampled May 2014 (64 cm/3000 g) and classified as maturing male (M1). (A) The presence of primary spermatocytes (1Sc) and spermatogonia (Sg) and late spermatids (lSt). Mitosis (arrows) and necrosis (circle) can be seen affecting spermatogonia (B) Presence of spermatozoa in spermatic sinuses, and degranulation of eosinophilic cells (ECG) (insert) in the connective tissue at the margins of testicular loops. *Abbreviations*: EGC=eosinophilic granular; 1Sc=primary spermatocytes; lSc=late spermatids; Sg=spermatogonia. (H&E)

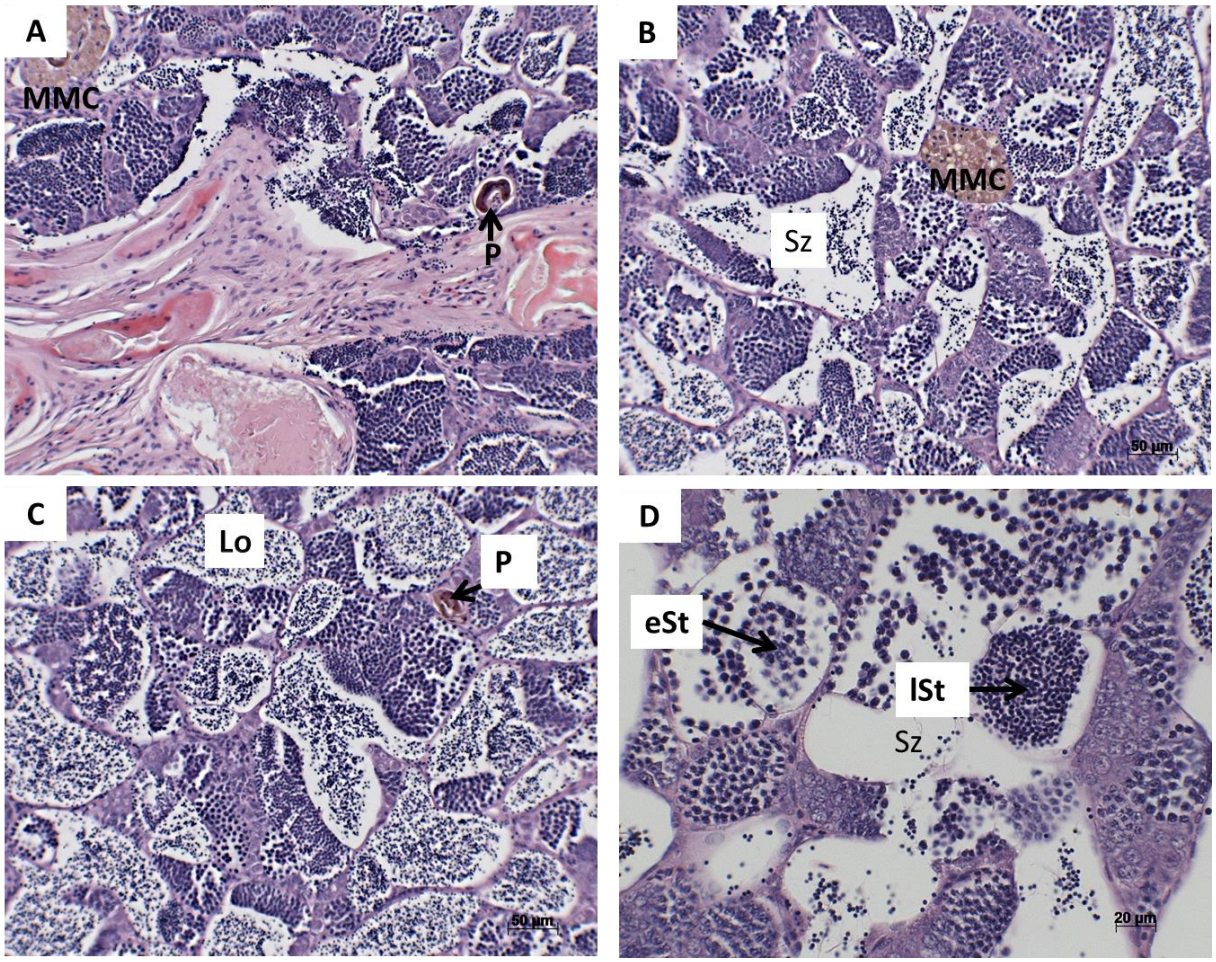

Figure 2.19. Early maturing (M1) testis of dusky grouper size 64 cm TL, sampled May 2014 (**A&B**) the centre of testis containing connective tissue and necrotic tissue surrounded by connective tissue and basophilic inflammatory cells. As well as Melanomacrophage centres (MMC) which can be seen and a parasite larva (P). (**C**) The testicular lobules (Lo) contain several stages of spermatozoa development but no sperm within lobules (**D**) early spermatids and late spermatids. *Abbreviations*: Lo=testicular lobules; MMC=melanomacrophage centres; P=parasite; Sz=spermatozoa; It=interstitial tissue; eSt=early spermatids; lSt=late spermatids. (H&E)

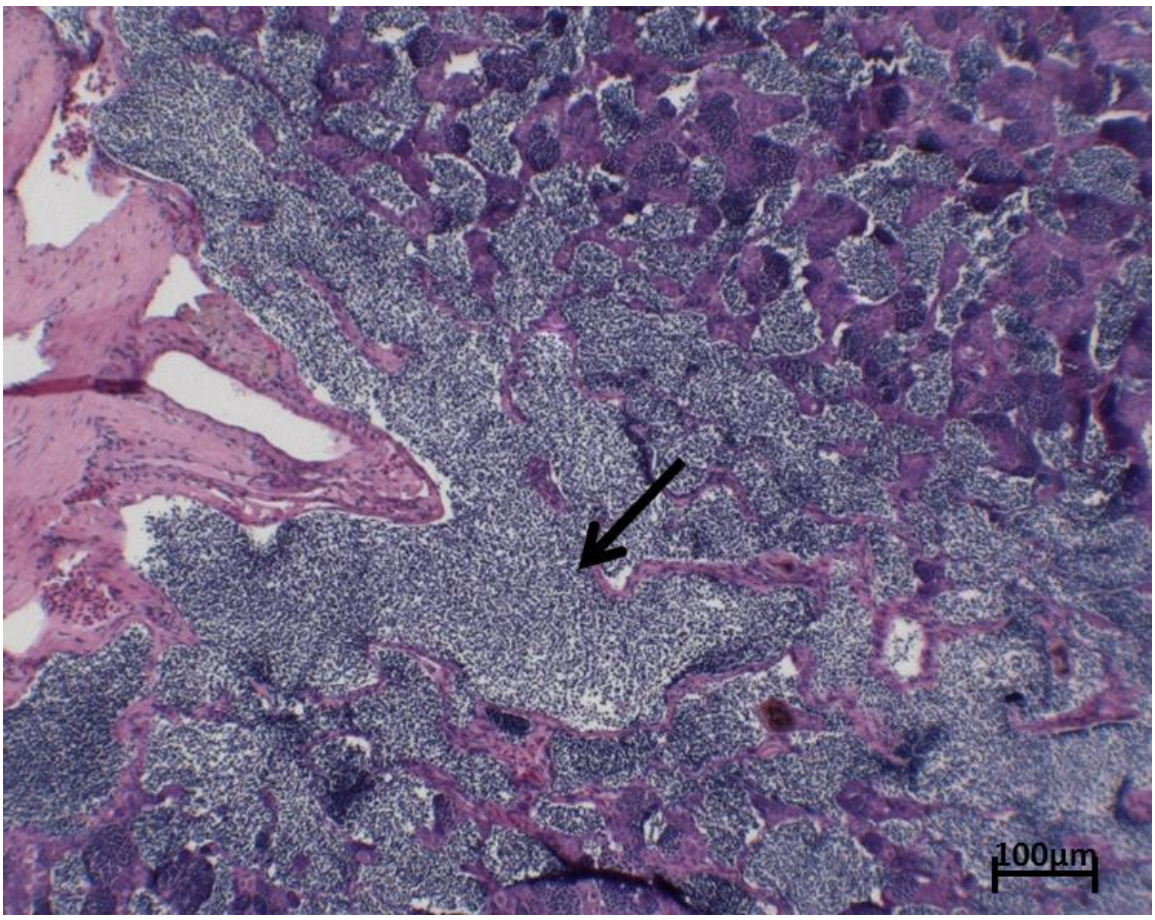

Figure 2.21. A ripe testis (M3), sampled from a dusky grouper in May showing free sperm as the predominant element of the sectioned tissue with numerous free spermatozoa in the large lumen of the lobules and in the spermatic sinuses (black arrow). (H&E)

2.4.5 Survey of the health status of sampled dusky grouper

2.4.5.1 Parasites

All fish sampled were seen to be infected with at least one parasite. An annotated list with the inspected organs, prevalence and infecting parasite identity is given in **Table. 2.7.**

Monogenean spp. showed 100% prevalence, followed by didymozoans affecting the gills (86%). *Philometra sp.* infecting the ovaries showed 52% prevalence while in the testis it is 22.2 %. Given the small size of the philometrid male and the infective larval stages and their intimate relationship to the tissue, it is possible that these stages may have been missed and thus the estimated prevalence may be an underestimate.

Table 2.7. The incidence, prevalence (%) and site of infection for sampled 50 female and male dusky grouper infected by a range of parasites, in addition to (*) 5 gonads separately sampled.

Parasite	Organ	Incidence	Prevalence %
Nematoda			
Philometridae			
Philometra sp. Suspected *Philometra inexpectata Philometra jordanoi*	Ovaries*	21/40	52.5
	Testis*	2/9	22.5
	Transitional*	3/6	50
	Eye socket	30/50	60
Trematoda			
Didymozoidae	Gill filaments	43/50	86
	Skin (dermis)	3/50	6
Trypanorhyncha (larval stages)	Head kidney	30/50	60
Monogenea sp.			
Pseudorhabdosynochus sp.	Gills	50/50	100
Protozoa			
Trichodina sp.	Gills	9/50	18
Zschokkella sp.	Bile duct	5/50	10
Myxozoan			
Myxobolus.	MMC kidney	6/50	12
Myxozoa sp.	MMC ovary	3/40	7.6
	Gallbladder	7/50	14
Crustacea			
Gnathia	Gills	3/50	6
	Skin	1/50	0.2
	Oral cavity	43/50	86
Not identifdied isopod	Tail	1/50	2
Copepoda		2/50	
Hatschekia	Gill filaments		4
Unknown aetiology			
Black calcified material	Dermis	36/50	72

2.4.5.1.1 The ovaries

2.4.5.1.1.1 Philometra infection of ovaries

Nematodes belonging to the genus *Philometra* were observed to infect ovaries with a prevalence of 68.9%. *Philometra* were identified using compound light microscopy and

scanning electron microscopy following a description by Moravec (2006). Male philometrids were minute, measured a few mm and were translucent to whitish. Worm identification was based on description of the caudal end, spicule and gubernaculum for which, transverse lamellae could be seen. By contrast the long (10- 30 cm) filiform gravid and sub-gravid females, had relatively short oesophagi. Females were characterised by their pink / red colour resulting from their haematophagous feeding habit (feeding on the host blood). RBC's were readily seen when ruptured females were observed using light microscope.

Philometra was found to infect the gonads of dusky grouper all year round. From the fifty-five gonads inspected for *Philometra* infection, 26 were found to be infected by *Philometra*: from which 21 ovaries (52.5%), 2 testes (22.5%) and 3 transient gonads (50%) were infected (**Table. 2.7**), giving an overall prevalence of 47.2%.

Ovaries sampled in March-May often contained small foci of necrotic tissue in sections of the ovarian lumen. These foci were surrounded by apparently normal ovarian tissue. No gonads of transient, male or juvenile fish were found to contain adult female *Philometra*, while adult male *Philometra* were present in the ovaries of adult females, males and transient fish. Juvenile female *Philometra* were found in the ovaries from April-June and live adult female *Philometra* were found in ovaries during the months of June, July and August. One dusky grouper sampled from the fish market had a parasite protruding from the genital opening. Upon further examination the ovary of this fish was heavily infected by *Philometra sp.* (**Fig. 2.22**).

Mixed parasitic infections of gravid ovaries of female dusky grouper were often observed, with trypanorynch larval cysts on the external surface of the ovary, and infection with adult

Philometra females grossly observable (**Fig. 2.23**). Adult female parasites were only observed in ovarian stages F2 to F5, (maturing to spawning ovaries), these being visible from the red coiled appearance of gravid adult nematodes, often seen between the ovarian folds and apparently filled with RBC's. In one ovary it was possible to count 10 adult *Philometra* females. Adult parasites were often fragile and well-embedded in tissue and were therefore easily ruptured when sampled (**Fig. 2.24**).

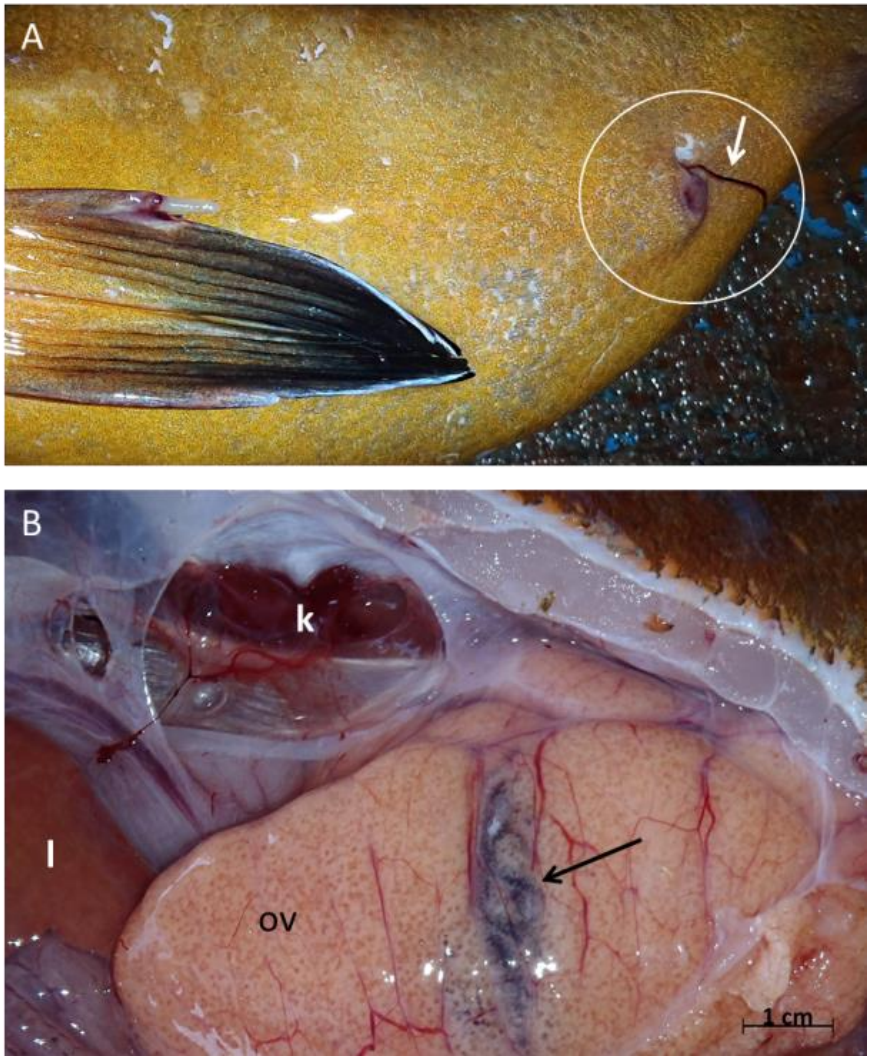

Figure 2.22. Parasitic infection of female dusky grouper during the spawning season, (**A**) a *Philometra sp.* (arrow) can be seen protruding from the genital opening (circle). (**B**) Same fish showing the infected ovary containing the parasite within (arrow). *Abbreviations*: k=kidney; l=liver; ov=ovary

Adult male *Philometra* were also found infecting ovaries and the largest number collected was 12 individual males from a single spawning ovary. Developing dusky grouper ovaries harboured adult males and sub adult *Philometra* females, containing eggs which had not yet developed embryos. These were found in the ovaries during the months April-May. Few testes were infected by *Philometra* sp. parasites, but in few there were 1-5 adult males. Adult females were never seen infecting the testis.

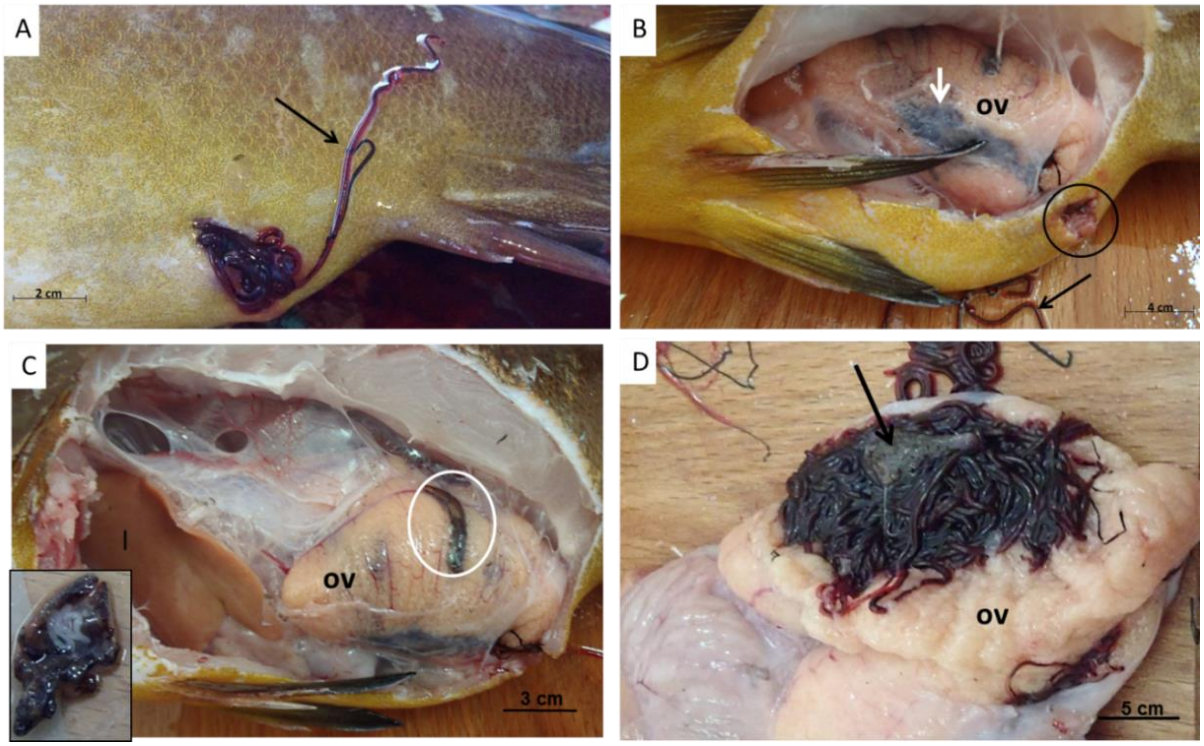

Figure 2.23. Mixed parasitic infection of gravid ovaries of a female dusky grouper infected with a *Philometra sp.* (**A**) Adult gravid female (arrow) seen protruding from the enlarged cloacal opening possibly due to traumatic injury. (**B**) The cloacal injury (circle) can be seen and the encapsulated *Philometra* sp. within the ovary (ov) (white arrow), a *Philometra* sp. worm can be seen on the dissection board (black arrow) (**C**) attached to the distal surface of the ovary (ov) a Trypanorynch larval cyst (circle) (insert), with a grossly normal looking liver (l). (**D**) Upon opening the ovary (ov) a mass of 10 gravid *Philometra* sp. females were causing localized necrosis to ovarian tissue seen as grey coloured tissue (arrow). *Abbreviations*: l=liver; ov= ovary

The ovaries of immature (juvenile) dusky grouper were found infected only by larval, immature and male stages of *Philometra sp.,* which did not appear to cause pathological change to the ovaries. The ovaries of the juvenile female dusky grouper were never found to harbour adult female *Philometra* sp.

Philometra infection had a major impact upon the structure and likely integrity of post spawning ovaries Most ovarian necrosis was observed from fish samples following the spawning season, where dead and decaying *Philometra* sp. were found in the ovaries in September, October and November and severe necrosis was observed from fish ovaries sampled in October and November. Some necrotic ovaries, in fish sizes ranging from 42-66 LT, contained a large mass of dead and decaying *Philometra* sp.. A few live male *Philometra* sp. were seen amongst some of the decaying parasites, but none in ovaries containing calcified necrotic material (**Fig.2.25**).

Based on the information presented, the species of *Philometra* (Table. 2.7) is tentatively proposed as *Philometra inexpectata* (**Fig 2.28 A&B**) and the adult male *Philometra* sp. resembles *Philometra jordanoi (López-Neyra, 1951)* (**Fig 2.28 C&D**), however, further information is needed to support these observations.

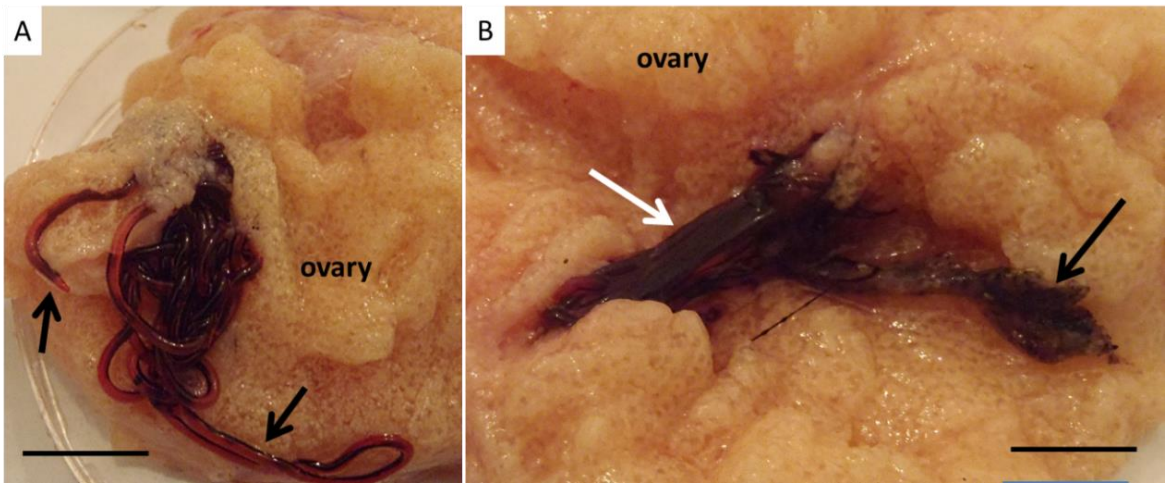

Figure 2.24. Ovary of a dusky grouper showing a female *Philometra sp.* (**A**) live female parasite, reddish in colour (black arrow). (**B**) Upon opening the ovary necrotic material (black arrow) could be seen in proximity to the aggregation of live parasites (white arrow), the parasite showed great fragility it easily ruptured upon manipulation. Scale bar: (A)=approximately 1 cm; (B)=approximately 1.2 cm

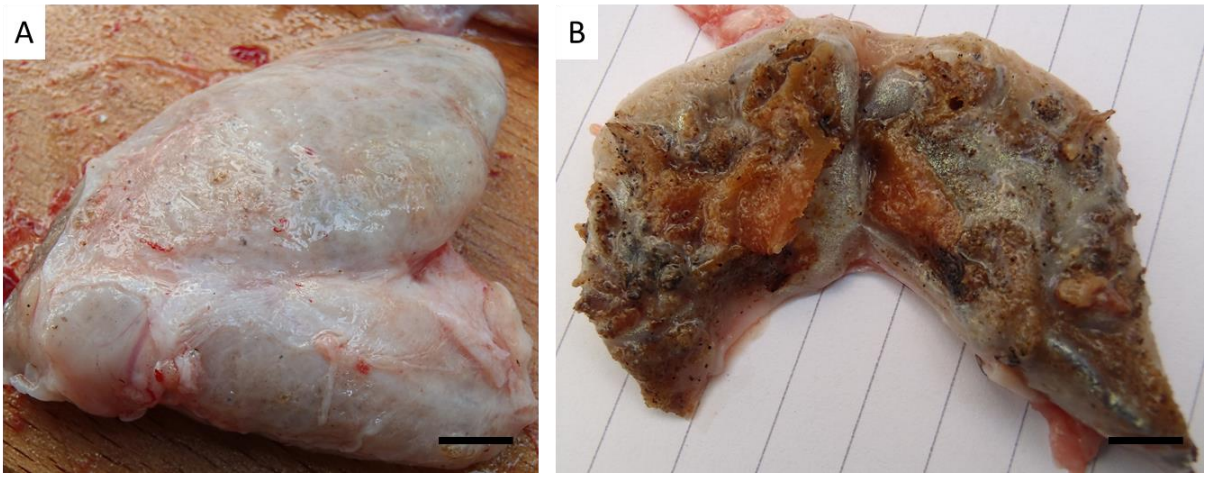

Figure 2.25. Gross morphology of an ovary from a dusky grouper sampled in October (**A & B**). A seemingly intact ovary is seen upon dissection to possess severe necrotic tissue damage. Parasitic castration could be assumed upon observing such lesions. Scale bar: approximately 1 cm

Live adult female *Philometra sp.* appeared to cause minimal necrosis, this being observed only in adjacent ovarian tissue surrounding the adult parasite and causing localised necrosis in the tissue surrounding the parasite (**Fig. 2.23, 2.24 & 2.26**).

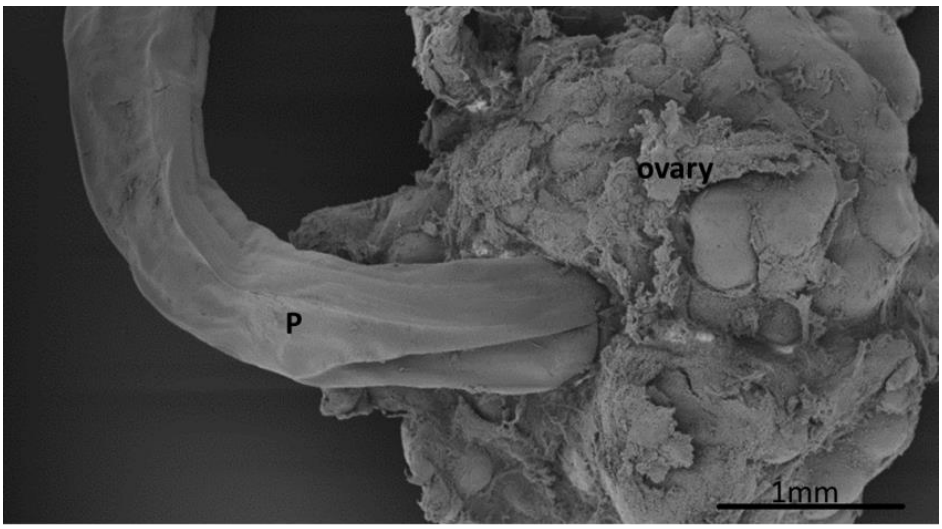

Figure 2.26. A scanning electron micrograph (SEM) of an adult female *Philometra* sp. (P) embedded within the ovary and causing localised necrosis (seen from histological sections). *Abbreviations*: P=adult female *Philometra.*

The ovarian necrosis appeared, in some fish, to lead to the loss of almost half of the ovarian tissue, as seen from the post spawning ovary of a 7 year old dusky grouper size 41 cm TL sampled in October 2013, which was replaced by fibrous material, grossly appearing leather-like (**Fig. 2.27A**).

Due to the state of the ovary it was not possible to identify parasites by gross examination or histology, so the cause of this necrosis could be related to a combination of atresia and *Philometrid* sp. decay. . From scanning electron microscope images, bacterial colonies could be observed in association with female and male *Philometra sp.*, sampled from ripe ovaries of a freshly killed fish. The bacteria were close to the oral opening of the female, and on the cuticle of the male parasite (**Fig. 2.28 B & C**), and the ovum of the host and some ova were also covered by bacterial colonies. Encysted viable trypanorhynch larval stages (**Fig.2.23C**) & (T**able.2.7**) and calcified unknown parasites were seen to be present in ovaries and testes, with ovaries more frequently and more heavily infected than testes. In three fish a

Myxozoan sp. was observed to be present in a MMC. Male *Philometra* ***sp.*** were present in ovaries of dusky grouper all year round (**Fig. 2.23**) (**Table. 2.7**).

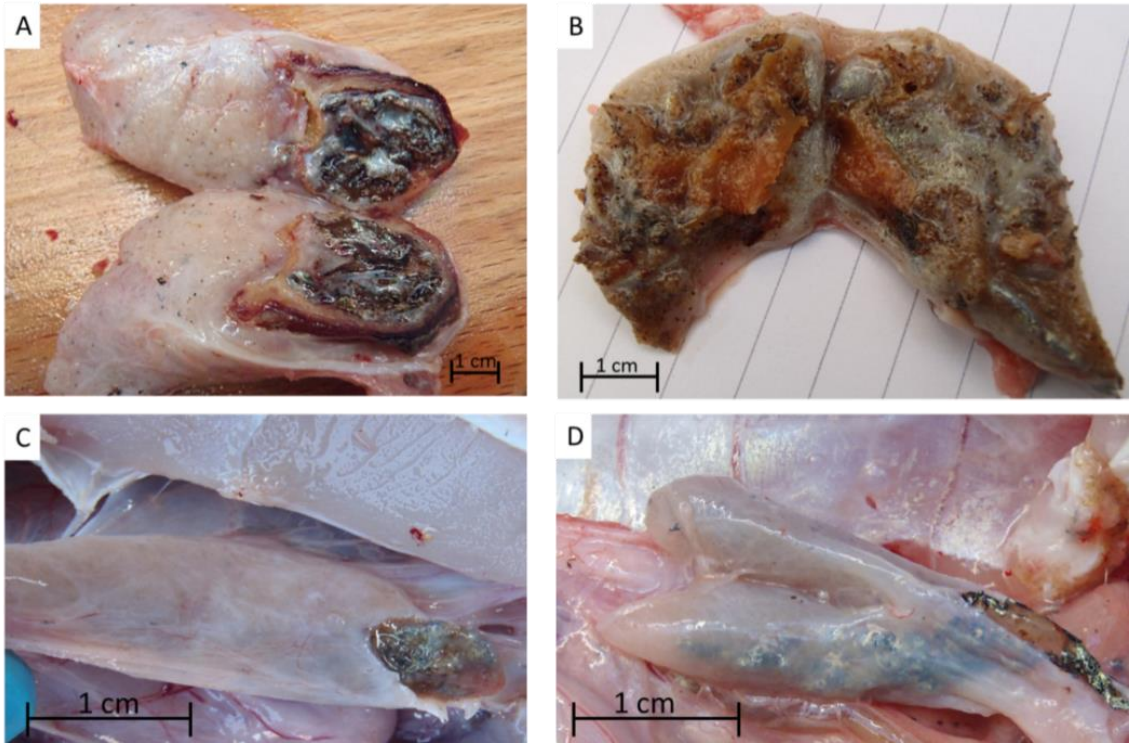

Figure 2.27. Several pathological changes observed to affect mature ovaries as a consequence of post-spawning atresia and possible *Philometra* sp. infection. (**A**) The ovary has almost leathery necrotic changes involving 1/3 of both ovarian lobes (Sampled October 2013). (**B**) The ovary of a post spawning grouper with extensive necrotic changes involving both lobes and extending throughout almost all of the ovarian tissue (Sampled October and November 2014). (**C &D**) necrotic changes to post-spawning ovary containing remnants of necrotic changes associated with decaying parasite (probably *Philometra* sp.) or due to atresia.

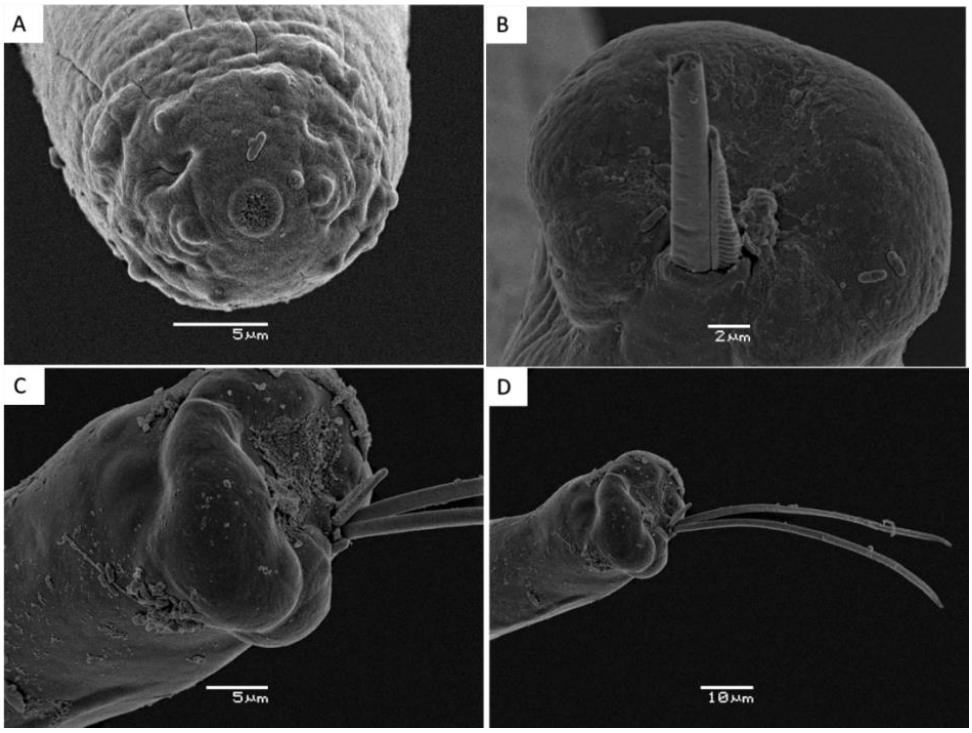

Figure 2.28. Scanning electron micrograph (SEM) of two adult male *Philometra* sp. parasites infecting the ovary of an adult dusky grouper sampled May 2014. (**A**) Anterior (cephalic) end almost apical view resembling a new species described by Moravec *et al.* 2016, as *Philometra inexpectata*. (**B**) Distal end, showing gubernaculum, and broken spikes, with a few bacteria attached on its surface. (**C & D**) Adult male *Philometra* sp. resembling *Philometra jordanoi*, with bacteria attached to its skin, showing gubernaculum and two spikes

Philometrid larvae survived for a minimum of 7 days. For 2/7 days the ovaries were kept in the fridge at 4-10 °C. Following ovarian dissection, the larvae survived for an additional 5 days in aerated seawater at 20-25 °C, vigorously moving in a pulsating motion. After 7 days work had to be terminated given the security situation in Tripoli. This initial work was conducted in order to study the life span and thus the viability of the free-living larvae in an aquatic environment.

2.4.5.1.2 The Skin

2.4.5.1.2.1 Skin lesions

From the 267 fish inspected, 57 fish were seen affected by skin lesions (**Fig. 2.6C**) which could be characterised in terms of a multi focal dermatitis. Further gross, histological and epidemiological descriptions of these lesions, as well as a possible aetiology, are provided Chapters 3, 4 and 5.

2.4.5.1.2.2 Didymozoid trematodes

In addition to fish bearing the above mentioned skin lesions, three of the fifty five dusky grouper sampled, while showing no evidence for external skin lesions, were nevertheless infected by didymozoid trematodes. These were not visible from the intact skin, but could be detected upon skin incision. Parasites were visible embedded within the dermis. Parasites were visible macroscopically as large coiled yellow filamentous worms, exceeding 20 cm in length and being frail and easy to break. Histologically the parasites seemed to cause no gross lesions in the host, with the host showing minimal tissue reaction to the live parasite (**Fig. 2.29& 2.30**).

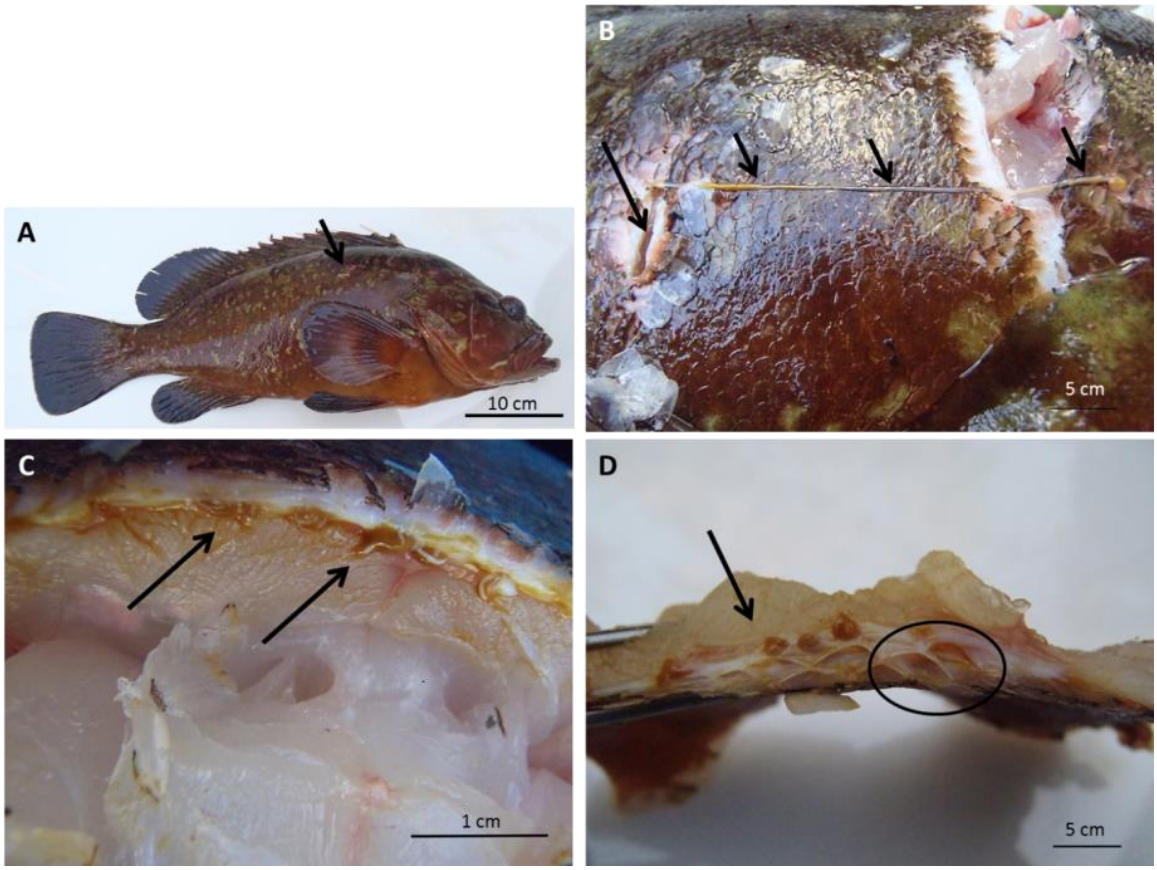

Figure 2.29. The skin of dusky grouper infected by didymozoid trematodes (**A**) adult dusky grouper sampled from local fish market showing wound induced b spear fishing (arrow). (**B**) From the wound (long arrow) a didymozoid trematode protruded, which was visible to the naked eye (3 short arrows). (**C**) A longitudinal cross-section revealing the yellow-coloured parasite subcutaneously (two long arrows) which upon rupture stained the surrounding flesh yellow. (**D**) Located dermally (circle) just above the underlying muscle (long arrow). Parasite is located in the dermis (circle).

Intact parasites were found to be filled with thousands of yellow, roundish, shelled eggs, ranging in size between 17.41-18.37 µm long and 9.7-10.6 µm wide and having a shell thickness ranging between 1.3-1.5 µm. All visible eggs were embryonated. In addition, it was also frequently observed (36 fish), that skin sections showed what appeared to be branch like streaks of black material, probably comprising calcified parasites. Affected fish sizes were 28-68 cm TL. These were only observed after dissection of the skin from the underlying muscle (**Fig. 2.31**).

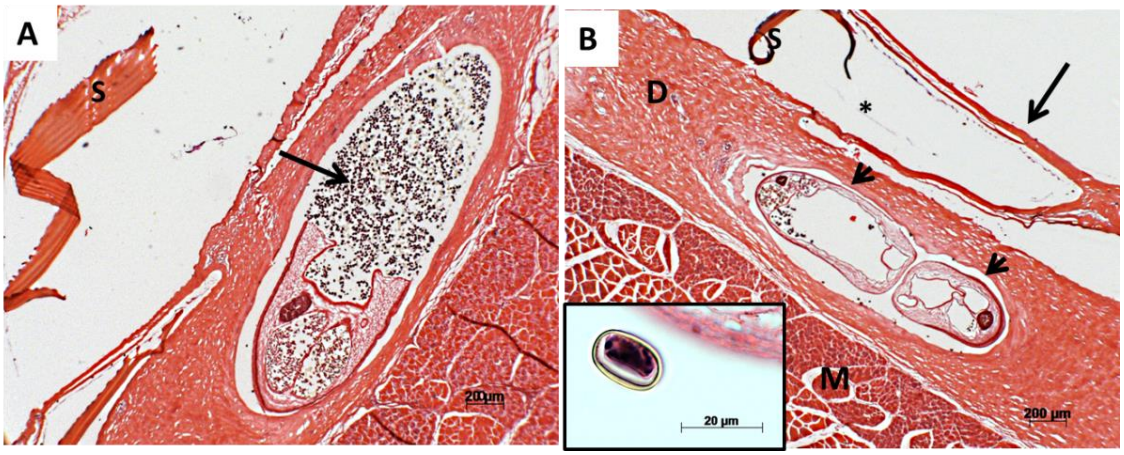

Figure 2.30. Histological sections of didymozoid trematodes (A) found within the dermis and filled with eggs (long arrow), (B) didymozoid trematodes in tissue showing lack of involvement of the dermis, often eggs (insert) are translocated during histological processing and sectioning (arrow head). *Abbreviations*: D=dermis; M=Muscle; S=scale; (*)=scale pocket (H&E)

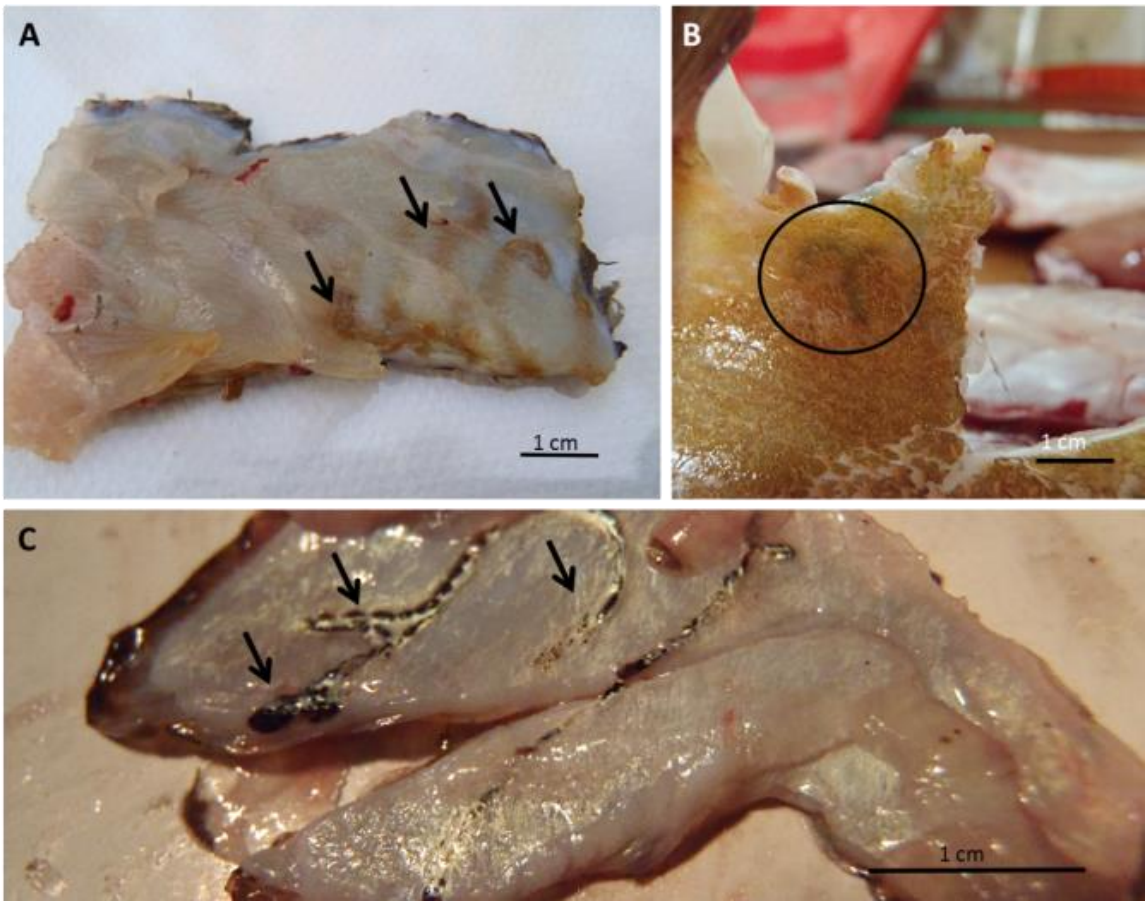

Figure 2.31. Abdominal skin of dusky grouper showing (**A**) yellow parasite embedded subcutaneously. (**B**) The abdominal skin of a dusky grouper showing a dark streak on the external surface of the skin when held in the light (circle), which when seen from the inner skin surface

comprises black calcified material (arrow), its form resembling the didymozoan parasite described earlier in (A)

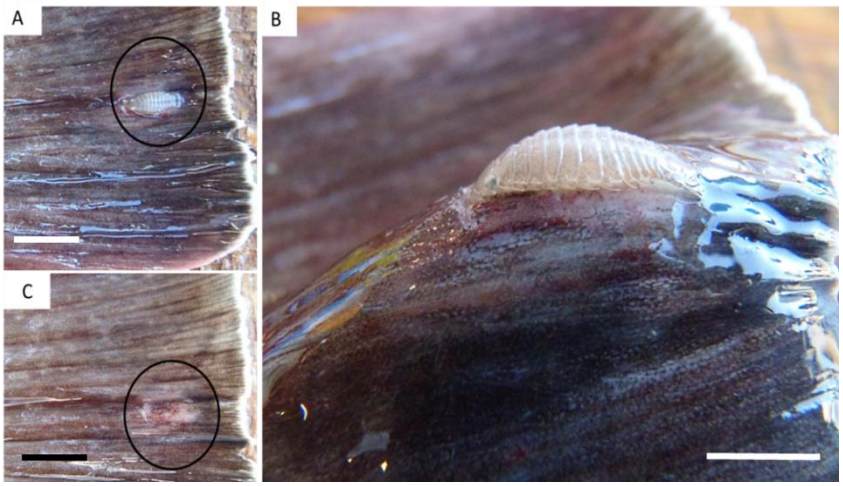

Figure 2.32. Isopod parasite found attached to the caudal fin of a dusky grouper, (**A** (circle) **&B**) showing attached isopod (**C**) evidence that the isopod causes localized erosion at point of feeding and attachment (circle). Scale bar: (A, C)=20 mm; (B) = 10 cm

2.4.5.1.2.3 Crustacean parasites

A single dusky grouper had a crustacean isopod attached to its caudal fin. The isopod appeared to cause mild erosion at site of attachment and feeding (**Fig. 2.32**).

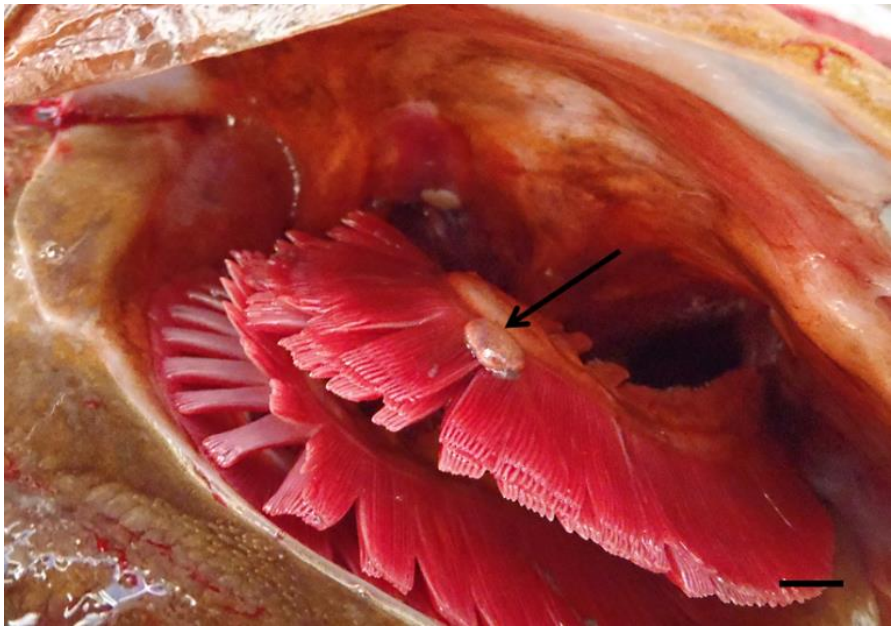

Figure 2.33. Didymozoid cysts attached to the gill filaments (Black arrow). Scale bar=10 mm

2.4.5.1.3 Gills and operculi

Gills were frequently affected by yellow and creamy-coloured cysts. These cysts contained gravid didymozoid digenetic trematodes with eggs. One or more cysts could often be seen affecting filaments on gill arches and pseudobranchs (**Fig. 2.33**). Histologically the cyst was seen to cause localised inflammation, characterised by an increased granular eosinophilic infiltrate surrounding the affected gill filaments. All fish were infected by at least one monogenean. Crustacean parasites were also found to cause localised inflammatory responses at the point of attachment, with hatschekiid copepods and gnathiid isopods both being observed to infect gills. Hatschekiids were observed to affect 2/50 sampled dusky grouper giving a 4% prevalence of infection.

Trichodina sp., were observed to infect gills with a prevalence of 18% of sampled individuals (**Table. 2.7**).

2.4.5.1.4 Oral cavity nostrils

Gnathiid praniza larvae (Crustacea, Isopoda) (**Table.2.7**), were frequently observed to infect the oral cavity of dusky grouper, with host sizes ranging from 28-92cm TL and an infection prevalence of 86% (**Table. 2.7**) as well as infecting the skin and nares (**Fig. 2.34**).

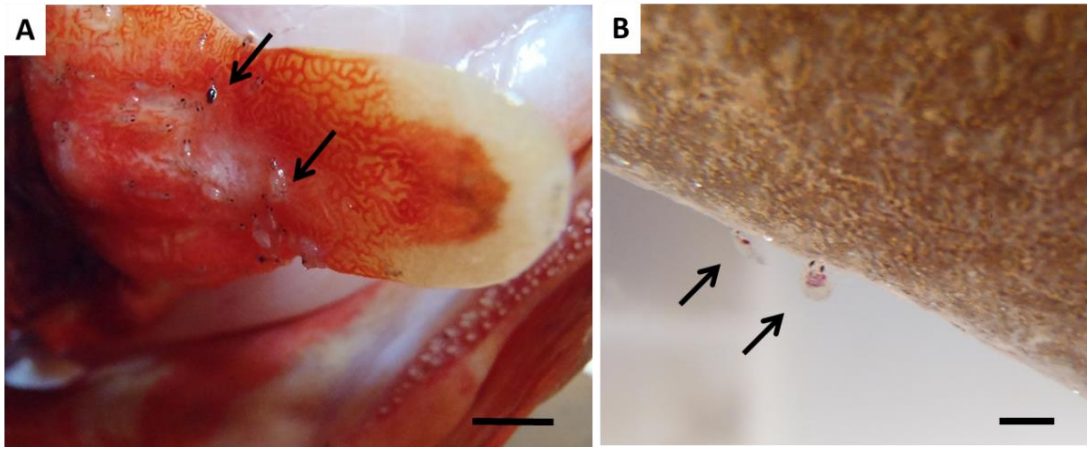

Figure 2.34. Picture showing praniza larvae of gnathiid isopods, (**A**) found attached to the dorsal aspect of the tongue (arrow) and (**B**) rarely attached to the skin (arrow). Scale bar: (A)=4 mm; (B)=2mm

2.4.5.1.5 Abdominal cavity

The abdominal cavity was often seen to be affected by calcified encysted parasites representing trypanorhynch cestode larvae (Cestoda, Trypanorhyncha). A group of trypanorhynch larvae were often seen in what might be a preferred site, just above the stomach roof (also the first area likely to be encountered following exit from the stomach), but they mostly seemed to infect the head kidney, having 60% prevalence overall (**Table. 2.7**). The maximum number of cysts observed in the abdominal cavity of a female caught July 2013, was 64.

2.4.5.1.6 Gall bladder

Although gall bladders exhibited no visible external signs of pathology, gall bladders from 7/50 fish contained myxozoan parasites (**Table.2.6**) resembling *Ceratomyxa* sp. (**Fig. 2.35**). Myxozoans in fresh or histological sections were identified according to the descriptions given by Dyková & Lom (2007) as a *Ceratomyxa* sp.

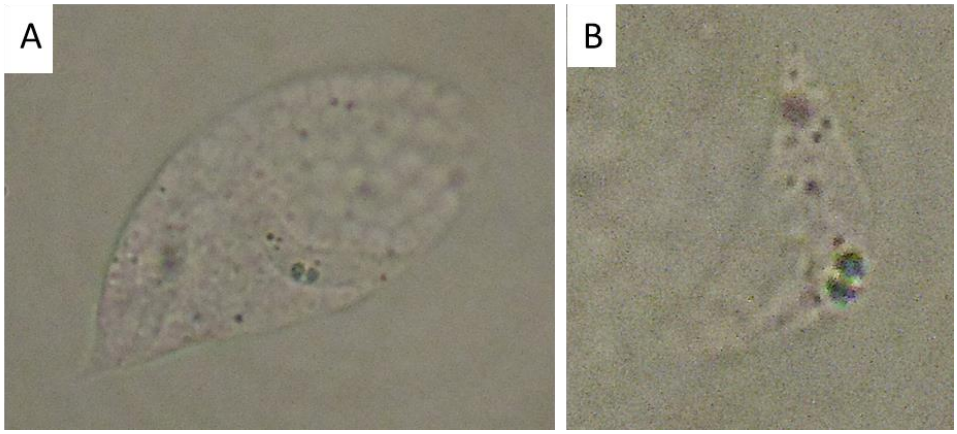

Figure 2.35. Wet mount from the gallbladder from an apparently normal dusky grouper infected by *Ceratomyxa* sp. (**A**) disporoblasts and (**B**) *Ceratomyxa* sp. spore (Image scale uncalibrated)

2.4.5.1.7 Eye and orbit

No parasites were observed in the lens, anterior or posterior chambers or retina of the eye. Between one and four gravid female philometrid nematodes, based on the diagnosis of *Philometra* (see Moravec 2006), were uni-laterally or bilaterally present and occasionally calcified. These were frequently observed in the superficial tissues of the underlying eye socket at a 60% prevalence of infection.

2.4.5.1.8 Liver

The liver was often found to be affected by multiple calcified parasites, some of which were surrounded by basophilic inflammatory infiltrate, and MMC's in the vicinity. The bile duct was often found to be infected by the myxozoan parasite *Zschokkella* sp., and occasionally the surrounding liver tissue was seen to show necrosis and a mild predominantly basophilic Inflammatory infiltrate. Fatty liver was rarely seen, and only observed in one fish, with liver cells showing rounded vesicles in the cytoplasm, and markedly shrunken nuclei.

2.4.5.1.9 Spleen

Enlarged spleens were often observed, especially in fish that were affected by moderate and severe skin lesions (**see Chapter 4**.), and these were interpreted to reflect inflammatory responses to infection.

2.4.5.1.10 Heart

Localised and diffuse myocarditis was seen in most hearts of sampled grouper, with few MMC's.

2.4.5.1.11 Kidney

The kidney was often seen to contain parasite cysts, but only one fish had grossly visible superficial cysts (**Fig. 2.38**). Histologically, it was not possible to identify the pathogen causing the cyst due to the advanced calcified state of the cysts, which took up a large portion of the tail kidney. MMC's were often seen in the kidney parenchyma and bacteria and myxozoa were often seen to be present (**Fig. 2.36**). The renal tubules contained hyaline eosinophilic droplets in a single apparently normal dusky grouper sampled in January 2015 (**Fig. 2.37**).

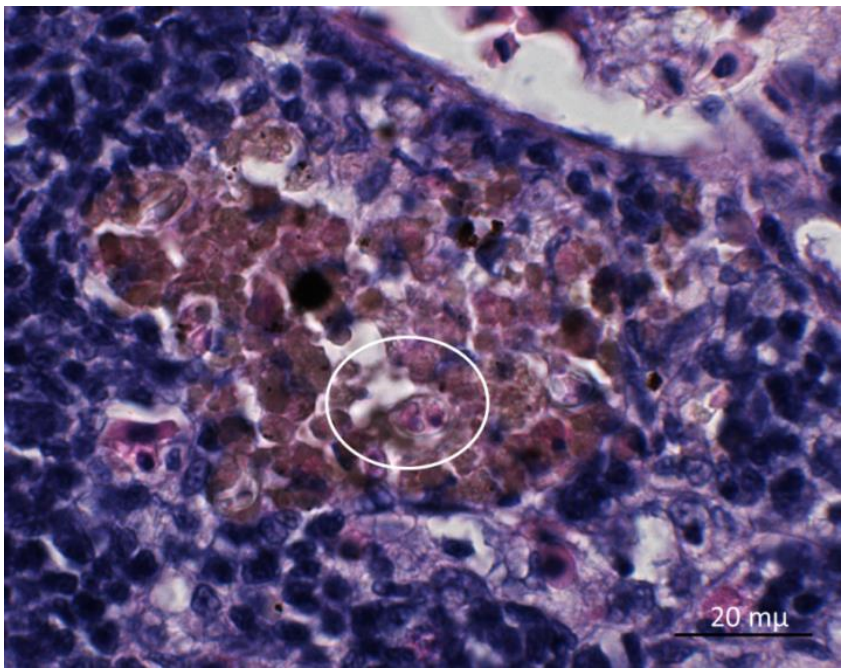

Figure 2.36. Myxozoan parasite (white circled) located within an MMC in the kidney of a dusky grouper. (H&E)

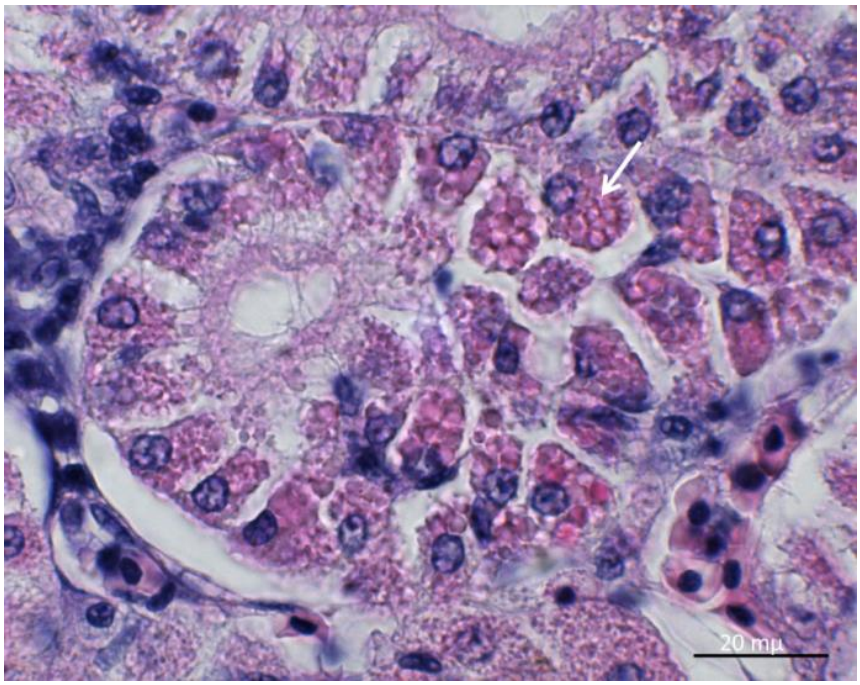

Figure 2.37. Eosinophilic hyaline droplets (white arrow) were found within renal tubules of an apparently normal dusky grouper sampled in January 2015. (H&E)

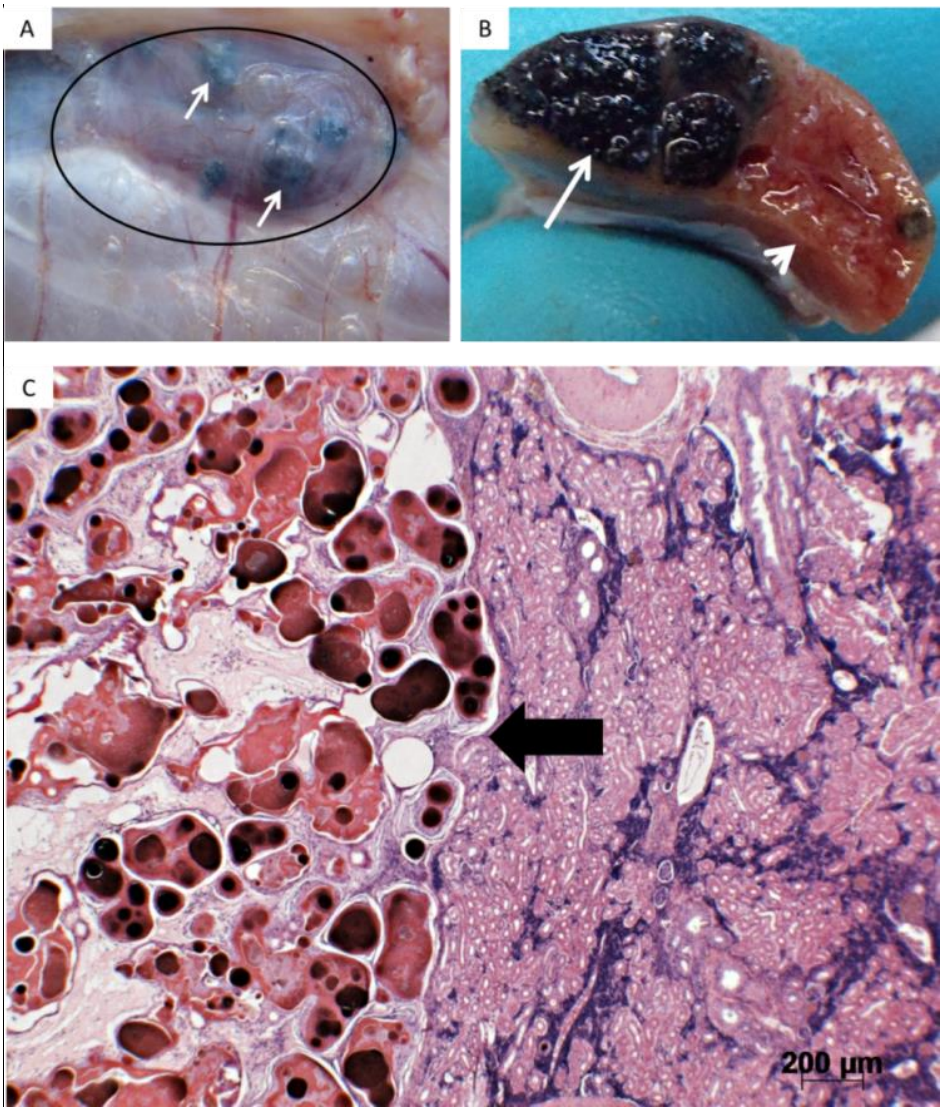

Figure 2.38. (**A**) Tail kidney (circle) of a dusky grouper seen with black cysts (white arrow). (**B**) Gross lesion of tail kidney showing normal tissue (white arrow head) of a dusky grouper seen with black cysts (white arrow) and involving more than half the kidney when observed in cross-section. (**C**) The cysts can be seen taking up a large portion of the kidney tissue (black arrow). Scale bar=1cm .(H&E)

2.4.5.2 Physical anomalies

Abnormal body morphologies were observed in three fish, which could either result from healed injuries or congenital or environmentally induced malformations (**Fig. 2.39**). The fish were adult females sizes ranged from 45-60 cm TL, and other then the morphological anomalies, showed no clinical signs. A single juvenile fish presented with a malformed operculum, which could also be a result of earlier injury (**Fig. 2.40**).

The spleen of one fish was in the form of two attached lobes, with no evidence of this affecting the fish, which was an adult of 54 cm TL. The cloaca and genital openings of most fish were a few mm apart, while in one single fish they were several cm apart (**Fig. 2.41**).

A single fish sold as dusky grouper (**Fig. 2.42**) had a morphology that did not resemble that of normal dusky grouper and not described for grouper present in the Mediterranean Sea. It is not clear if this represented a new species, the consequences of cross-breeding or a congenital or environmentally induced malformation.

Figure 2.39. Abnormal body morphology due either to injury or congenital malformation and involving the dorsal fin (white arrow)

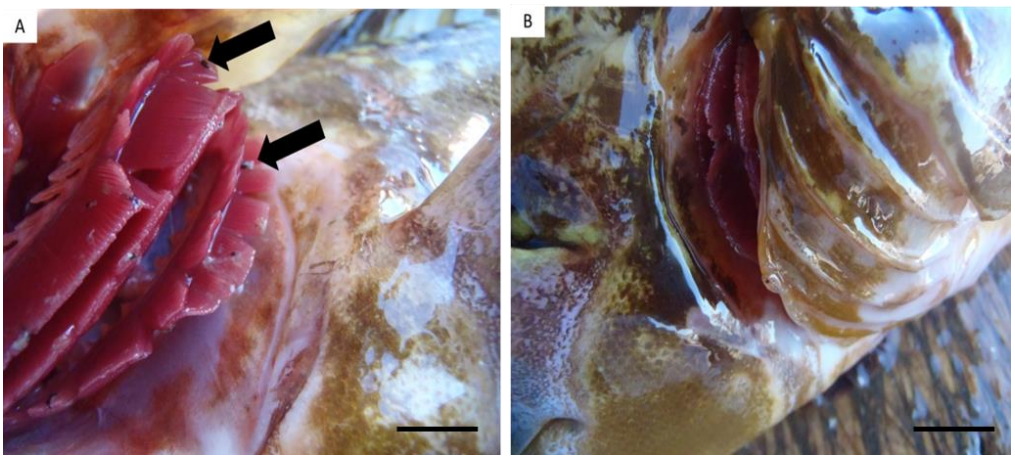

Figure 2.40. (A&B) Isopod parasites (arrowed) are infecting the gills of a fish showing a malformed or injured and healed operculum of a juvenile dusky grouper. Scale bar: (A)=1 cm; (B)=3 cm

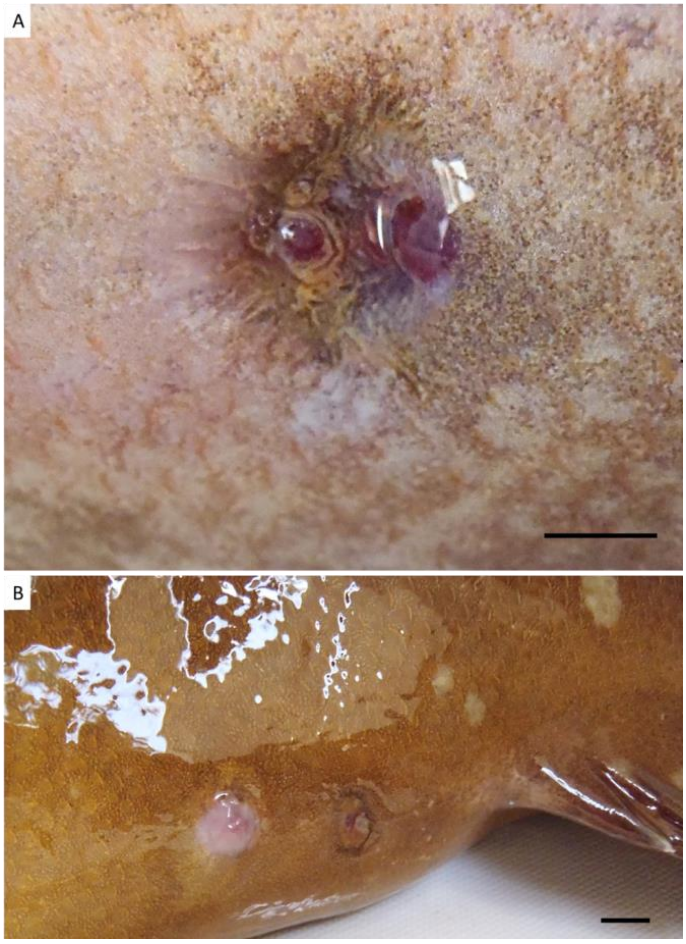

Figure 2.41. Anomaly of cloacal opening in dusky grouper. (A) Normal cloacal openings comprising urogenital papilla and anus only a few mm apart. (B) A variant morphology showing well-defined anal and genital openings, several cm apart. Scale bar (A) =1cm; (B)=2cm

Figure 2.42. It is not clear if this fish specimen, frequently observed to be offered as dusky grouper, is in fact a malformation or a result of cross-breeding. Note humped nape, pointed snout and the indentation between the dorsal aspect and sharp, almost 90 degree inflection, to form the caudal peduncle

2.5 Discussion

Grouper are being fished to extinction worldwide (Sadovy de Mitcheson, 2013). The present study shows that in Libya there is evidence of uncontrolled fishing, with no minimum size limit being adhered to and no restrictions concerning seasonal capture, including the spawning season. This is evidenced by the fact that dusky grouper are available all year, with sizes available at the fish market ranging from 20-92 cm TL, of which undersized fish ranging from 20-29 cm TL comprise 16% of the total offered for sale. This is despite the national minimum dusky grouper size regulation being 30 cm TL and there being a closed fishing season between June and July (Reynolds *et al.* 1995). The daily demand for dusky grouper no doubt contributes to the grouper being offered, which in turn leads to increased fishing activities exploiting grouper of all sizes. The current political disruption in Libya may also contribute to this state of affairs, with protection of grouper no doubt assuming a lower priority against other demands for intervention.

Some grouper have been fished almost to extinction in some parts of the world *e.g.* the critically endangered Goliath grouper *Epinephelus itajara* (Lichtenstein, 1822) (IUCN 2016). Only following drastic measures such as a harvest moratorium in Florida in 1990 (Porch *et al.* 2006) and more than 20 intervening years, have indications of population recovery in this species been observed, accompanied by the inevitable calls for the lifting of fishing bans and the reopening of recreational fishing activities (Shideler *et al.* 2015).

This fishing practice might, if continued, have an adverse effect on fish recruitment, affecting spawning potential and causing stock depletion for populations, which will place further pressure on the already endangered dusky grouper (IUCN, 2004) in the region. Scuba

spearfishing is being extensively practiced in Libya (Lamboeuf 2000) and might perhaps be subject in the future to the requirement for spear fishermen to acquire a license and bag limits, as has been already implemented in some countries e.g. France (Pawson *et al*. 2008). With approximately 300 spear fishermen supplying the studied fish market, the introduction of some form of monitoring system would help to control a fishing practice that has been deemed detrimental to coastal fish populations, particularly given its highly selective nature (Coll *et al.* 2004). Thus, implementing a ''spearfishing license'' system and setting up a surveillance programme could help both to protect existing grouper populations and to ensure the presence of a sustainable fishery for future generations of commercial and recreational fishermen.

Currently, the closed months for dusky grouper fishing in Libya are June and July, although from histological assessment of gonadal development in the present study it has been possible to determine that the spawning season in Libya extends from May to September. By October, all studied fish were post-spawning or transitional, while fish captured in May and September were at stage F3. Although these findings derive from a limited number of 55 sampled fish, this still suggests the possibility of extending the closed months to include May-August. The status of fish in September remains to be properly assessed, as the results for that month are based on only one sampled individual.

Similarl to the descriptions given by Marino *et al.* (2001), only in one female was testicular tissue observed to be intermingled with ovarian tissue. In most individuals, testicular tissue is found as islands at the borders of the lamellae. Histological analysis of cross-sections of dusky grouper ovaries showed an asynchronous ovarian development. This seems to agree with the findings of Reñones *et al.* (2010), however, not enough fish were sampled to

compare with the finding of Marino *et al.* (2001), who suggested that the dusky grouper is a group-synchronous fish. The spawning season is assessed by the presence of hydrated oocytes and or post ovulatory follicle (POF), stages F4 and F5 by Reñones *et al.* (2010). In the present study POF were seen from July to early September, however, no hydrated oocytes were seen in the present study.

With largely missing data from September, it is not possible to definitively identify the end of the spawning season. Given that all sampled ovaries in October showed signs of post-spawning atresia, this month can be considered post spawning season. Marino *et al* (2001) observed transitional individuals occurring in May through November. In the present study, gonads undergoing sex change occurred in fish sampled in October, but also in a single fish sampled in January.

Following observation of histological evidence for deterioration of ovarian tissue in the same timeframe as development of testicular tissue, Marino *et al.* (2001) proposed a sequential protogynous (female to male) nature for the dusky grouper sex change. These observations of histological transition were also mirrored in the current study. While there is no established seasonality for transition of females to males, in the current study transitional males were captured only in October and January, corresponding to the hypothesised post-spawning period.

Reñones *et al.* (2010) observed the greatest numbers of transient males to occur in the same month, with transitional fish and males beginning to appear at sizes of 52.1 cm TL and 58.4 cm TL respectively. Larger sizes were obtained in the present study with the smallest transient and male sizes being 58 cm TL, and 64 cm TL respectively. Given the number of

sampled individuals in the present study, this result might be an artefact of small sample numbers and requires further investigation for confirmation.

The age of the dusky grouper, established using whole otoliths correspond to the findings of Reñones *et al.* (2007), but because we were only able to retrieve otoliths from female's considerable further work need to be done to the age structure.

In accordance with Gemmill *et al.* (1999) who suggested that parasitic nematodes often synchronised their maturity with maturation of their hosts, adult female *Philometra* sp. in the present study were only seen in developing spawning ovaries. This suggests synchronisation of the parasite with its host spawning season in gonad infecting *Philometra* sp., as observed by Perez *et al.* (2009), who found that adult female *Philometra carolinensis* worms only infected the ovaries of adult female spotted seatrout *Cynoscion nebulosus* during the spawning season.

The most clearly visible pathology associated with *Philometra* was observed in ovaries infected by decaying adult female *Philometra sp..* Live adult female *Philometra* sp. infecting the ovaries, despite feeding on host blood, seemed to elicit minimal to almost no inflammatory reaction from the host, agreeing with the observations of Perez *et al.* (2009) and Bakenhaster *et al.* (2014). Tissue immediately adjacent to parasite aggregations showed the greatest necrotic changes, while the remaining ovary usually remained unaffected. Concurring with observations by Moravec *et al.* (2003), adult female *Philometra* sp. was only found in spawning ovaries, and these authors also indicated that ovarian necrosis was probably associated with decaying adult females. Bakenhaster *et al.* (2014) similarly found that most visible damage is done by the dead or decaying adult female *Philometra sp.*.

Inflammatory reaction following the death and decay of female *Philometra sp.* was observed to induce ovarian fibrosis by Mohamed *et al.* (2010), which might cause several pathological changes including inflammation, granuloma formation, hemorrhage and parasitic castration (Ramachandran 1975; Clarke *et al.* 2006; Moravec & Buron 2013).

Although both testes and ovaries were found to be infected by nematodes, encysted parasites or calcified cysts, only ovaries collected in October and November were observed to be affected by the necrotic changes. Given the extensive scarring often observed, with involvement of almost the entire ovarian tissue, parasitic castration can be assumed in a few cases as an outcome of the infection in some individuals. The extent of necrosis to the ovarian tissue observed in some grouper made it difficult on occasion to evaluate the stage of host sexual maturity. The speed of ovarian recovery from necrosis induced by *Philometra* sp. infection remains unknown, as is the likelihood of successful regeneration for the following spawning cycle.

The inflammatory reaction of the host to *Philometra* sp. infection varies. Williams *et al.* (2011) observed, in wild crucian carp *Carassius* (L), an inflammatory reaction to gravid migrating female nematodes embedded in the fins, while no tissue reaction was seen to the unfertilised females and males in the swim bladder. All dusky grouper assessed were subject to multiple parasitic infections and all fish had one or more histopathological changes associated with these infections. Fish showed considerable variation in the intensity of infection, which might be attributable to health status, season, and aspects of parasite life cycles.

Bakenhaster *et al*. (2014) described foci of unidentified rod-shaped bacteria in the ovaries of *Philometra* sp. infected fish. Similar findings were observed in the present study, were colonies of rod-shaped bacteria were seen in SEM images to be attached to live adult female worms, males and ovarian tissue, giving a high likelihood of involvement in the observed necrotic reaction. While the extent of necrotic reaction displayed by ovaries varied from one fish to another, a combination of parasitic and bacterial infection might explain this variation in some post spawning ovaries, this finding needs further sampling programmes for confirmation.

Eosinophilic granular cells (EGCs) are often associated with parasitic infections (Hogan *et al.* 2008; Holzer *et al.* 2008), and Reñones *et al.* (2010) observed the EGCs to increase during gonadal transition from female to male. Similar findings were observed in this study. Melano-macrophage centers (MMCs) are often used to characterise the developmental stage of ovaries, providing an indication of past spawning activity induced by ovarian atresia (Reñones *et al.* 2010) and MMCs are also an indicator of inflammation and the response of the immune system to infectious agents (Agius & Roberts 2003). MMCs were frequently observed in the current study and although they were often observed in association with atresia and the presence of *Philometra* sp. infection and necrotic changes, it was not possible to ascribe abundance of MMCs to either condition.

Given that most scarring was observed in ovaries sampled September - November, and given the extent of pathological changes to the ovaries, especially post-spawning. it might be preferable for broodstock choice, and determination of sexual stages of wild fish for culture purposes, to consider catching fish from March to July, preferably from March to April, during which period most *Philometra* sp. were juveniles and no gravid *Philometra* sp. were

seen within the ovaries. This could avoid fish already subject to post spawning atresia and necrosis associated with decaying *Philometra* sp. infections, which can influence assessments of stage of maturation of the fish. Sampling in that period could decrease the possibility of ovarian necrosis associated with *Philometra* sp. infection, which, following application of antiparasitic drugs, would eliminate the parasitic infection before its full growth to adult female and therefore protect potential broodstock

Depending on the stage of atresia, it was occasionally difficult to differentiate the necrosis and atresia caused by normal ovarian post-spawning events from that induced by the parasitic infection. Parasites are often surrounded by fibrous tissue, with necrotic foci containing cysts. *Philometra* sp. are often found enclosed in cysts following death (Bakenhaster *et al.* 2014), a common finding during *Philometra* sp. infection to the ovaries.

Despite some ovaries sustaining extensive pathological changes, it seems that it had no visible effect on size/weight ratio, between sampled fish. Fertility was not determined in the current study, in terms of gonadosomatic index, thus the comparison between infected and non-infected ovaries could not be drawn.

Philometra sp. has previously been observed to protrude from the anal region in *Diagramma pictum* (Moravec & Justine 2015), and this was also observed for dusky grouper in the present study. It remains unclear whether the female *Philometra* sp. migrates to the genital pore for release of the larvae directly into the aquatic environment, or responds to the death of the host by seeking exit. This behaviour was also observed during sampling of male *Philometra* sp., which either specifically migrated or alternatively simply “leaked” from the cut surface of gonadal tissue into the containing Petri dish when left to stand for few

minutes. The period of free-living larval survival in the present study, although disrupted, showed similarities with that recorded for other philometrid larvae such as those of *Philometra obturans* (Moravec 1978), although this needs further investigation, including recording of *Philometra* sp. larval survival at various temperatures.

Other parasites such as gnathiid isopod larvae were seen infecting dusky grouper more frequently during the summer months than in the other seasons, and adult fish were more likely to be seen with the parasites infecting the oral cavity.

With the exception of only one small fish, which was severely infected with isopods on the gills, most gnathiid infections were associated with the oral cavity and nostrils, with no visible pathology to the host. This is in agreement with Genc's (2007) description of gnathiid larvae infecting wild dusky grouper. In this particular fish the operculum covering the gill was deformed, however, the finding of isopods associated with juvenile fish needs further sampling of the same or a similar size group for confirmation. Given the low number of sampled fish smaller than 30 cm TL (*n= 3*) the finding of smaller fish being less infected might be inaccurate. Future sampling might, however, not be advised given dusky grouper size limits in Libya (Reynolds *et al.* 1995).

Cultured grouper are known to be affected by parasitic infections, which often have an adverse effect on production (Nagasawa & Cruz-Lacierda 2004 Hoa & Ut 2007). Monogeneans are known to cause mortalities in several cultured grouper species *e.g. Neobenedenia melleni* (Capsalidae) has been described by Sanches (2008) to cause significant economic loss when infecting cultured dusky grouper of ~20 cm and ~142 g, leading to loss of appetite, erratic swimming, skin lesions, blindness and mortalities. The gills

of several dusky grouper in the present study were also seen infected by Capsalidae, however, no significant pathological changes could be detected.

In the present study encysted didymozoans were frequently seen to affect skin and gills of dusky grouper. Didymozoans are frequently described to affect wild and cultured grouper (Abdul-Salam *et al.* 1990; Nagasawa & Cruz-Lacierda 2004). In the present study, upon filleting the fish, the didymozoan and their yellow eggs discoloured the muscle, which might reduce the quality and commercial value of the fish. Similar observation were made by Ohiekezie *et al.* (1992), who described the reduced marketability of the white grouper *Epinephelus aeneus* (Geoffroy Saint-Hilaire, 1817) affected by a didymozoan. While Ohiekezie *et al.* (1992) reported 43% prevalence, in the present study a prevalence of 6% was observed. Despite the low prevalence in the present study, the impact that such infections could pose for grouper culture should be kept in consideration.

Calcified, encysted parasites representing trypanorhynch cestode larvae were often seen to affect the abdominal cavity of dusky grouper during the current study. In agreement with similar finding by Soliman *et al.* (2011) these parasites did not seem to cause significant pathological changes.

Zschokkella sp. are described to affect many fish species (Dyková & Lom 2006) and may cause necrotic changes to the tissue surrounding the parasite in heavy infections (Ferguson 2006; Bruno, Nowak & Elliott 2006; Dyková & Lom 2006). Similar findings were seen in the present histological investigation, where *Zschokkella* sp. infection was frequently observed in the lumen of the bile duct. In the present study MMCs were observed in the stroma of haemopoietic tissue containing myxozoans. Agius & Roberts (2003) described melano-

macrophage centres to represent a frequent aspect of the tissue response to parasitic infections *e.g. Ichthyophonus*. Dyková & Lom (2006) similarly described MMCs in the kidney of roach containing spores and sporogonic cells of *Myxobolus* sp..

Katharios *et al.* (2007) observed 100% mortality of cultured sea bream infected by *Ceratomyxa diplodae*, which had undergone hormonal treatment to stimulate sex change into males, raises the possibility of similar outcomes for dusky grouper, which also receives hormonal treatments for broodstock (Glamuzina *et al.* 2000; Marino *et al.* 2003; Sarter *et al.* 2006). In the event of heavy infection with *Ceratomyxa* sp., this might be yet another risk factor to be taken into consideration in the event of using dusky grouper broodstock from the wild.

Grouper culture is mostly capture-based (Leong 1998; Pierre *et al.* 2008), with broodstock generally captured from the wild. Hence, the health issues identified during this study need to be considered if broodstock are to be sourced from the wild, since parasites naturally found in wild fish populations may similarly cause disease in cultured fish of the same species (Scholz 1999). Overall, the parasites and pathologies observed in the current study provide insights that should be considered during future sampling of wild fish for broodstock and in the event of future culture of the dusky grouper in Libya.

For a fish that is characterised by a long life, slow growth and late maturation (Tupper & Sheriff 2008), ensuring lack of disruption of the spawning season by extending the period of closed-season fishing bans and introducing a limited maximum size for fishing should be considered. In this respect, extension of the fishing ban to include the period of May to August / September, instead of the present June-July fishing ban, given the length of the

spawning season established in this study, seems a logical approach. Being a batch-spawner, as observed by various authors from the Mediterranean (Marino *et al.* 2001; Reñones *et al.* 2010) and Brazil (Condini *et al.* 2014), extension of the closed season from May to August / September would give the fish sufficient time to regenerate. Increasing the imposed minimum capture size limit of 30 cm TL (Reynolds *et al.* 1995) to 45 cm TL, would bring limits into line with the that imposed by EU Council Regulation 1967/06 [Management measures for the Sustainable Exploitation of Fishery Resources in the Mediterranean Sea - Chapter 5, Article 15] (MEPA 2011). Given that the ovary size correlates positively with size of specimens (Marino *et al.* 2001) making larger fish more fecund than smaller fish, a further maximum size limit preventing capture of fish above 70 cm TL, would allow the larger and more fecund individuals to spawn, which in turn would increase the likelihood of fish recruitment. These closed season changes, bag size limits, and maximum and minimum size limits, would be temporary measures, put in place pending further assessment of the sustainability of dusky grouper fishing along the Libyan coastline and could be removed or adjusted according to findings. These measures could help to ensure the longer term sustainability of Libyan dusky grouper populations, ensuring maintenance and increase of catches for commercial and artisanal fisheries, not only for the present but also for further generations.

It is recognised, however, that the implementation of such measures, even under normal conditions (a country with relative stability) is often difficult. The artisanal structuring of grouper fisheries (Sadovy de Mitcheson *et al.* 2013), combined with the current political instability and unrest might make approaches to control dusky grouper fisheries, *e.g.* imposing size limit, bag size limit and closed season, highly unlikely to be implemented in Libya at the present time. These considerations will need revising once the political unrest in

Libya stabilises, at which point the assessment of the current dusky grouper fisheries would be required. Although ultimately the aim of such regulations would be to safeguard an endangered fish species, the more pressing issue of providing food security and availability to the consumer takes precedent during an ever-worsening conflict.

Study constraints and limitations

Constraints from working in an active fish market

Often fish were processed on site, given that transporting the fish to a laboratory might take considerable time during the rush hour, followed by more than an hour to process. Given that the work was often carried out in the fish market, although the salesmen had kindly offered space for sampling, the lack of microscopes meant that samples would only be processed upon arrival to the laboratory. Not wanting to alarm consumers or salesmen by performing extensive necropsies in public, sampling had to be done with speed. This limited capacity to conduct time-consuming tasks in terms of documenting, photographing, observing and sampling the different organs as described above.

Travel restrictions during the sampling period

During the period of the present study, Libya experienced considerable political unrest. As a result of the deteriorating situation, fish sampling was unexpectedly curtailed in the summer of 2014, this unfortunately being at a critical point in the field investigations. At this time, fish were also scarcer in the market as a direct result of fuel shortages, which forced fishermen to stay on land. Sampling was resumed in October of the same year during a

period of relative stability and in the midst of further water and fuel shortages and extended power cuts; the last sampling trip was conducted at the end of November to the beginning of January 2015.

Chapter 3

A novel use of social media to evaluate the occurrence of skin lesions affecting wild dusky grouper, *Epinephelus marginatus* (Lowe, 1834), in Libyan coastal waters

Manuscript accepted in Journal of Fish Diseases on the 30-Jun-2016. Currently in press.

A novel use of social media to evaluate the occurrence of skin lesions affecting wild dusky grouper, *Epinephelus marginatus* (Lowe, 1834), in Libyan coastal waters

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***Running title**:* Skin lesions of dusky grouper

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Abstract

The social media network Facebook™ was used to gather information on the occurrence and geographical distribution of dusky grouper dermatitis (DGD), a skin lesion affecting the dusky grouper, *Epinephelus marginatus*. Dusky grouper are common targets for spear fishermen in the Mediterranean and by monitoring spearfishing activity in Libyan waters, it was possible to document skin lesions from their entries on Facebook. Thirty two Facebook accounts and 8 Facebook groups posting from 23 Libyan coastal cities provided a retrospective observational dataset comprising a total of 382 images of dusky grouper caught by spearfishing between December 2011-December 2015. Skin lesions were observable on 57 / 362 fish, for which images were of sufficient quality for analysis, giving a minimal prevalence for lesions of 15.75%. Only dusky grouper exceeding an estimated 40 cm total length exhibited lesions. The ability to collect useful data about the occurrence and geographical distribution of pathological conditions affecting wild fish using social media networks, demonstrates their potential utility as a tool to support epidemiological studies and monitor the health of populations of aquatic animals. To our knowledge, this represents the first time that such an approach has been applied for assessing health in a wild population of fish.

Key words: Facebook, YouTube, dusky grouper dermatitis, DGD, disease surveillance

Introduction

The majority of Libya's population reside in its coastal cities, with over 2 million people living in the capital Tripoli. With a 1,770 km coastline, much of the country's fish derives from capture fisheries. In 2013, for which the last figures are available, the total tonnage was 36,000 mt, valued at US$ 100 million (FAO FishStatJ 2015). While most fishing activities are artisanal, fishing operators are obliged to hold a fishing permit issued by the Libyan Secretariat of Marine Wealth (Reynolds, Lamboeuf, Ben Abdallah, Abukhader, Abdulbari & Nafati 1995). Spearfishing, however, remains an uncontrolled fishing practice, whose captures are not visible in formal landing statistics. The dusky grouper is considered a high quality food fish and is caught by artisanal means as well as by spearfishing in Libya (Kasem, Ben Abdallah, Treky & Mosa 2009). A field study of local fish markets in Tripoli, conducted between 2013-2015, revealed that approximately 300 spear fishermen were supplying a local fish market in Tripoli (unpublished data).

The dusky grouper, *Epinephelus marginatus* (Lowe, 1834) [syn. *E. gigas, E. guaza*] (Perciformes, Serranidae), is a protogynous, monandric, hermaphroditic teleost endemic to the Mediterranean Sea with an extensive distribution (Heemstra & Randall 1993). The dusky grouper is currently listed as being endangered by the IUCN (EN A2d) (Cornish & Harmelin-Vivien 2004; IUCN, 2016).

As a K-strategist, female dusky grouper in the western Mediterranean reach sexual maturity at about 5 years of age at 38.6 cm total length (TL) (Reñones *et al.* 2010). Males, however, reach sexual maturity around 7 years of age at 58.4 cm TL (Reñones *et al.* 2007). The maximum recorded size for this species being 120 cm TL (Heemstra & Randall 1993). The

oldest recorded individual was 61 years old (Reñones *et al.* 2007). While a number of grouper species are important as commercially landed or sport fish species (Leong, 1998), the dusky grouper is considered to be the highest quality food fish and in Libya it is commonly found at fish markets priced at 10-34 Libyan Dinars (LD) kg^{-1} (*i.e.* US$ 7.3 – 24.8). Given its perceived value, flesh quality and large size, increasing pressure is being placed on local dusky grouper populations by spearfishing (Reñones, *et al.* 2007), a fishing method with dusky grouper as one of its main targets (Morales-Nin, Moranta, García, Tugores, Grau, Riera & Cerdà 2005).

Dusky grouper dermatitis (DGD)

DGD is a condition characterised by the presence of skin lesions affecting dusky grouper sized between 42-92 cm TL (Rizgalla, Bron, Shinn, Herath, Paladini & Ferguson 2016) and is described from fish that were caught by spearfishing and sold at a fish market in Tripoli between 2013-2015. The observed skin lesions are of unknown aetiology, and were classified according to their gross and histopathological presentation into either mild, moderate or severe dermatitis (Rizgalla *et al.* 2016).

Grossly, skin lesions were evident either as reddened patches or as whitish lesions with haemorrhagic ulceration and scale loss. The lesions could comprise multiple foci, and were unilateral or bilateral. They affected both the head and flanks, ventral and dorsal aspects, and the caudal peduncle. Lesions on the head were frequently seen on the skin covering the operculum posterior to the eye (Rizgalla *et al*. 2016). No evidence was found for a bacterial, fungal or viral aetiology of the lesions but the presence of blood flukes suggested the possibility of parasite involvement (Rizgalla *et al*. 2016). In 2014, a dusky grouper presenting

similar skin lesions was reported from Benghazi (32°11'N; 20°03'E), situated approximately 1,050 km to the east of Tripoli, suggesting that these lesions were not confined to the population of fish around Tripoli but might instead affect dusky grouper over a much wider geographic range (pers. obs.). Attempts to further investigate the epidemiology and occurrence of the lesions using conventional methods such as literature searches, and questioning of fishermen at local fish markets failed to provide sufficient epidemiological data for analysis.

Online Social Networks (OSNs) provide a platform for individuals to self-promote (broadcast) and maintain relationships (Underwood, Kerlin & Farrington-Flint 2011). This offers a constant stream of information posted online (Lima & de Castro 2014) that is shared among groups with common interests, helping to create and strengthen social links (Ellison, Steinfield & Lampe 2007). The increasing scale of such networks brings a commensurate expansion of access to information. Facebook™ provides an example of the scale of these networks, with the site hosting 1.65 billion active users per month (Facebook 2016) and therefore comprising the largest network of active users (Olmstead, Mitchell & Rosenstiel 2011), with 350 million new images uploaded daily (Henschen 2013). By comparison, the video-sharing website YouTube™ has 1 billion users, while the search engine Google™ supports c. 3.5 billion searches a day (Anon, 2015a, b).

The extensive connectivity and broad knowledge base that such activity builds has attracted the attention of academia and industry alike (Boyd & Ellison 2007). OSNs have thus been widely used by researchers, who have employed data obtained through user completion of survey instruments, often cross-validated against data obtained through direct access to Facebook user profiles (Ryan & Xenos, 2011; Golbeck, Robles, Edmondson & Turner 2011).

With the internet providing an increasingly powerful resource for disease surveillance in relation to human and animal pathogens, it has been extensively used by governmental and non-governmental entities to collect, as well as to disseminate information (Brownstein, Freifeld & Madoff 2009; Bernardo, Rajic, Young, Robiadek, Pham & Funk 2013). Bernardo *et al.* (2013) conducted a structured review of the research use of social media between 2002-2011, including Facebook, Twitter™ and Google, for surveillance of infectious diseases. The review concluded that despite possible biases, disease surveillance through use of social media can be used to support existing surveillance programmes. Images and comments posted by individuals online, coupled with use of online survey instruments or direct questioning, can prove very effective during the investigation of disease outbreaks. For example, a case was described by Stuart Chester, Taylor, Sandhu, Forsting, Ellis, Stirling & Galanis (2011) in which a campylobacteriosis outbreak affecting participants in a bike race, led to the organisers notifying the CDC. Following examination of images and information posted by participants, the ''mud'' appearing in images was analysed and subsequently identified as the source of infection.

Recreational and sporting aspects of spearfishing, coupled with the scope for competitive comparison of catches, mean that spearfishing individuals seek out others with similar interests, with whom to share their relative experiences and details of their catch. The aim of the current study, therefore, was to use a variety of search engines, social networks and video sharing websites, e.g. Google, Facebook and YouTube, to seek out information regarding dusky grouper caught by spearfishing in Libyan waters and in the neighbouring countries of Tunisia, Algeria and Egypt, with the purpose of determining the prevalence and geographical distribution of skin lesions in this region.

Materials and methods

Generic data collection via internet searches

The search space used to collect data comprised the following sites and databases: Google, Facebook and YouTube. For all search engine, social network and video-sharing website queries, a defined list of query terms was employed to interrogate the databases in order to collect data concerning grouper, spear fishing activity and skin lesions. The query terms, employed in a variety of combinations, were: “grouper”, ‘’fishing’’, “dusky grouper”, ‘’Libya” “Libyan”, “Mediterranean”, “spearfishing”, “diving”, “wound”, “skin”, “lesion” “scratches” “fish” and their equivalents in Arabic.

Facebook

Facebook was used to conduct a retrospective search, using images of fish included in spearfishing posts, to survey for presence of lesions on captured dusky grouper. The earliest relevant entry discovered comprised a personal Facebook page from 2011. This provided a study period beginning in December 2011 and spanning four years to December 2015.

Two approaches were used for data collection. The first approach involved following Facebook groups having an interest in spearfishing, these being individually identified and having all their postings screened for content relating to the capture of dusky grouper, particularly content with appended images, which could be examined for the presence of skin lesions. The second approach involved following active members posting pictures and

footage of dusky grouper fishing activities and tracing them back to their personal Facebook profiles to see whether they had posted further materials of relevance. Facebook friends of members who commented on a particular piece of relevant content (e.g. a “like” vote), were also traced and followed, with priority being given to those that also posted personal images of dusky grouper caught within the specified timeframe set for this study, or posted images of spearfishing in their personal Facebook profile picture posts.

Google and YouTube

A similar approach, with the use of the same key words, was taken to search for relevant material, images and video footage of dusky grouper fishing activity within Libya using the Google search engine, from which spearfishing web sites were found. The same approach was applied to the YouTube video sharing website, which provides access to content comprising video footage of grouper fishing activities in Libya and the surrounding region.

Estimating the size of fish posted on Facebook and the occurrence of lesions

Information concerning dusky grouper capture that was posted on Facebook was grouped into five size categories of 20-29 cm, 30-39 cm, 40-49 cm, 50-59 cm and >60 cm (**Table 1**). Where the weight and / or total length of the grouper were not specified, these parameters were estimated. This was achieved through scaling against known-size recognisable objects within each photograph, e.g. branded plastic bottles, equipment *etc.*, or where reference objects were not evident, the height of the fisherman (assuming an average height of 168-170 cm), the length of their arm (assuming a standard 1 m length) and /or the size of their feet (estimated at between 30 to 35 cm) were used as a scale to estimate the size of each fish specimen, providing it was held sufficiently close to the body.

Categorising the spearfishing methods used

Information regarding the spearfishing methods was also collected and used to categorise fishermen. These data included: when the dive took place (day or night), whether they used air tanks (e.g. scuba diving using up to four tanks for a dive period) or if air pumped from the surface was used (as reported by two Facebook users), whether the dive started from the beach by foot or from a boat etc., the dive depth, and the number in each fishing party.

Processing dusky grouper catch data from Facebook

To avoid the duplication of information, the process of downloading relevant images of dusky grouper fishing activity, with and without evident skin lesions, proceeded from the earliest posting on the Facebook page towards the most recent entry. Data were collected and sorted by closest city, with relevant data used for an estimation of lesions in relation to the total number of fish landed per person, the method of fishing used, the time of year, water depth and the temperature, where provided.

An archive of all dusky grouper pictures accessed was kept for this study and checks were applied to the photographs posted between accounts / groups to ensure that the statistics presented were based on unique records. The size of fish, and the species of grouper were not always possible to assess, thus some fish records / postings were discarded as the quality of the images was not sufficient to obtain accurate data.

Permission to use selected pictures and footage for the current work was obtained by directly contacting Facebook members, some of whom were Facebook "friends of friends". Contributors are noted at the end of this document in the acknowledgments section.

Results

Facebook groups with special interests in spearfishing and scuba diving in Libya

Within the study period, 12 Facebook groups were found, six with open access and six accessible only by members. All the groups had an interest in fishing by free diving and by scuba diving in combination with spearfishing. The largest group had 29,626 members during the study period and 32,077 members as of the 4th March 2016, drawn from across Libya and the neighbouring countries of Tunisia, Egypt and Algeria. The smallest interest group had only 7 members, with the oldest group (closed access) being formed in 2010 and the most recent formed in 2015 (closed access). For closed access groups no data could be directly accessed beyond their entry as interest group. Active groups often included associations of pre-existing friends who shared information through real-world connections offline, *i.e.* outside the network space. Although the largest social group nominally comprised 29,626 members, from the activity on the pages it would appear that no more than ~200 members actively engaged in posting fish pictures and video footage.

Images of specimen fish were frequently accompanied by information relating to the location where the specimen was caught, including nearest city, depth, grouper weight and the time of the dive. In the group with 29,626 members, it was compulsory for members to provide a description of the fish, the fishing method used, the location, the depth, diving method and the time, in addition to keeping postings strictly related to fishing and hunting.

Location was often subsequently omitted from more recent postings, as several members openly voiced their fears that advertising their catch rate and size of specimens in specified areas might lead to increased blast / dynamite fishing activity, thereby posing dangers to recreational divers.

Other Facebook groups did not show details relating to their members or provide information regarding fishing / catch locations. Sometimes this information was derived *post hoc* by following particular members and subsequently locating a city. Postings where the location of the fish was unknown were not included within the study.

Individual Facebook pages and social networks

From 200 Facebook users that were followed, most chose privacy settings that allowed access to their postings only by friends, but since they often posed with dusky grouper in their Facebook profile photos (PPs) it was possible to include them as individuals with an interest in spearfishing. Thirty two timelines with open access were, however, fully accessible. The information gathered from open access Facebook groups often allowed tracking of the fishing activity of other individuals, nominally inaccessible due to their restricted privacy settings. The remaining 168 Facebook users provided regular posts within the spearfishing Facebook groups but they offered no access to their personal Facebook pages. Some Facebook users provided personal information regarding their place of work, age, marital status, residence *etc*. Some of this information was useful in identifying potential fishing activity in waters close to coastal cities, and in neighbouring Tunisia, Egypt Algeria and Morocco. All the spearfishing activity posted on Facebook from Libyan-based

groups appeared to involve men, typically aged between 16-40 years old, and to a lesser extent those aged 40-50+.

Spearfishing in Libya

Spear fishermen reporting catches could be divided into three nominal categories, “professional”, “seasonal” and “recreational / sport”, according to the diving method they used, their catches and the comments made by Facebook members. Diving depths ranged from shallow (ca. 10 m) to deep (ca. 50 m); the size of the dive party varied from solitary dives to groups of seven spear fishermen; the duration of the dive ranged from an hour up to eight hours. Weather-permitting, the spear fishermen operated all year round, with certain operators using dive computers and professional underwater cameras to document their dive activity. It was often mentioned in comments that for diving from December to February, the water was particularly cold and that grouper were seldom seen. In March, however, the spear fishermen commented on the increased sightings of dusky grouper. Most postings including appended images used photographs captured using mobile phones.

Frequency of posting for fishing activity

Providing a precise estimate of fishing activity is difficult given the central issue of access to personal Facebook pages. The available evidence suggests, however, that recreational spearfishing activity increased on Thursday and Friday of each week, which are typical rest days in Libya as they comprise the national weekend. While there appears to be daily spearfishing activity by professional fishermen, the flow of information on Facebook from recreational fishermen is more constant than from the professionals.

Dusky grouper skin lesions seen on Facebook pages

Within the timeframe set for this study, *i.e.* December 2011-December 2015, the commentaries and photographs of grouper and spearfishing activity shared between networks of members and friends could be followed, and by doing so, the prevalence of skin lesions in dusky grouper captured from populations along the Libyan coastline could be estimated.

The earliest record of lesions documented on Facebook, appears to be from a picture of dusky grouper caught in the area of El Alose taken in January 2008, which was posted in 2013 and showed that two (>60 cm TL) of the three dusky grouper portrayed had evident skin lesions. From a total of 387 dusky grouper photographs of fish measuring between 20-100 cm TL that were posted on Facebook, 25 fish obtained from 11 separate images, were subsequently discarded as they were not appropriate for inclusion within the analysis, either because the images did not show the skin condition clearly or it was not possible to estimate the size of the fish. Of the analysable 362 fish, 156 fish ranging in size between 20-39.5 cm TL showed no observable lesions, while 57 of the remaining 206 fish, ranging in size from 40 to ~100 cm in total length, showed evident skin lesions, giving an overall prevalence of 15.75% (**Table. 1**). Skin lesions were compared to the morphological description of DGD provided by Rizgalla *et al* (2016). These fish were caught in water depths ranging from a few metres to up to 50 m. From video footage posted on Facebook and YouTube of four separate dusky grouper from the west (Tajoura and Misratah) and centre of Libya (Zwetina), three of the fish can be seen to have skin lesions posterior to the eye, while one has relatively extensive skin lesions below the dorsal fin. The swimming behaviour of all five videoed dusky grouper appears to be unaffected.

Table 1. Relationship between the size of dusky grouper, *Epinephalus marginatus*, (*i.e.* total length in cm) and the prevalence of skin lesions on 362 analysable specimens landed in Libyan coastal waters between December 2011 and 2015. After assessment and exclusion of unreliable images (n=25), only data on 362 of the 387 dusky grouper captured were used for analysis. (*) 3 fish from El Alose were shown in an image, of which 2 fish had skin lesions. These fish were captured in 2008 but pictures were only posted in 2013 with comments on fishing date and area.

Total length (cm)	20-29.9	30-39.9	40-49.9	50-59.9	>60	Total
Total no. fish	31	125	122	54	30	362
Total fish with lesions	0	0	26	20	11*	57

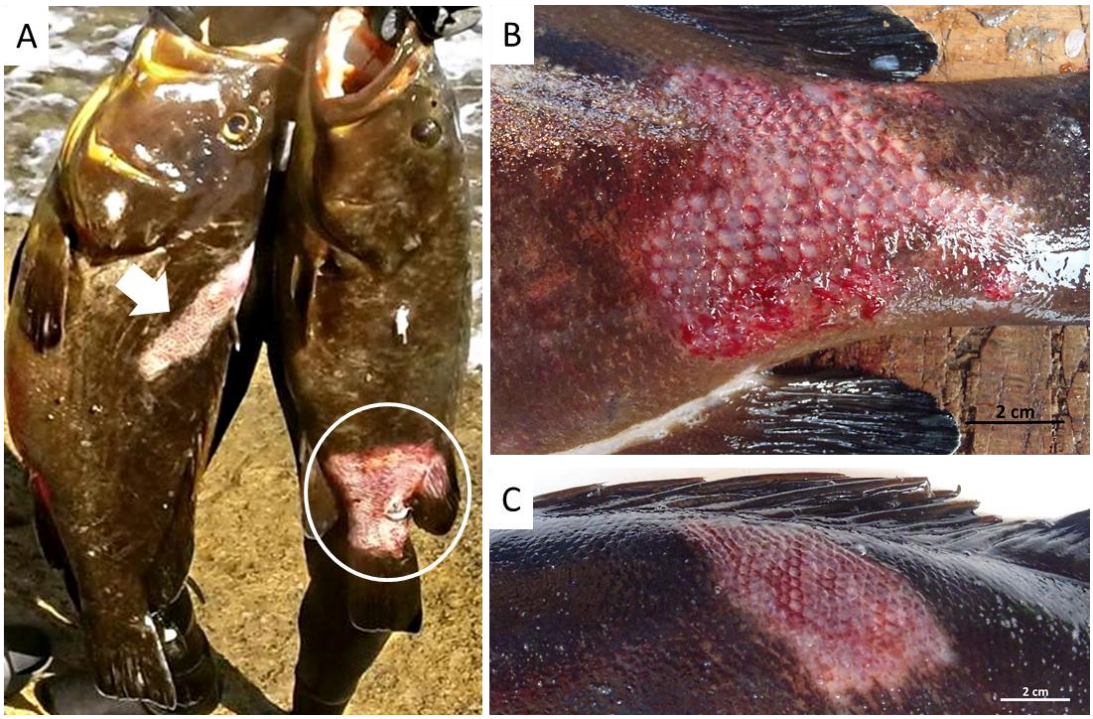

Figure 1. Pictures of dusky grouper, *Epinephalus marginatus*, with skin lesions as posted on a private Facebook page (**A**), and sampled from a local fish market (**B and C**). (**A**) Two dusky grouper caught by spearfishing showing skin lesions below the dorsal fin (arrow) and caudal peduncle (white circle). Note the similarity in the morphological presentation of the lesions with (**B**) a dusky grouper with a skin lesion affecting the caudal peduncle and (**C**) the skin lesion below the dorsal fin. *Image A used with the permission of Mr. El Shagmani*

Observed lesions were located posterior to the eye, below the dorsal fin (Figure 1), around the abdomen, on the caudal peduncle and around the cloaca. **Figs 1-3** show a comparison of the lesions described in Rizgalla *et al*. (2016) with those apparent from images posted on Facebook, showing similarities in the gross morphologies observed. These images include

healed and severe lesions posterior to the eye, acute lesions affecting the caudal peduncle, acute lesions affecting the flanks and healing lesions below the dorsal fin. In addition, a DGD healing lesion affecting the isthmus below the jaw is compared to an image posted on Facebook of an acute lesion similarly affecting the isthmus (**Fig. 4**). From the posted images, apart from the dusky grouper, no other fish species caught at the same time were seen to be affected by skin lesions. Fish showing no sign of lesions included members of the families Carangidae, Mugilidae, Mullidae, Serranidae and Sparidae.

Epidemiology and geographical distribution of dusky grouper showing lesions

Some captured images were associated with information about the location of capture. In addition, a number of uploaded pictures included the time and date on the photograph. Some of the spear fishermen on Facebook had the location option switched on, actively broadcasting where they were and documenting their precise location at the time they submitted their posting. Thus it was possible to map the prevalence of grouper with skin lesions to coastal cities. The current study found dusky grouper with lesions in the vicinity of 19 of the 23 Libyan coastal cities from which Facebook postings were made (**see Fig. 5 and Table 2**). The 26 dusky grouper caught in the waters off Athrun, Ras Al Helal, Karsa, and Derna ranging in size from 40-70 cm TL, however, displayed no observable lesions.

The prevalence of skin lesions for individual cities was calculated only from cities with entries of 10 fish or more, thus only entries from Tajoura, Misratah, Sirt, Tripoli and Benghazi were used (**Fig. 5 and 6**). Dusky grouper pictures posted by Facebook members from Tajoura showed the highest prevalence of lesions with 25% skin lesions, followed by Misratah 20%,

Sirt with 18.5 %, the capital Tripoli with 15.5 % and Benghazi with 7.6%. The reasons for such variability remain unclear (**see Fig. 6 and Table 2).**

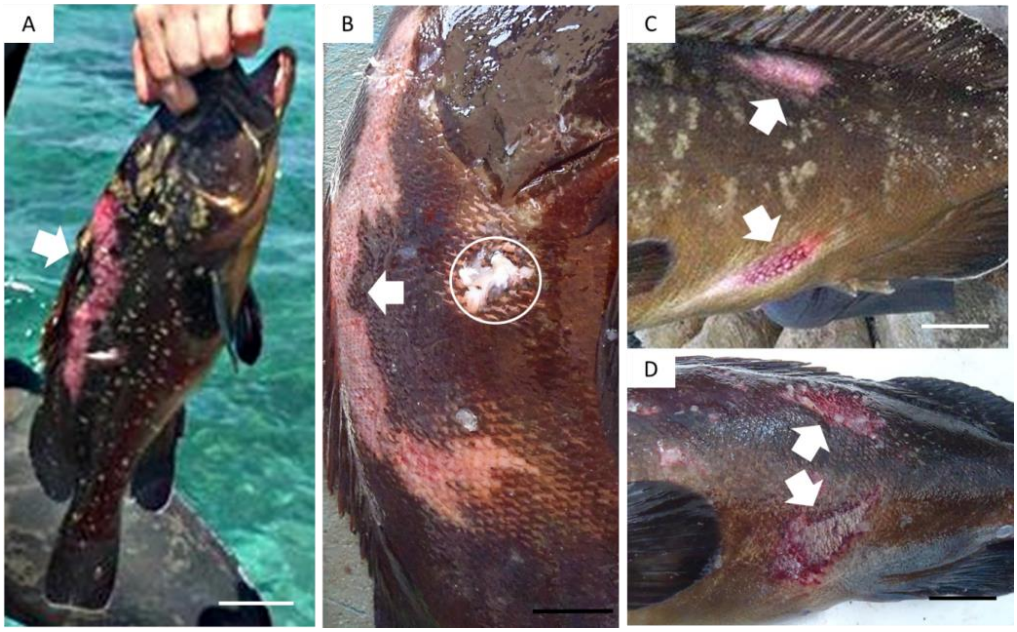

Figure 2. (**A**) Dusky grouper, *Epinephalus marginatus*, posted on a Facebook group (arrow) showing severe lesions. (**B**) Note the almost identical skin lesion affecting a dusky grouper sampled from a fish market in Tripoli that had been caught by spearfishing, evident from the penetration wound caused by the harpoon (circle) and showing a fused extensive skin lesion below the dorsal fin at a healing stage (arrow). (**C**) Dusky grouper posted on a Facebook group page with one lesion below the dorsal fin and one on the abdomen. (**D**) Dusky grouper sampled from a fish market in Tripoli showing multiple lesions similar to those in (C) one below the dorsal fin and one on the abdomen (white arrow). Note the almost identical position of the lesions. Scale bar: A & C & D=2cm; B=4cm. *Image A used with the permission of Mr. Fozi and image C used with the permission of the Facebook group Libyan Youth*

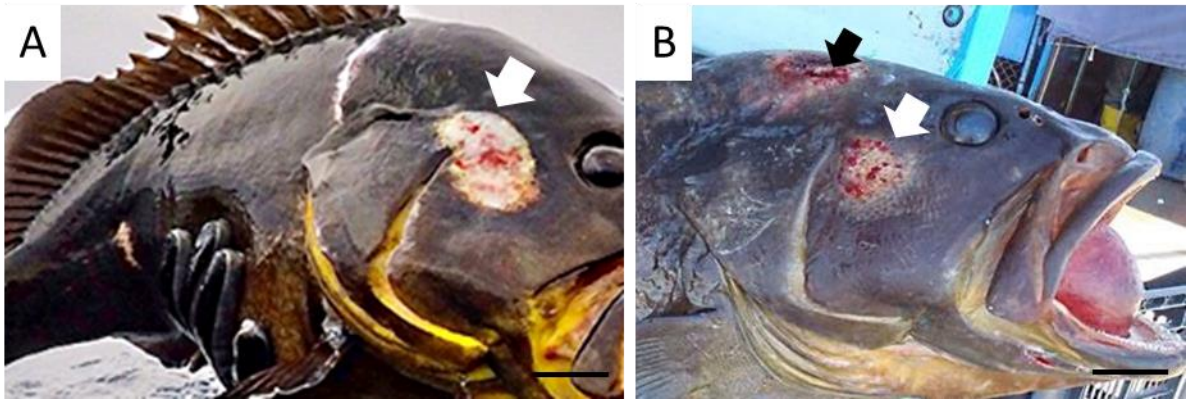

Figure 3. (A) Dusky grouper *Epinephalus marginatus* caught by spearfishing as posted on Facebook with a healing lesion posterior to the eye. (B) Dusky grouper picture taken from a fish market in Tripoli showing multifocal skin lesions, one posterior to the eye (white arrow) and one on the head (black arrow). Scale bars: A & B=3cm. *Image A used with the permission of Mr. El Shagmani*

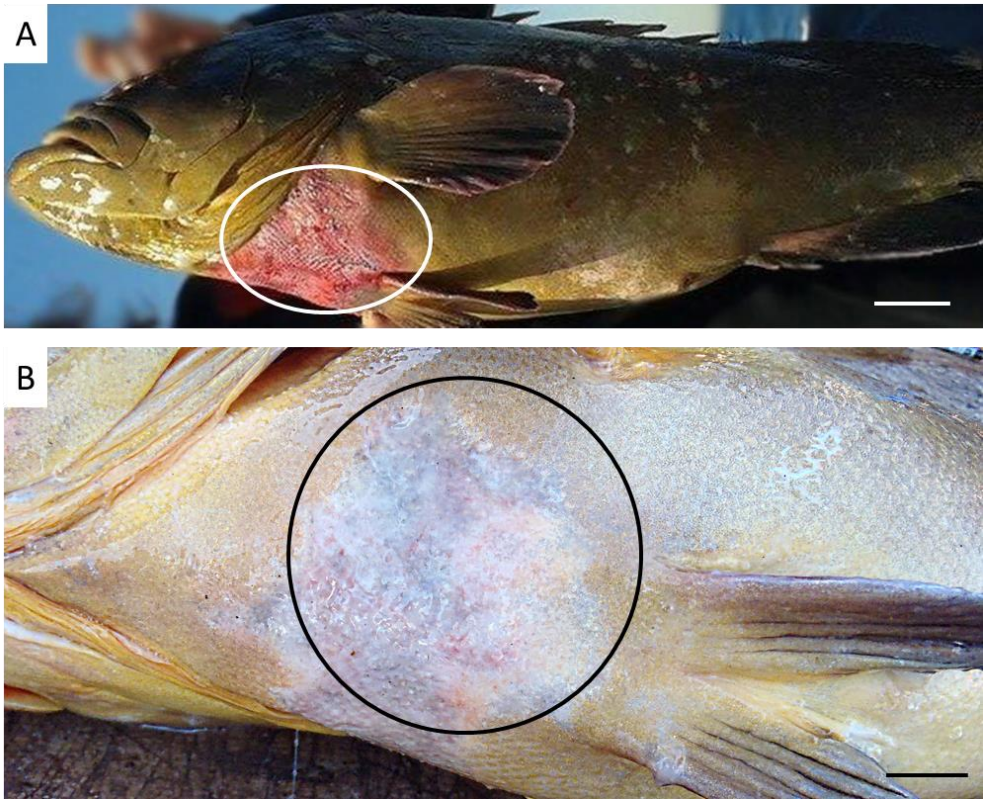

Figure 4. (**A**) Picture of a dusky grouper, *Epinephalus marginatus*, showing severe skin lesions affecting the isthmus of the jaw (white arrow). (**B**) A healed skin lesion on isthmus of the jaw (black arrow) of a dusky grouper sampled from a fish market in Tripoli. Note the darker discolouration against the background typical of a healed lesion. Scale bars: A=5cm; B=2cm. *Image A courtesy Mr. Ben Salem*

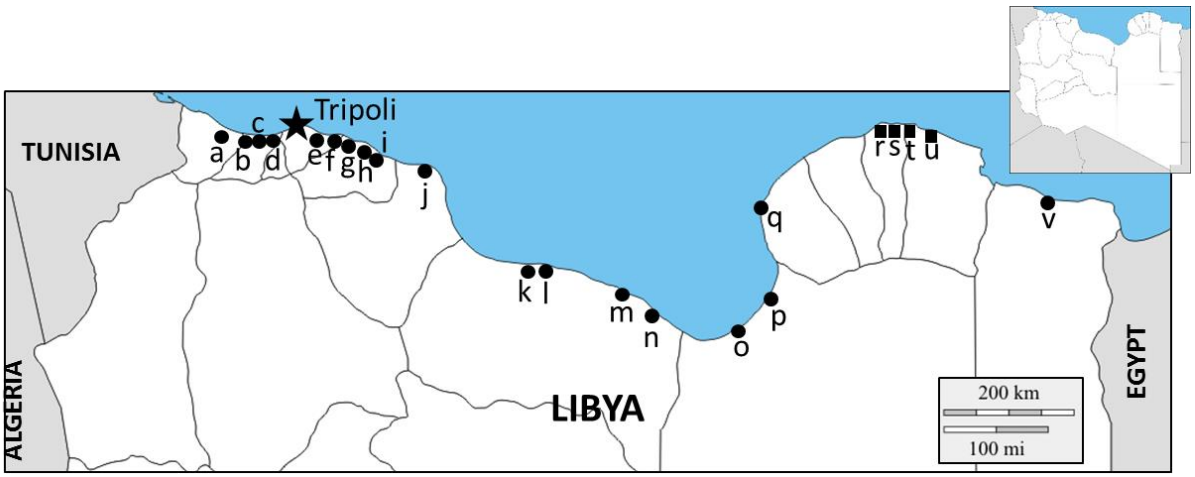

Figure 5. Records of the occurrence and prevalence (%) of skin lesions on dusky grouper, *Epinephalus marginatus*, caught by spearfishing in Libyan coastal waters obtained from an analysis of Facebook™ image postings between December 2011-December 2015. Cities marked by a black circle denote capture regions where lesions were seen on fish, while cities denoted by a black square represent regions where no lesions were seen on captured fish. Lesion prevalence is only estimated for capture reports of 10 or more fish. Tripoli /Nadi Bahri 15.9%. Key: a = Sabrata 4.7%; b = Surman; c = Mutrod; d = Zawia 7.4 % ; e = Tajoura 25%; f = Garaboli; g = El Alose; h = Celine; i = Al Khums; j = Misratah 20.5 %; k = Sirt 18.5%; l =Sulatan ; m = Ben Jawad; n = Ra's Lanufn; o = Al Burayqah; p = Zwetina; q = Benghazi 7.6 %; r = Ras Al Helal; s = Athrun; t = Karsa 0%; u = Derna 0%; and, v = Tobruk

Facebook members' reactions to dusky grouper skin lesions

In Libya, it is widely believed by the fishermen and divers that observable lesions can be attributed to the attachment and feeding activity of remoras, *Remora remora* (L.) (Echeneidae), known locally as the ''Golfat''. Hence, although the skins lesions do not go unnoticed, they do not generally elicit concern within the Facebook community. There has, however, been some limited speculation regarding the cause of the lesions. In one post written in 2014 and then re-posted in 2015, it was suggested that the cause of the lesions was due to viral nervous necrosis resulting in a loss of equilibrium and with the resulting physical damage leading to secondary bacterial infection. A second post in October 2014,

suggested that the lesions were due to a fungal infection and cautioned readers not to eat "infected" fish, while other postings suggested cutting out infected areas *etc*.

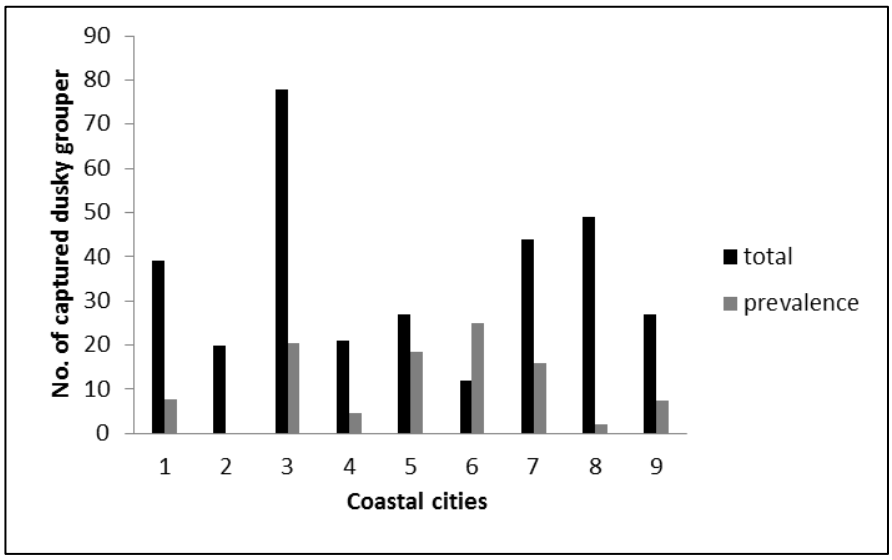

Figure 6. The prevalence of skin lesions on dusky grouper, *Epinephalus marginatus*, caught in the coastal waters of Libya during the study period and posted on Facebook. Only cities where 10 fish or more were landed are shown. Fish are categorised according to the nearest major town or city to the capture site. Total fish number is indicated by the black bars and the prevalence of skin lesions by the grey bars. Key: 1 = Benghazi 7.6 %; 2 = Karsa 0%; 3 = Misratah 20.5 %; 4 = Sabrata 4.7%; 5 = Sirt 18.5%; 6= Tajoura 25%; 7 =Tripoli /Nadi Bahri 15.9%; 8 = Tobruk; 9 = Zawia 7.4 %

Of the relevant videos available on YouTube (n = 3), 2/3 were posted by the same group members who were active on Facebook and in 2/3 cases, the same video footage had already been posted on their private Facebook pages or within the Facebook common interest group.

Several pages from similar interest groups posting pictures of dusky grouper were found in Tunisia (2), Algeria (5), Egypt (1), Turkey (1), Italy (1) and Spain (1). While most of the dusky grouper displayed were 60-80 cm TL, no lesions were seen except for two fish measuring >70 cm TL that were spearfished in Algeria and one in Turkey measuring 70 cm, which had evident lesions close to the cloaca and on the caudal peduncle (pictures not shown).

Table 2. The number of dusky grouper, *Epinephalus marginatus*, with and without skin lesions, caught in the coastal waters of Libya. Fish are categorised according to the nearest major town or city to the capture site. DG = dusky grouper.

City	Lesions/ DG examined	Prevalence %
Athrun	0/3	0
Al- Khums	2/6	33.3
Al Burayqah	1/7	14.2
Benghazi	5/39	7.6
Jadabia	0/1	0
Ben Jawad	4/8	50
Celine (Villa Seleen)	1/1	100
El Alose	2/3	66.6
Garaboli	3/7	42.8
Karsa / Derna	0/20	0
Misratah	16/78	20.5
Mutrod	1/1	100
Ra's Lanufn	1/2	50
Ras Al Helal	0/3	0
Sabrata	1/21	4.7
Sirt	5/27	18.5
Sulatan	1/1	100
Surman	1/1	100
Tajoura	3/10	25
Tripoli / Nadi Bahri	7/44	15.9
Tobruk	1/49	2
Zawia	2/27	7.4
Zwetina	2/2	0

Spearfishing in neighbouring countries and the prevalence of dusky grouper skin lesions

Discussion

This study has demonstrated that social media and other allied digital resources can be used as a valuable supplementary source of epidemiological data for examining the health of fisheries. This agrees with the findings of previous authors reporting on findings in other sectors, and who have also recognised the utility of online social media networks for disease surveillance. For example, Corley, Cook, Mikler & Singh (2010) demonstrated strong correlations between posted blogs reporting flu symptoms and CDC ILI surveillance data for influenza epidemics.

Assessing the population size and health of wild fish populations is challenging and problematic even under optimal conditions (Peeler, Murray, Thebault, Brun, Giovaninni & Thrush 2007). From the images of 362 separate dusky grouper used in this study, 57 fish landed in waters close to 19 Libyan cities were found to have skin lesions while 26 dusky grouper caught in the waters off Athrun, Ras Al Helal, Karsa, and Derna, had no observable lesions. These latter fish were captured at the same time as dusky grouper with lesions were landed elsewhere in Libyan waters and were of similar sizes. The reasons why no fish with lesions were observed from these areas are not clear and it may be either that there is an absence of lesions on fish in this area or that fishermen in this region are selective about what they post or capture. The accurate estimation of prevalence is further constrained by small sample sizes for any given area. More accurate estimation therefore requires further investigation including direct contact with Facebook users to enquire whether dusky grouper with skin lesions are seen in their area and whether individuals might be prepared to provide images for assessment. The digital domain can offer a rich repository of visual material (Vilnai-Yavetz & Tifferet 2015), which can be important as visual material is considered a key

resource in describing and characterising skin lesions affecting fish (Peeler, Ryder, Thrush, Mewett, Hulland & Feist 2014), with use of images avoiding errors inherent in purely verbal descriptions. When the morphology of the skin lesions in the pictures posted on Facebook are compared with the DGD skin lesions described in Rizgalla *et al*. (2016), there are close similarities in their gross morphologies, potentially indicative of common pathology / aetiology. Further similarities between the two datasets reside in the size of fish that are affected by lesions, *i.e.* 42 cm+ TL in Rizgalla *et al*. (2016) and >40 cm TL from the images posted on Facebook. In both cases the only species that appears to be affected is dusky grouper and observed lesions were present on landed fish throughout the year. While the DGD lesions are often severe, these do not appear to impair feeding (Rizgalla *et al*. 2016) or normal swimming behaviour (video footage posted on Facebook).

The small number of postings showing dusky grouper with lesions, for fish caught in Algerian and Turkish waters require further investigation to determine their significance and the possible wider distribution of DGD elsewhere in the Mediterranean.

The popularity and commercial value of dusky grouper as a target for spearfishing (Reñones *et al.* 2007) contribute, among other factors, to the frequency and quality of the information that is posted on social network sites. This is evident from the competitive nature of recreational spearfishing within and between different interest groups as seen from the largest Facebook group of over 29,626 members. Members from a broad geographical distribution regularly reporting catches and supplying photographs, provide a unique resource for observing visible markers of fish health with associated metadata, including details relating to the site / conditions of capture and the size of fish. In the absence of this social network site, this information would be unavailable unless gathered from those

individuals willing to declare their participation through the use of surveys (Mann, Scott, Mann-Lang, Brouwer, Lamberth, Sauer & Erasmus 1997; Morales-Nin *et al.* 2005).

Spearfishing is a relatively inexpensive activity requiring simple equipment (*i.e.* diving fins, a mask and a trigger harpoon). From the current study, spearfishing in Libya is predominantly practiced by the younger male population, 16-40 years old, drawn from diverse economic and social backgrounds and, to lesser degree, by men aged 40-50. A similar observation was made by Morales-Nin *et al*. (2005) who noted that 90% of the recreational fishermen around the island of Majorca were men with a mean age of 46 ± 2 years. No female participation in the Libyan spearfishing groups is evidenced from Facebook or from the YouTube postings. In terms of widening the social network web (Boyd & Ellison 2007), Facebook friendships in a spearfishing context could sometimes be formed between people more than 1,000 km apart and crossing borders into the neighbouring countries of Tunisia, Egypt, Algeria and Morocco.

With over 200 identified Facebook members indicating that they spearfish as a regular activity, it is clear that this fishing method is widely practiced and is capable of exerting increased pressure on an already vulnerable coastal fish species. Although spearfishing is banned in Marine Protected Areas (MPA) within the waters of some Mediterranean countries (Coll, Garcia-Rubies, Moranta, Stefanni & Morales-Nin 1999), spearfishing is common throughout the Mediterranean Sea. Similarly, spearfishing in Libya is an uncontrolled fishing practice as it is elsewhere in the Mediterranean. More information is needed to assess levels and impact of recreational fishing (Camilleri, Carpentieri, Cervantes, Charilaou, Darmanin, Dimech, Guijarro, Moguedet, Perez Gil, Vassiliades, Vassilopoulou & Vigneau 2007), in order to improve management practices and conservation of protected species.

Study constraints and limitations

The passively generated data derived from Facebook postings, while providing a considerable improvement on the pre-existing total lack of data, nevertheless suffer from a range of problems including a lack of consistency given the sporadic and unstructured provision of information, an unknown fishing effort and a high probability of unshared data. As it is not possible to see the entire body surface in each photograph, it is highly likely that the actual prevalence of skin lesions in dusky grouper is higher than the estimated prevalence provided by this study. The lack of parallel samples for histopathological evaluation prevents direct investigation and characterisation of lesions and the potential for determining their aetiology.

Spear-fishermen may participate and post updates on one or more social network sites and so caution must also be exercised in ensuring that the same fish is not double counted when it appears on different sites. Through the analysis of photographs in the current study, the authors were aware that on several occasions the same fish pictures were sometimes re-posted on different sites and sometimes months apart. To minimise errors in determining the prevalence of lesions, cross-checking is required, involving the initiation of searches at the time at which the individual(s) activated their accounts, and then the checking of all subsequent postings.

Some Facebook users have a “friends only” setting to ensure privacy and hence access to these pictures was not possible. Within certain open groups, pictures of fish can be accessed but occasionally the source/author of the photograph is anonymous. To address these issues, information can be obtained by reading the comments linked to the photographs or

by following those individuals recording a “like” vote for the image to their own Facebook pages. By employing this strategy, it was possible to identify some of the original authors/publishers of the work and to subsequently determine the location of capture. Although this method was successful on this occasion, it proved to be an extremely time-consuming task.

While the information posted on Facebook may not reflect the actual fishing activity of each individual, there may be considerable potential to form a network of people potentially willing to join monitoring programmes and to take part in controlled surveys. Such people are commonly described as “citizen scientists” and comprise individuals volunteering to collect and / or process data relevant to scientific enquiry (Silvertown 2009). Volunteers might be recruited using the Facebook groups and profiles already identified in the current study as a basis, in order to create a Facebook network which could be used to provide disease surveillance information.

Potential sensitivity and the ethics of using public information from social media

Despite the information being in the public domain it is important to ensure that the privacy and anonymity of individuals are not infringed and that confidentiality is upheld (Zimmer 2016). Consent has been sought from the owners of any images used in the current thesis, while the ethical conflict of mining data from the online activity of individuals who might inadvertently post personal information remains an issue that has split the scientific community (Gehner & Oughton 2016; Zimmer 2016). Until the issue of using divulged information in the public domain is clarified in a wider contest, it falls back on the researcher

to ensure the safeguarding of the privacy of each individual's information, as proposed by the Norwegian National Committee for Research Ethics of 2014, regarding internet research.

In conclusion, this study has shown that under conditions where a visible pathological marker is present in photographs, social network sites may be employed to conduct preliminary epidemiological studies in situations where other sampling or survey strategies may be difficult or impossible. In data collection terms, such a survey method can also be extremely cost-effective although it may be manpower-intensive. While mining data from these social network sites represents an innovative approach to the collection of data that might otherwise be regarded as inaccessible.

There are, however, limitations to the usefulness of these sites, as is the case for conventional survey methods, in that the information available is limited to the users' interest in specific fields and subjects, and their willingness to cooperate with researchers.

The information posted on social network sites and allied sources of information and media represents an extensive and varied resource that can support epidemiological studies in particular contexts. These sites increase the number of observers of certain conditions, increasing the likelihood of obtaining data on short-lived events, *e.g.* fish stranding / mortality events, over wider areas that, in most cases, are not possible to access within the remit of funded research. The current study, for example, demonstrates that information relating to the incidence of skin lesions on dusky grouper, an endangered species, over an area extending the entire 1,770 km coastline of Libya, during a period of civil and political unrest has been possible by mining data posted on a range of social network sites.

Acknowledgements

We wish to thank the Libyan Ministry of Higher Education and Scientific Research for the generous PhD scholarship funding that helped to support the research work described in this paper. Mr. Ben Salem, Mr. El Shagmani, Mr. Fozi and the administrators of the Facebook group Libyan Youth are thanked for allowing the use of grouper pictures posted on their Facebook pages, and special thanks are also due to Mr. Khalaf and Mr. Gashout.

Chapter 4

Dusky grouper dermatitis (DGD)

Paper I

Rizgalla J., Bron, J.E., Shinn A.P., Herath, T.K., Paladini, G., Ferguson, H.W. (2016) Ulcerative dermatitis in wild dusky grouper *Epinephelus marginatus* (Lowe) from Libyan waters. Journal of fish diseases (Article in press)

Aspects of this paper were presented as:

Oslo Epi 2016, Rizgalla J., Shinn A. P., Ferguson H. W., Paladini G., Herath T., Jayasuriya N., Taggart J., J. Ireland, Bron J. E. (2016) Use of the Facebook social media network to establish the distribution and prevalence of dusky grouper dermatitis (DGD) in Libyan waters. (Talk - accepted)

PhD conference, 2015 Stirling (poster) entitled ''Novel observations of dermatitis in Libyan wild-caught dusky grouper (*Epinephelus marginatus*) (Lowe)''

4.1 Introduction

Background:

As reported in **Chapter 2**, one of the major features of dusky grouper offered at a local fish market in Tripoli was the presence in some individuals of characteristic skin lesions. Using social media networks and other web-based resources, it was further demonstrated in **Chapter 3**, that similar skin lesions had been widely observed by spear-fishermen operating along the Libyan coastline in the vicinity of 19 identified cities or had been passively included in their photographs. The work described in the present chapter, therefore, sought to investigate more closely the prevalence and morphological / histopathological characteristics of the skin lesions, as well as their possible aetiology.

4.1.1 Normal skin structure of teleost fish

In order to describe and understand the pathology and pathogenesis of the observed lesions, it is important to have an understanding of the normal skin structure. Since this has been minimally studied in dusky grouper, a description of the general structure of skin in teleosts is provided below.

In general the skin of bony fish comprises an outer epidermis and an inner dermis, with a layer of well-vascularized loose connective tissue, the hypodermis separating this from the underlying muscle fibres (Elliot 2003; Elliott 2011) (**Fig. 4.1**)

The epidermis consists of a few to several cell layers (Henrikson & Matoltsy 1968 a, b & c) composed mainly of epidermal cells, also known as malpighian cells. Depending on their location within the epidermis, these are divided into basal layer epithelial cells, mid-layer epithelial cells, and surface layer (Henrikson & Matoltsy 1968 a, b & c ; Elliott 2011). Mucous

cells, which are unicellular glands, initially arise within the mid-layer of the epidermis and as they move towards the surface, they increase in size, in the process maturing into large oval to goblet-shaped cells filled with mucus which is eventually secreted onto the skin surface (Henrikson & Matoltsy 1968b). Once this happens, they die (Van Oosten 1957). Melanophores and wandering and resident inflammatory cells can be seen in normal skin layers such as aggregates of lymphocytes which are seen close to the base of the epidermis (Esteban & Cerezuela 2012).

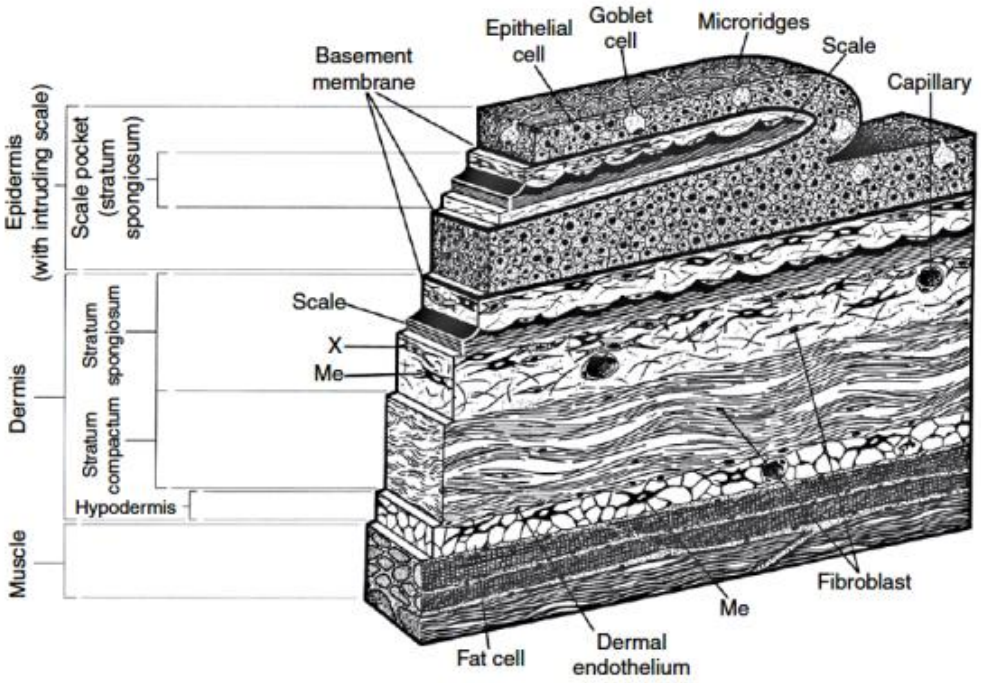

Figure 4.1. Depiction of a three-dimensional skin section of a teleost fish, the coho salmon *Oncorhynchus kisutch*, showing the main morphological features of both the dermis and epidermis and the general structure of each compartment. Abbreviations: Me, melanophore; X, xanthophore (both are chromatophores or pigment cells). Taken from Elliott (2011) page 272.

Faílde *et al.* (2014) also described the presence of lymphocytes at the base of the epidermis as a morphological feature in normal skin of turbot (*Scophthalmus maximus L.),* however

their function remains unknown (Ferguson 2006). An acellular *basal lamina* or basement membrane to which the basal layer epithelial cells of the epidermis are firmly attached, sits between the epidermis and underlying dermis and is composed of laminin-entactin/nidogen complexes and collagen IV.

The dermis, which is vascularised, is two-layered and comprises an inner *stratum compactum* and outer *stratum spongiosum.* These are formed by fibrous connective tissue with a large amount of collagen fibres, nerves, scales, pigment cells, and wandering inflammatory leukocytes *e.g.* macrophages, and granulocytes (Elliott 2011; Esteban 2012). The dermis is also the site of the lateral line system, which is a key provider of sensory information about the fish's environment.

Teleost fish scales are structures consisting principally of collagen fibrils and calcified zones (Schönbörner *et al.* 1979), and are embedded in the dermis, with the free part extending into the epidermis, which covers the scale by several layers of epidermal cells (Hawkes 1974) (**Fig.4.1**). In addition to the mechanical protection they provide, scales improve the structural toughness of the skin.

Embedded in the dermis is the mechanosensory lateral-line system (Goulet *et al.* 2008), which opens onto the outer skin surface through a number of pores (Bleckmann.& Zelick 2009). The lateral line serves a number of different functional requirements, including provision of the sensory information needed to maintain the position of a fish in a school, avoid obstacles, detect the presence of predators or prey and generally maintain the equilibrium of the fish while undertaking feeding, defensive, schooling, reproductive, and parental behaviours (Bleckmann.& Zelick 2009). The sensory structure of the lateral line

comprises a series of superficial and canal neuromasts (Kasumyan 2003; Goulet *et al.* 2008). The neuromast organs themselves consist of sensory hair cells, surrounded by supporting cells, all of which take the form of a cupula (Blaxter 1987). The lateral line also has chemoreceptive functions.

Beneath the dermis sits the hypodermis, a thin layer comprising deep arteries, veins and nerves, embedded in adipose tissue and collagen fibres. In this layer originates the blood supply and innervation of the dermis (Elliott 2011).

4.1.2 Inflammatory responses of fish skin to injury and invading pathogens

The skin's intimate interface with the aquatic environment (Henrikson & Matoltsy 1968) makes it vulnerable to the attachment and invasion of pathogens (Ferguson 2006). The limited range of pathological responses to injury, however, can make a diagnosis difficult, especially when the flushing action of the surrounding water often removes the pathogen. Skin lesions in fish can be divided into deep and superficial, depending on which part of the skin is primarily involved (Ferguson 2006).

Invasion by neutrophils and macrophages is one of the characteristic early inflammatory responses against parasitic infection in fish (Richards *et al.* 1996). Eosinophilic granulocytes (EGC's) are seen in blood vessels, pavementing (adhering to the blood vessels wall), in preparation for extravasation. Eosinophilic granular cells can also, for instance, be found attached to the outside of migrating cercarial stages of the blood fluke *Sanguinicola inermis* (Richards *et al.* 1996), while eosinophilic granular cells, sometimes in the process of degranulation, can be seen in the vicinity of damaged parasites (Shin *et al.* 2009) Eosinophil-mediated tissue inflammatory responses in helminth infection. Korean Journal of

Parasitology 47, 125–132.Macrophages are also often seen in the early stages of lesions; these have the function of clearing out or ridding the tissue of necrotic debris including that from melanophores which can be seen as brown/black inclusions (Roberts & Bullock 1976).

During acute parasitic inflammation, neutrophils, eosinophils and lymphocytes can be found forming the inflammatory response towards migrating gravid female *Philometroides sanguineus,* affecting wild crucian carp *Carassius carassius* (Williams *et al.* 2012).

4.1.3 The epidermis

Inflammatory responses (dermatitis) in the avascular epidermis are even more limited than in the rest of the skin, and are much more restricted than those seen in mammals (Roberts & Bullock 1976, Alvarez-Pellitero 2008). Unlike the mammalian epidermis, even the most superficial epidermal cells of fish are still viable and are capable of mitosis (Bullock *et al.* 1978). One of the earliest pathological changes associated with inflammation is loss of cell-to-cell junctions, the end result of which is spongiosis (Roberts & Bullock 1976). In severe skin lesions, the epidermis can slough with complete loss of the basement membrane, forming ulcers which leave the underlying dermis prone to invading organisms and deprive the body of its osmoregulatory barrier.

Healing of ulcerated skin starts with early attempts at reinstatement of the lost osmoregulatory barrier (Ferguson 2006) by rapidly multiplying adjacent malpighian cells. Studies on healing of ulcerative dermal necrosis (UDN) lesions in Atlantic salmon, *Salmo salar* (L.) reported the migration of malpighian cells from the margins of lesions to cover the ulcer, firstly with single cells, but followed by finger-like projections of piled up cells (Roberts & Hill 1976). Healing of injured skin can occur in a matter of hours (Esteban & Cerezuela

2012), this being a temperature-dependent process (Anderson & Roberts 1975). In UDN, time taken for healing of ulcers increased by 3 days during winter (Roberts *et al.* 1971).

4.1.4 The Dermis

In mammals, one of the earliest responses to injury in the vascularised dermis is increased blood flow, seen grossly as reddening and in histological sections as congestion of dermal vessels. Cell injury triggers the blood vessels to become more permeable then normal, allowing plasma to seep into the surrounding tissue. This is followed by the recruitment of leukocytes into the affected tissue, providing a cascade of different inflammatory cells each with its own unique roles and functional capabilities (Slauson & Cooper, 1990). Fish display a similar inflammatory response to injury to that observed in mammals (Finn & Nielson 1971).

In the event that scales and scale pockets are involved in the inflammatory process during the formation of ulcers, scales can be seen grossly poking out of the skin without epidermal cover as in “no-mucous skin disease” affecting rainbow trout (*Oncorhynchus mykiss)* (Ferguson *et al.* 1995). In those species that have them, loss of scales is often observed in severe skin damage, usually signalled by the presence of multinucleated osteoclasts (Ferguson 2006). The resulting “space” created by this loss can be filled by inflammatory infiltrate as seen in strawberry disease (SD) (Ferguson 2006) or red mark syndrome (RMS), both seen in rainbow trout (Oidtmann *et al.* 2013). Alternatively, inflammatory infiltrate can be absent from scale pockets as in “puffy skin” of rainbow trout (Maddocks *et al.* 2015).

4.1.5 Skin lesions in cultured grouper

Skin lesions often associated with bacterial infections in cultured grouper include those due to vibriosis (Nagasawa & Cruz-Lacierda 2004). In Malaysia cage-cultured greasy grouper

Epinephelus tauvina have skin lesions and corneal opacity during *Pseudomonas* spp. Infections, but this is accompanied by subacute bacterial septicaemia in internal organs, including diffuse pericarditis, multifocal endocardial and hepatic vein thrombosis and embolism. Mortalities can reach 60% (Nagasawa & Cruz-Lacierda 2004).

Loss of equilibrium was seen in grouper affected by vibriosis, an important disease affecting cultured greasy grouper, malabar grouper *E. malabaricus,* orange-spotted grouper *E. coioides,* and *duskytail grouper E. bleekeri* in Asia (Yii *et al.* 1997; Nagasawa & Cruz-Lacierda 2004; Harikrishnan *et al.* 2011). In addition to exophthalmia, corneal opacity and bloody discharge from the abdomen, skin ulcers are also seen, occasionally turning haemorrhagic (Nagasawa & Cruz-Lacierda 2004).

Vibriosis has often been associated with a disease called ''red boil disease'' in cultured grouper (Tendencia & Lavilla-Pitogo 2004) caused by *Streptococcus* sp. infection (Arthur & Ogawa 1996). Lesions include the formation of red boils (furuncles) on the skin which eventually rupture (Leong 1998), haemorrhagic liver and spleen, and yellow fluid from the intestine (Tendencia & Lavilla-Pitogo 2004; Qin & Yan 2010). Lymphocystis disease virus (LCDV), belonging to the genus Lymphocystivirus, is another viral disease affecting grouper in which the skin is involved (Huang *et al.* 2015). Histologically characterised by massively hypertrophied dermal fibroblasts (cytomegalic) producing wart-like lesions the nodules are composed of very large cells that contain viral inclusion bodies in the cytoplasm (Huang *et al.* 2015).

4.1.6 Skin lesions infections in wild dusky grouper

Despite the lack of information regarding normal skin structure in dusky grouper, a number of observations of skin lesions have been made for this species. In particular, dusky grouper have skin lesions when affected by nodavirus; this is seen in several regions of the Mediterranean Sea. Such lesions have been observed in Italy (Marino & Azzurro 2001; Vendramin *et al.* 2013), Algeria (Kara *et al.* 2014) Tunisia (Haddad-Boubaker *et al.* 2014) and Libya (IOE) (Anonymous 2013, Al-Attar *et al.* 2009; Al-Attar *et al.*2013). These lesions are typically confined to the head region as well as other clinical signs including corneal opacity, extended air bladder and loss of equilibrium (Marino & Azzurro 2001; Kara *et al*. 2014; Haddad-Boubaker *et al.* 2014).

Grouper with these skin lesions typically were observed floating on the water surface, and displaying abnormal swimming behaviour. Gross lesions included a distended abdomen, a swollen air bladder, and skin lesions mostly on the head, corneal opacity and an empty stomach (Marino & Azzurro 2001; Vendramin *et al.* 2013; Kara *et al.* 2014; Haddad-Boubaker *et al.* 2014). More pronounced skin lesions were observed by scuba divers on large dusky grouper swimming normally during the nodavirus disease outbreaks in Algiers (Kara *et al.* 2014). In these instances the lesions observed on the head were attributed to mechanical damage (Kara *et al.* 2014) and to the lateral recumbence of the fish affected by nodavirus (Marino & Azzuro 2001). In addition to the dusky grouper, gold blotch grouper (*Epinephelus costae*) (Vendramin *et al.* 2013; Kara *et al.* 2014) and the European sea bass (*Dicentrarchus labrax*) (Vendramin *et al.* 2013) were among the other fish species seen to be similarly affected.

Bacteria were isolated from dusky grouper during mass mortality amongst wild fish in Egypt and Libya (Soliman *et al.* 2011). *Pasteurella damsela* subsp. *piscicida* (= *P. piscicida*) was the dominant isolate from Libyan grouper mortalities, but *Vibrio* and *Aeromonas* spp. were also isolated. In another study, *Pasteurella damsela* subsp. *piscicida* (= *P. piscicida*) was recovered in pure culture from liver spleen and kidney of dusky grouper, sea bass and sea bream during an outbreak of mortality (Marzouk *et al*. 2009). Aside from erosive or ulcerative dermatitis, dusky grouper showed other clinical signs including peripheral hyperaemia, an inflamed haemorrhagic vent and abdominal distension, while internally, congestion and severe inflammation of gastric and internal mucosa were seen (Marzouk *et al.* 2009). Other pathological changes included unilateral or bilateral corneal opacity (Soliman *et al.* 2011).

Mycobacterium has been associated with mass mortalities of dusky grouper in Egypt. *Mycobacterium marinum* was recovered in pure culture from pooled liver and spleen of 5 fish (Eissa *et al.* 2011). Eissa *et al*. (2011) reported multifocal granulomatous inflammation in liver of the examined fish, a pathological change entirely consistent with mycobacterial infection in fish. Mycobacteriosis is typically a progressive chronic disease affecting many fish species (Austin & Austin 2007) that is characterized by the formation of multiple granulomatous nodules throughout internal organs *e.g*. liver, spleen, kidney and intestine (Austin & Austin 2007; Noga 1995). In addition, fish may be emaciated and suffer from exophthalmia, and may have dermal ulcerations.

4.1.7 Skin lesions of unknown aetiology affecting cultured fish

A characteristic pattern of lesion distribution can be observed in ulcerative dermal necrosis (UDN) affecting Atlantic salmon *Salmo salar* (L.) with lesions mostly affecting the skin on the head (Roberts 1971). Lesion distribution sometimes reflects underlying tissue structure. The

lateral line, for instance, is often directly or indirectly subject to pathological changes, as in chronic erosive dermatopathy (CED), a disease of unknown aetiology causing focal ulceration of the skin overlying sensory canals of the head and trunk of Murray cod, *Maccullochella peelii peelii* (Mitchell). This condition is associated with the use of waterholes (Baily *et al.* 2005); similar lesions are seen in sharpsnout sea bream, *Diplodus puntazzo* (Walbaum) reared in saline borehole water (SBH) (Katharios *et al.* 2011). Although the lumen of the lateral line is seen to contain hyperplastic and necrotic epithelium (Baily *et al.* 2005), the fish recover once removed from the waterhole / borehole water (Baily *et al.* 2005; Katharios *et al.* 2011).

4.1.8 Aims

The work described in this chapter sought to characterise the lesions observed in dusky grouper in the fish market survey, thereby providing a morphological and histological baseline description of the skin lesions and at the same time attempting to establish their aetiology and their relationship to previously reported lesions in dusky grouper and other marine finfish species.

4.2 Materials and Methods

4.2.1 Sampling

As reported in **Chapter 2**, field surveys were undertaken in Libya from October 2013 to January 2015 in order to investigate the health status of wild-caught grouper offered for sale at local fish markets. During that survey, skin lesions affecting the captured dusky grouper were observed to be a common finding.

Two hundred and sixty seven dusky grouper, caught by fishermen using a variety of methods including spear-fishing and baited hook capture and measuring 20-92 cm in total length (TL) (200 g to >15 kg), were examined for gross skin lesions. For more detailed laboratory-based investigations, 26 fish with gross skin lesions of differing severities, as well as five fish with no observable skin lesions, were investigated more extensively. Live fish were either sampled directly at the local fish market or after their transport in aerated water to a nearby laboratory where they were euthanised by gill exsanguination and severing of the spinal cord. Digital pictures (Olympus Tough TG-810 Digital Camera) were taken of sampled fish as well as measurements for morphological description and categorisation (**Fig. 4.2**).

4.2.2 Parasitology

After samples were taken for histology, skin scrapes were taken using a sterile blade from normal and ulcerated skin, or from skin showing pathological change. The scrapes were transferred onto a glass microscopic slide, cover-slipped and inspected using a compound microscope. Skin used for skin scrapes was not subsequently used for histological sampling.

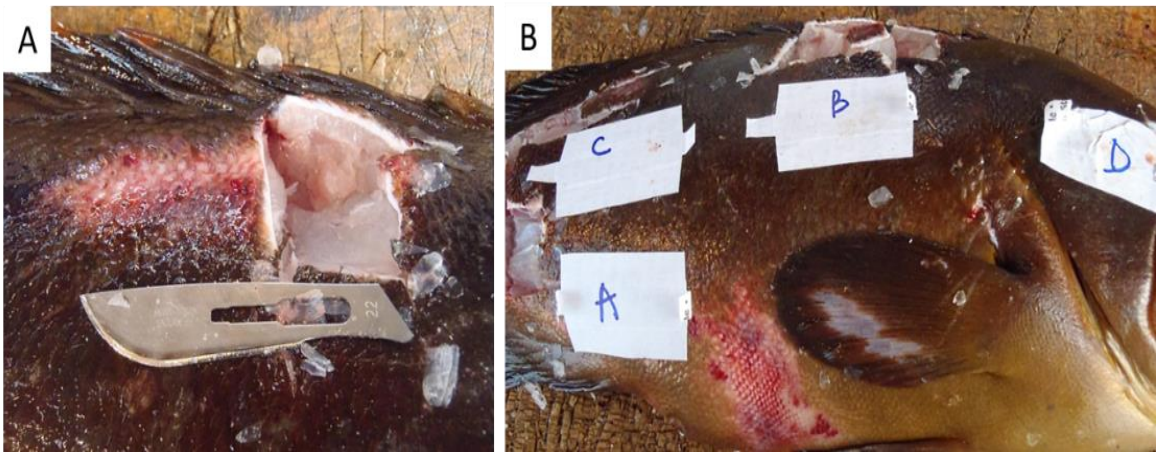

Figure 4.2. Sampling method (**A**) where skin sections are taken from the centre and margins of the lesion (**B**) and giving each lesion a code and taking skin without lesions from the same fish to serve as controls

4.2.3 Bacteriology

As part of the more detailed examination, incisions were made in apparently normal skin and in areas showing lesions, using sterile blades. Samples were taken, from the centre and margin, of each skin lesion, using sterile disposable plastic loops. Samples were also taken from the spleen and posterior kidney, again using loops. The loops were used to inoculate tryptic soy agar (TSA) with 2% NaCl, a general purpose culture medium, thiosulfate-citrate-bile salts-sucrose agar (TCBS), a selective medium used to isolate *Vibrio* spp., and marine *Cytophaga* agar (MCA), a medium used to isolate *Cytophaga*-like bacteria such as *Tenacibaculum maritimus,* which causes fin rot in cultured grouper. Inoculated media were incubated for up to 10 days at room temperatures from 18-24°C, reflecting ambient seawater temperatures.

4.2.4 Morphological measurements

4.2.4.1 Gross morphology

Pictures were taken of the skin lesions and categorised according to type (mild, moderate, severe, or healing), position (unilateral or bilateral), distribution (localised or diffuse), shape

(*e.g*. round, oval, irregular, elongated), the appearance of margins (smooth, irregular) and position on skin section in relation to its position and classified as e.g. ventral, dorsal, caudal, posterior etc. The lesion colour was described according to presentation and (or) in relation to the surrounding normal skin tone e.g. pale, haemorrhagic, pale, mottled *etc.*

The term "normal" was given to skin that had no evidence of gross lesions and resembled the skin colour of the dusky grouper in accordance with the description provided (Zabala *et al.* 1997b). Skin lesions were divided into stages based on the morphological and histological presentation into e.g. mild, moderate, severe and healing.

4.2.5 Histopathology

Samples for histopathology were taken from lesions as well as from apparently normal skin, eyes, gills and all the internal organs including liver, spleen, heart, intestine, pyloric caeca and gonads. All tissues were placed in 10% neutral buffered formalin and fixed for at least 24 h prior to further processing. Gonads were assessed for sex and stage of maturity as described by Marino *et al.* (2001). The internal organs including ovaries were examined for pathological changes, and the stomach was opened and examined for evidence of feeding activity.

For light microscope observations, samples were routinely processed following an overnight cycle using chloroform as the clearing agent (Shandon Citadel 2000 tissue processor, Thermo Fisher Scientific Inc.). The tissues were embedded in paraffin wax, and trimmed with a Leica microtome using disposable microtome blades (MX35 Premier Microtome blades 34°/80mm/ Thermo Fisher Scientific Inc.) at 20µm. All organs except skin and eyes were soaked in distilled water for 5-10 min at room temperature, excess water removed and

placed for 1-2 min on a cold plate then 5 µm thick multiple serial sections were cut. Skin and eye tissue blocks were pre-treated and decalcified in a rapid decalcifier (RDC™ and RDF™ cellpath) for 1 to 2 hr. Tissue blocks were then rinsed with running tap water, dried and processed as above.

It was observed that "decal" often caused artefactual epidermal separation; this was remedied by allowing skin blocks to soak in distilled water for up to 30 mins, then dried and placed on a cold plate and processed as above. Tissue blocks had to be re-placed in water to soak and the blades had to be more frequently changed, but by not using decal the epidermis was better preserved, especially when dealing with moderate and severe lesions where epidermis was often fragile.

Cut sections were then placed on a hot plate (Raymond Lamb) at 40°C, allowed to dry and then arranged in a staining rack and placed in an incubator (Windsor incubator) at 66C° for one hr for the internal organs and at least 2 hr to overnight for skin and eyes. Incubation for less time would cause the skin to be lost during staining, especially skin with lesions.

Dewaxed sections were routinely stained with haematoxylin-eosin stain (H&E) (Drury & Wallington 1980). Special stains used for selected slides included the following: Martius scarlet blue (MSB) (Drury & Wallington 1980) was used for fibrin and collagen staining, periodic acid–Schiff (PAS) (Drury & Wallington 1980) to detect polysaccharides such as glycogen, Giemsa to visualise bacteria and parasites, Gram to detect Gram-positive and Gram-negative bacteria, and Ziehl Neelsen acid-fast stain for the detection of acid fast bacteria. When required, appropriate positive and negative control slides were also used. As

a positive control, tissue affected by *Mycobacterium marinum* from seahorse *Hippocampus hippocampus* was used.

4.3 Results

4.3.1 Normal dusky grouper gross description

The colour of the normal dusky grouper skin showed variation from dark brown, to pale yellow (**Fig. 4.3**). For a more detailed description of normal skin see **Chapter 1**. The skin was often darker on the dorsal and lateral parts of the body and pale and yellowish on the ventral aspect. The skin colour from fish collected during the four annual seasons showed variability, but no differences in males or females were observed. For a detailed description of skin colour variation in dusky grouper see **Chapter.1**.

Figure 4.3. Normal dusky grouper adult female (45 cm TL), without skin lesions, skin is shiny with no excessive mucus production; eyes are bright and fins intact. Colour is mottled yellow on a brown background with a yellow abdomen. Scale bar=5 cm

4.3.2 Survey results

4.3.2.1 Fish species affected

Out of all of the fish species offered at the fish market (belonging to over 11 genera (**see Chapter 2**), only dusky grouper with sizes ranging from 42-92 cm total length (TL) were seen

to have skin lesions. The lesions were seen all year round, across multiple sampling trips, from 2013-2015, July-August 2013, October 2013, April-May 2014, June-July 2014, October 2014, November-December 2014, December-January 2015. No sampling trip was conducted in the month of February; thus no results were obtained for that month.

4.3.2.2 Gross lesions

From the two hundred and sixty seven dusky grouper that were examined, fifty five fish (42-92 cm TL) were observed to have gross skin lesions (**Table. 4.1 and Fig. 4.4**), twenty six of which were sampled for more detailed analysis. Smaller fish ranging between 20-41 cm TL showed no evidence of skin lesions.

Table 4.1. Number of gross lesions within each size group of sampled dusky grouper measured in cm total length

Size/cm TL	20 - 41	42 - 50	51 - 60	61 - 70	71 - 80	81 - 92
Total fish	122	42	63	22	8	10
No. fish with Lesions	0	13	21	15	4	2

Skin lesions were found in dusky grouper that were landed throughout the year, in water temperatures ranging from 15 to 27°C. All twenty six fish with grossly evident skin lesions were sexually mature fish, including mature females, transitional state individuals and mature males (for maturity state definitions see Marino *et al.* 2007).

The skin lesions varied in size, comprising single or multiple foci, and were unilateral or bilateral, a diagram of the major sites of lesion presentation is provided in **Fig. 4.5**.

The mildest lesions comprised focal or multifocal, reddened, circular zones progressing to a more haemorrhagic appearance and affecting, in severe cases, up to 40% of the skin.

Skin lesions affected both head and flanks, ventral and dorsal aspects, and the caudal peduncle as well as a few healed lesions at the isthmus (**Fig. 4.6 & 4.7 & 4.8**). Lesions on the head were frequently seen on the skin covering the operculum posterior to the eye (**Fig. 4.6**).

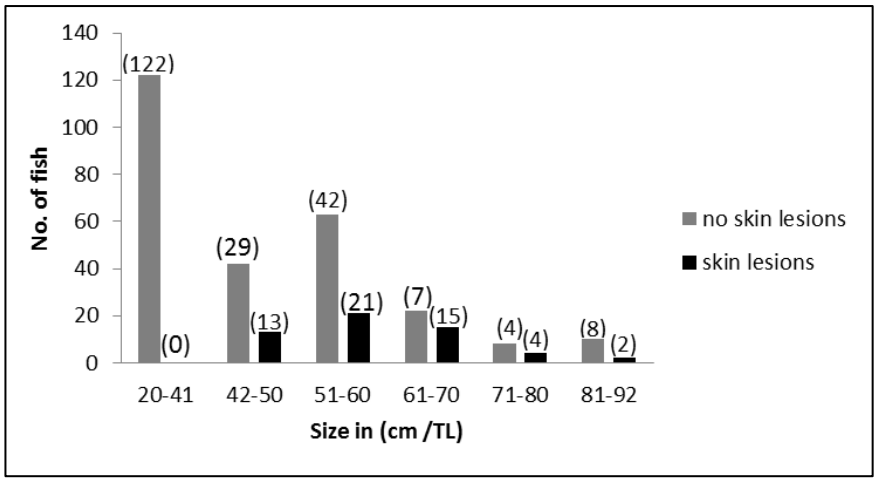

Figure 4.4. Diagram representing 267 dusky grouper for the field survey ranging from 20-92 cm total length (TL) and showing the total number of grouper with skin lesions in each size category

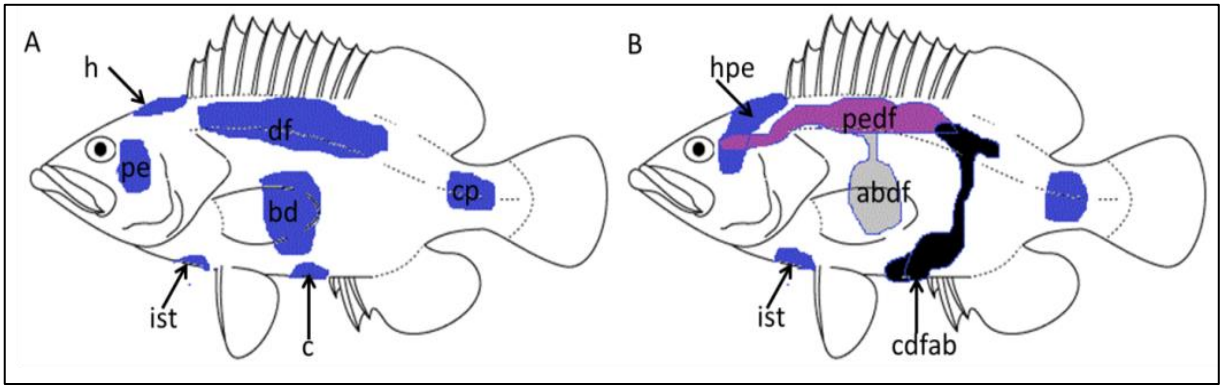

Figure 4.5. **A&B** Diagram showing the various locations of lesions on the body. (**A**) Shows lesion locations, and (**B**) Shows where lesion coalescence was observed. Colour coding is used to distinguish nominal zones. *Abbreviation*: h=head; df=adjacent to dorsal fin; ist=sthmus of the jaw; pe=posterior to the eye; abd=abdomen; cp=caudal peduncle; c=cloaca; pedf=posterior to the eye and adjacent to the dorsal fin; cdfab=cloaca adjacent to dorsal fin and abdomen; hpe=head and posterior to the eye; abdf=abdomen and adjacent to dorsal fin

The most severe cases comprised focal or coalescing ulcers with loosely attached scales and/or scale loss (**Fig. 4.7 & 4.8**), and the lesion seemed to be entirely superficial, affecting only the skin and with the underlying muscle remaining intact (**Fig. 4.9**). Some fish had visible penetrating injuries to the trunk or head as a consequence of having been captured through spear-fishing (**Fig. 4.8 & 4.9**) and some fish had hooks still embedded in the stomach, and oesophageal wall.

Healing lesions were recognised by their initial pallor and subsequent dark colouration with respect to normal skin (**Fig. 4.6 & 4.8**). Healed leisons could also be assessed by passing a finger over a healed skin section and testing for tissue softness / elasticity.

Crabs, fish or pieces of octopus, the latter often used as bait, were found in the stomach of most fish. Other than a frequently enlarged and slightly dark spleen in fish with large haemorrhagic lesions **(Fig. 4.9C**), all the remaining organs examined displayed no gross pathological changes. Some of the fish had calcified metazoan parasites, and encysted trypanorhynch (Cestoda) larval stages in the abdominal cavity and head kidney, and during the spawning season, gravid ovaries were sometimes infected with red coiled nematodes, an appearance typical of *Philometra* sp. (Nematoda). The gills were often infected by monogenean parasites and gnathiid isopod larvae (praniza) were attached in the oral cavity and to gill arches and filaments. These findings are described in more detail in **Chapter 2.**

The gills were often infected by monogenean parasites (*Pseudorhabdosynochus* sp.) and gnathiid isopod larvae attached in the oral cavity and attached to gill arch and filaments. The fish were often found alive, and were kept alive for several hours in aerated water. On

occasions the stomach was protruding from the mouth, and eyes bulging out, a consequence of bringing the fish to the surface too quickly with the resulting sudden change in pressure.

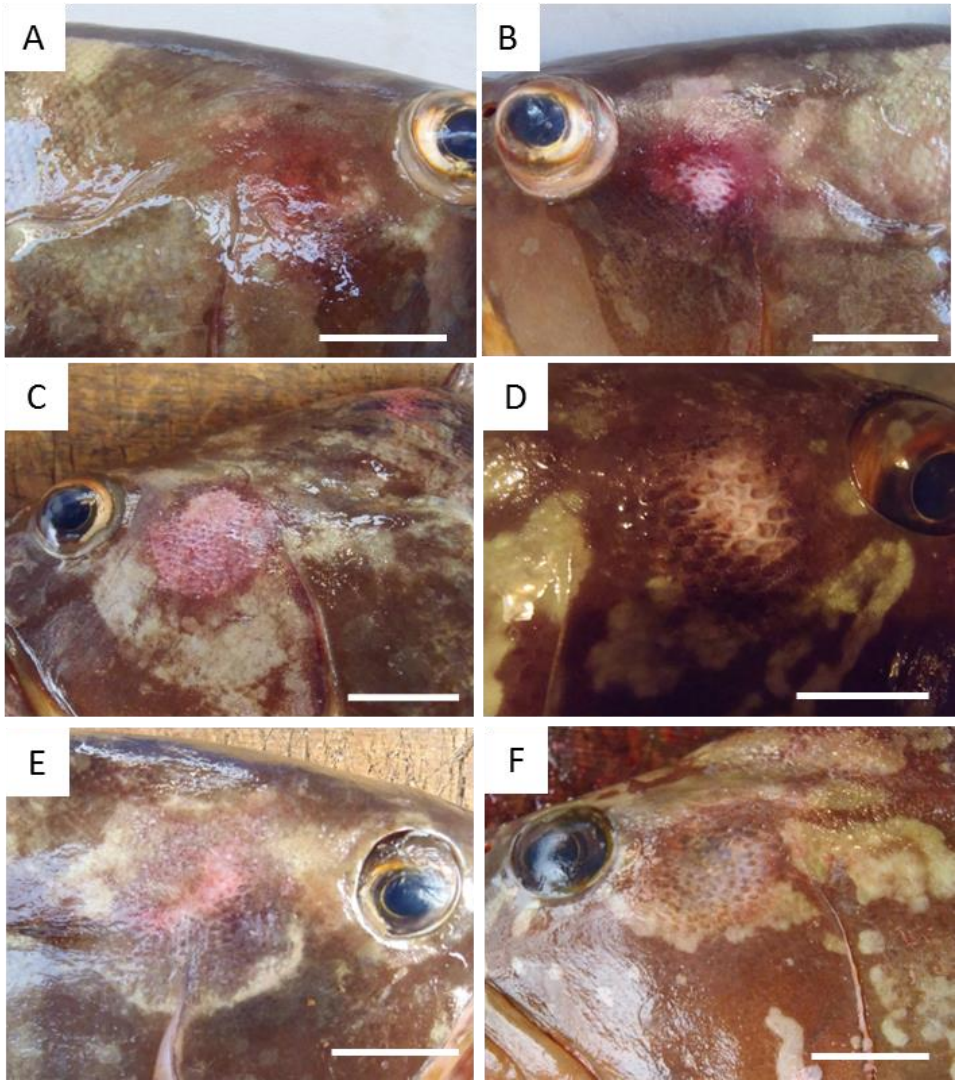

Figure 4.6. Gross appearance of dusky grouper dermatitis (DGD) lesions affecting the opercular skin posterior to the eye. (**A**) Haemorrhage of a mild lesion. (**B**) Loss of pigmentation in late moderate lesions giving rise to a halo-like appearance with hyperaemic margins and a de-pigmented centre. (**C**) Depigmentation in a severe lesion. (**D**) Healing lesion showing an unpigmented centre and healing margins. (**E**) Centre of a lesion showing persistent inflammation and healed margins. (F) Healed lesions showing pale areas when compared to adjacent skin areas. Scale bars=5 cm

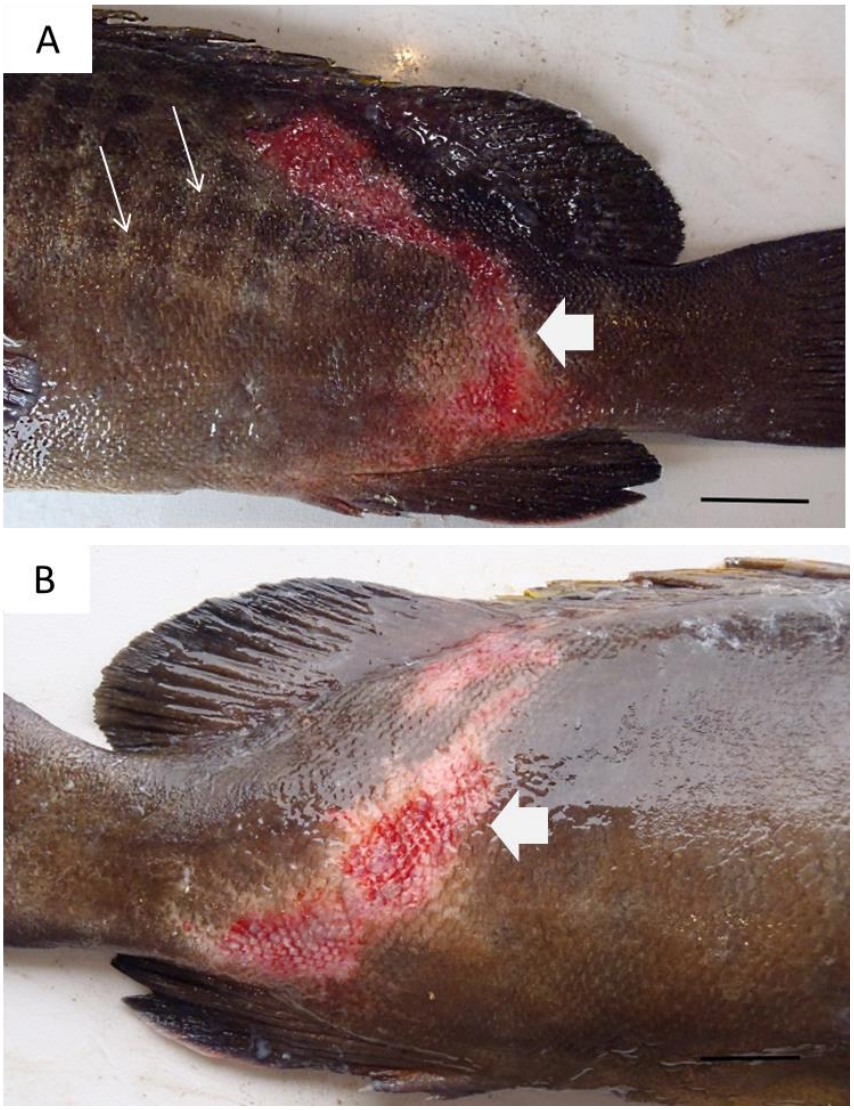

Figure 4.7. Gross appearance of dusky grouper dermatitis (DGD) showing severe bilateral lesions (**A & B**) affecting the same fish on either side of the trunk, almost mirroring one another (short white arrow). Discolouration on the trunk of (long white arrows) (seen in A) is due to pressure from the box in which the fish was placed after it was caught. Scale bars=(A) 5 cm; (B) 2 cm

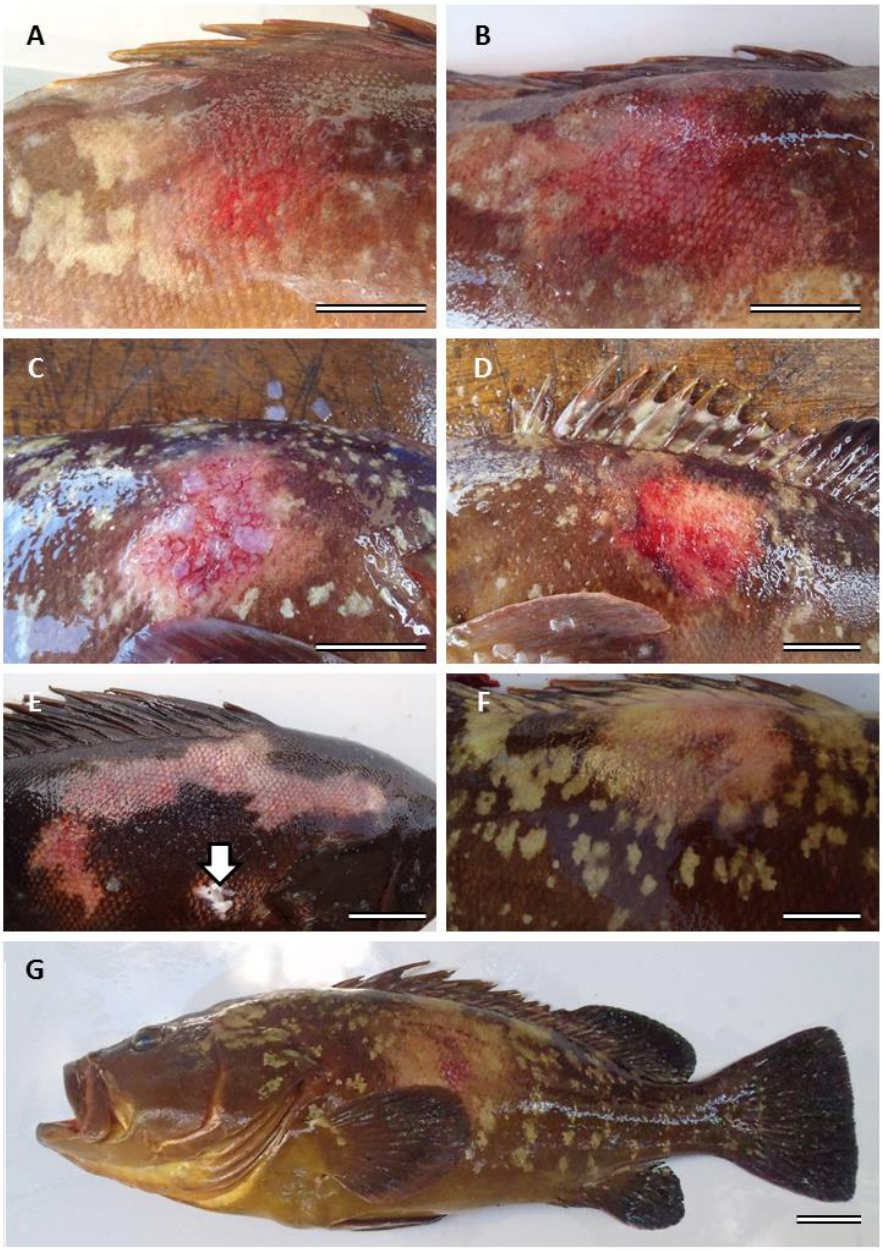

Figure 4.8. Gross appearance of dusky grouper dermatitis (DGD) lesions affecting the trunk of fish across a range of severities. (**A**) Mild haemorrhagic lesion on the flank of fish just below the dorsal fin. (**B**) Severe haemorrhagic lesion. (**C**) Lesion with scale loss, haemorrhagic centre and pale margins. (**D**) Ulcer formation with haemorrhagic margins a pale centre and protruding scales. (**E**) A fused, healing but still partially haemorrhagic lesion on the dorsal flank also bearing an evident injury caused by spear-fishing (white arrow). (**F**) A large, oval, circumscribed area of healing lesion on the dorsal flank. (**G**) Pallor associated with a healing flank lesion. Also note the active centre and pale margins. Scale bars=5 cm

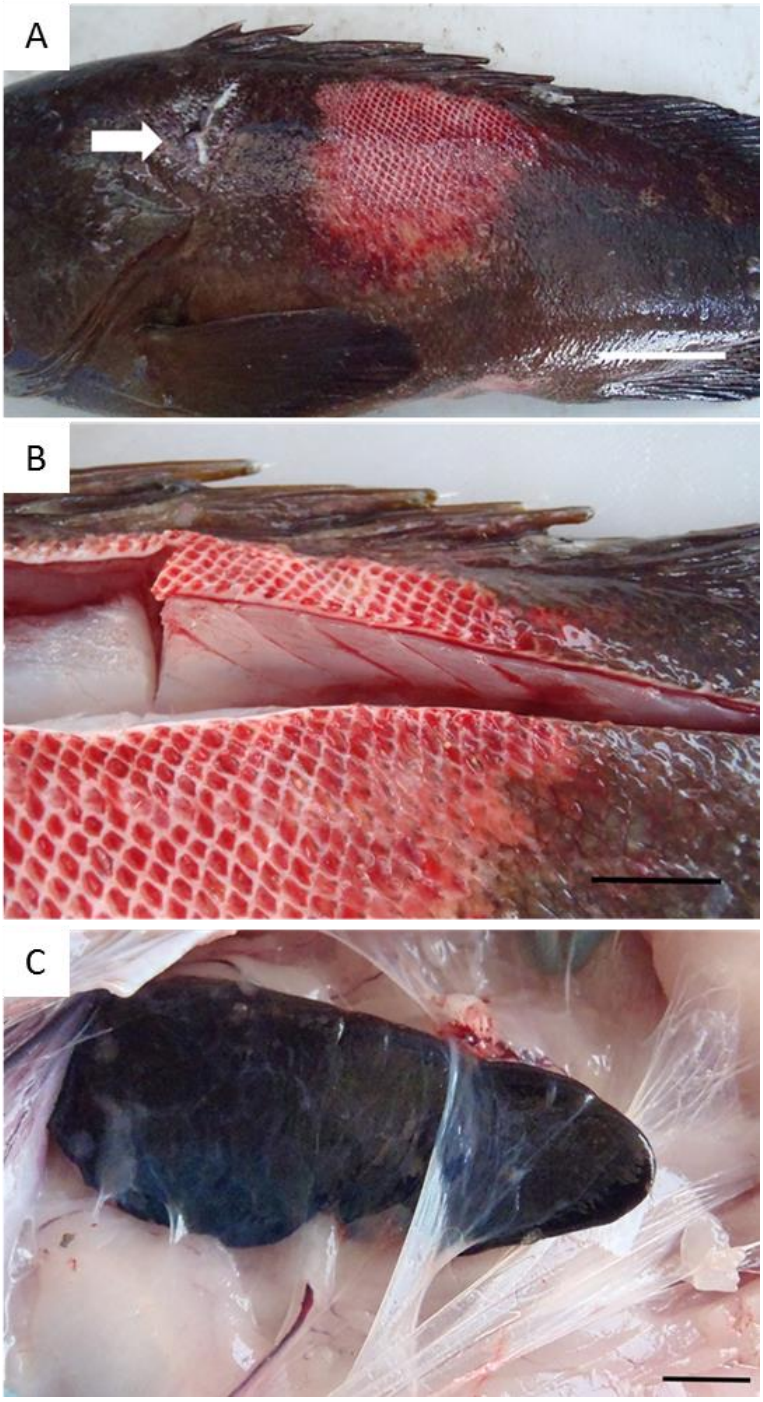

Figure 4.9. (**A**) Gross appearance of dusky grouper dermatitis (DGD) severe lesion affecting the trunk of fish, large roughly round area of ulceration and haemorrhage extending from just next to the base of the dorsal fin to the flank. Also bearing an evident injury caused by spear-fishing (white arrow). (**B**) Incision into the lesion shows the superficial nature of the ulcer involving only the skin while the muscle remains intact (**C**) same fish showing an enlarged and slightly dark spleen. Scale bars: A=5 cm; B & C=1 cm

4.3.2.3 Bacteriology

Mixed yellow, white and creamy bacterial colonies were cultured from skin swabs with no predominant colony type being apparent. There was no bacterial growth from spleen or kidney swabs. No bacteria were detected from Gram and Giemsa staining of the histology slides. Ziehl-Neelsen stain acid fast stain for all stages also provided negative results (**Fig. 4.10**).

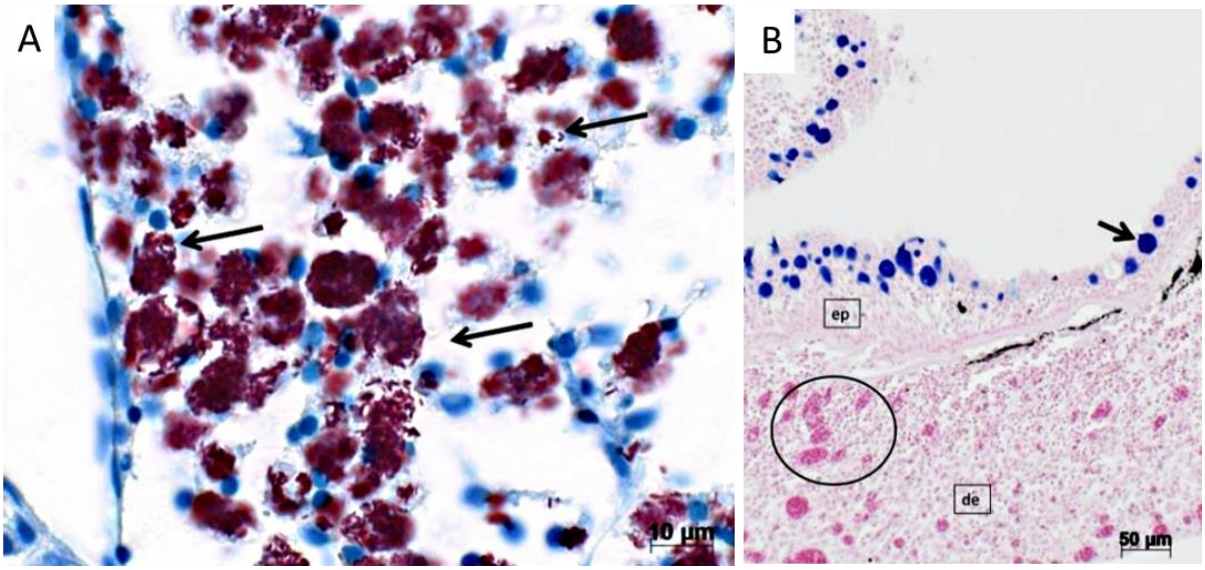

Figure 4.10. Ziehl-Neelsen staining showing (**A**) positive control of a tissue affected by *Mycobacterium marinum* from seahorse *Hippocampus hippocampus*. Notice magenta staining of rod-shaped cluster of bacteria (arrow) indicating acid-fast positive. (**B**) Negative staining of dusky grouper skin section of a late moderate skin lesion of the dermis (de) with congested blood vessels (circle) and epidermal (ep) mucous cells staining blue (arrow). *Abbreviations*: de=dermis; ep=epidermis. (H&E)

4.3.2.4 Histopathology

Lesions principally involved the epidermis and the dermal stratum spongiosum. In severe lesions, the stratum compactum and deeper tissues were affected (**Table. 4.2**).

Table 4. 2. Description of DGD key histological findings of mild, moderate, severe and healing lesions in dusky grouper.

Stage		Tissue changes
Mild		
	Epidermis	Few metazoan eggs, within the mid-epidermal layers, mainly lymphocytic response. Hyperaemia of superficial dermal vessels along with mild infiltration of perivascular eosinophilic granular cells and lymphocytes and the appearance of melanin-containing cells within the epidermis
	Dermis	Gravid adult blood flukes* within blood vessels of deep and superficial dermis, perivascular inflammatory infiltrate. Aggregates of embryonating metazoan eggs*, in upper stratum spongiosum, in proximity to the BM within blood vessels, and superficial capillaries. Eosinophilic granular cells, lymphocytes, vasodilation and congested superficial blood vessels
Moderate		
	Epidermis	Diffused spongiosis, vesicles, lymphocytes, macrophages and RBC's
	Dermis	Necrosis and fibrous exudate, staining bright pink with H&E, scale necrosis, some scale debris and giant cells.
Severe	Epidermis	Spongiosis at the margins of the lesions with sloughing centre and with loss of basal cells
	Dermis	Breaching of BM, aggregation of mixed inflammatory cells and RBC's. A few giant cells and disorganised dermal collagen bundles
Healing		
	Epidermis	Thin epidermis comprising few layers, and a few scattered melanin aggregates within the epidermis
	Dermis	Re-instatement of normal architecture and organised collagen bundles, and presence of newly-formed scales.

*The significance of the presence of the blood flukes to the pathogenesis / staging of lesions has yet to be determined. Only 5 fish with mild lesions were observed in total during the study, all of which contained digeneans. Only a single fish with moderate lesions showed the presence of eggs (no parasite).

4.3.2.4.1 Mild lesions

Mild lesions comprised hyperaemia of superficial dermal vessels along with mild infiltration of perivascular eosinophilic granular cells and lymphocytes and the appearance of melanin-containing cells within the epidermis.

Malpighian cells undergoing mitotic change were found scattered along the epidermis. Melanocytes, which in unaffected skin formed an uninterrupted band lining the basal layer, were clumped into aggregates, along with melanin-containing cells within the epidermis.

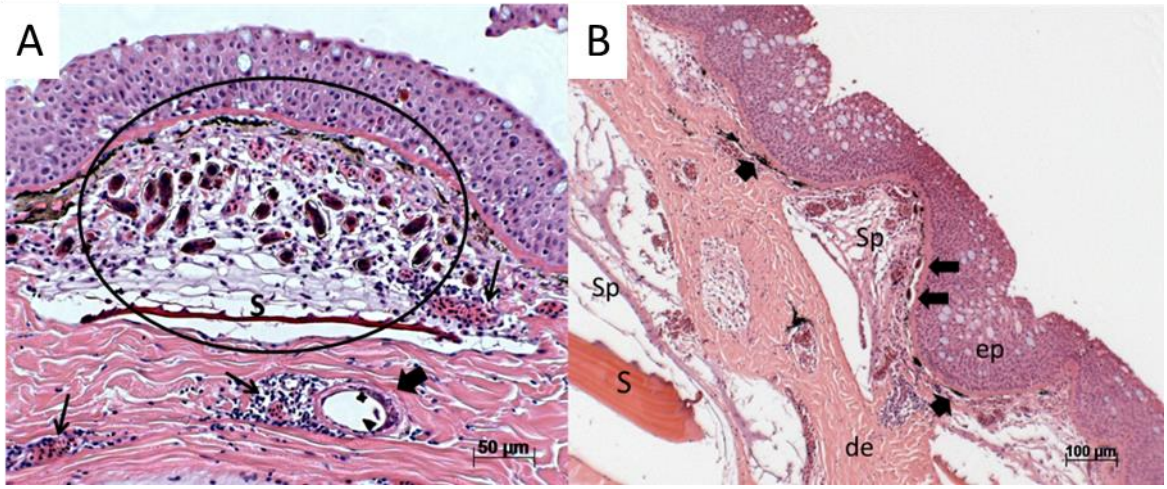

Figure 4.11. Histological sections of dermis and epidermis of dusky grouper affected by mild DGD. (**A**) Inflammatory response in the dermis involving the scales and scale pockets (black arrow) showing perivascular inflammatory infiltrate surrounding the metazoan parasite containing three egg fragments (thick black arrow) and congested blood vessels and distended scale pocket (black circled). (**B**) lesion affecting the skin covering the operculum posterior to the eye. There is also clumping of melanin granules (short black arrow). Metazoan parasite eggs can be seen in proximity to the basement membrane zone (BMZ) (black arrow) where there is congestion of the superficial blood vessels in proximity to the scale pockets. *Abbreviations*: de=dermis; ep=epidermis; s=scale; sp=scale pocket). (H&E)

Embryonated digenean trematode parasite eggs were seen in five fish with mild lesions and in one fish showing an early moderate lesion. These parasites eggs were located in the epidermis, within distended scale pockets and in blood vessels of the *stratum spongiosum* and *stratum compactum*, or within the connective tissue of both the *stratum spongiosum* and *stratum compactum* (**Fig.4.11**) and at the roof of scale pockets in proximity to the basal layer. These eggs were accompanied by an eosinophilic inflammatory response (this will be further discussed in Chapter 5) and a mixed mononuclear inflammatory infiltrate comprising

lymphocytes and macrophages (**Fig. 4.11A**) whilst deeper vessels in the hypodermis were congested. Of the six fish with embryonated digenean trematode parasite eggs, five fish were infected by adult gravid blood flukes carrying similar or identical eggs to those seen in dermal blood vessels, where their presence elicited a mild inflammatory response.

4.3.2.4.2 Moderate lesions

Histopathological investigation found marked inflammation of scale pockets and an increase in lymphocytic infiltration of the epidermal *stratum basale* with pronounced spongiosis of the epidermis and the presence of moderate numbers of melanin-containing cells. Dermal oedema and haemorrhage were also noted, along with increased perivascular inflammatory cell infiltration. Additional features included disorganisation and necrobiosis of connective tissue as well as the presence of activated osteoclasts in proximity to degenerating scales (**Fig. 4.14**). Hypodermis was sometimes involved, with the congestion of blood vessels and the presence of mononuclear inflammatory cells. The lateral line was found partially occluded with hyperplastic and necrotic epithelium (**Fig. 4.12 & Fig. 4.15**) when stained with (MSB) which shows the disruption of connective tissue (**Fig. 4. 13**).

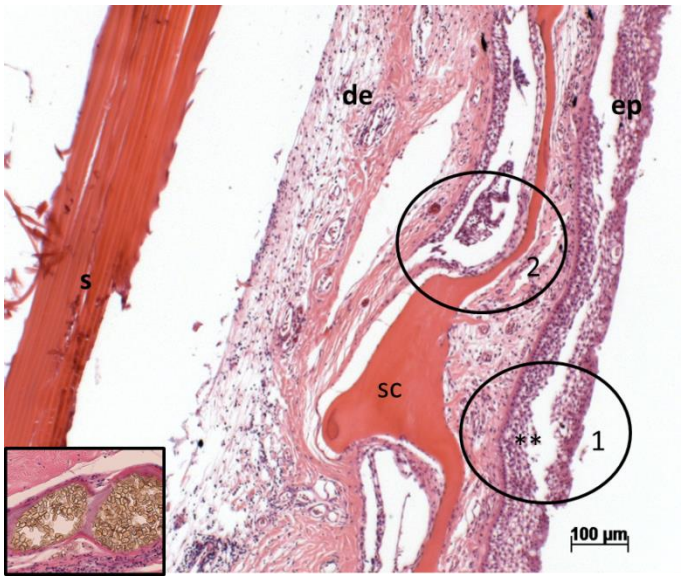

Figure 4.12. Moderate skin lesion in the region of the lateral line on the dusky grouper trunk showing scale resorption (S) and spongiosis of the cells lining the lateral line (black circle 2). Epidermal spongiosis (black circle 1) and separation of the epidermis (**), which is a sectioning artefact reflecting the fragility of the epidermis with the absence of melanocytes at the basement membrane, with few scattered melanin containing cells. Occasionally didymozoan parasites were seen within the dermal connective tissue (insert). *Abbreviations*: de=dermis; ep=epidermis; s=scale; sp=scale pocket. (H&E)

The epidermal spongiosis involved the entire mid-thickness of the epidermis, rendering the epidermis fragile and causing it often to separate during sectioning (**Fig. 4.12**). The basement membrane zone (BMZ) seemed to be a focus for the inflammatory process with signs of discontinuation of the basement membrane (BM), melanocyte clumping and a thickened wave-like appearance of the BM with weak PAS staining.

The spongiotic areas, developing into vesicles in the mid-section of the lesions, contain few lymphocytes and very few macrophages. As part of the process of necrosis, basal cells underwent hydropic degeneration and apoptotic cells were seen containing round fragmented chromatin and red blood cells. Lymphocytes and macrophages were seen infiltrating between Malpighian cells (**Fig. 4.16**), as well as clusters of bacterial colonies, which were seen in the superficial dermis, mixed with necrotic tissue (**Fig. 4.16E**).

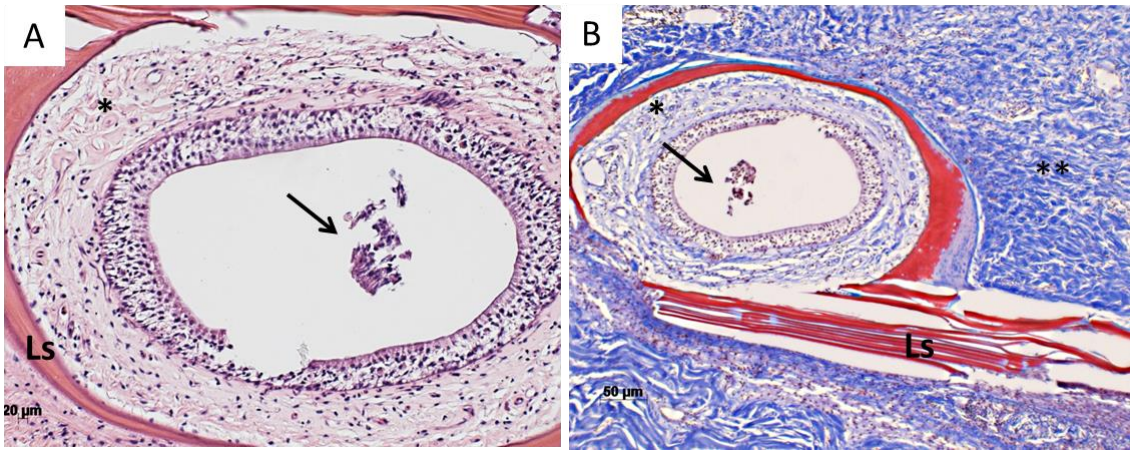

Figure 4.13. Skin lesions taken from the flanks of dusky grouper (**A**) stained with H&E and (**B**) stained with MSB. Shown are a lateral line scale (Ls) undergoing resorption, spongiosis of epithelial cells lining the lateral line canal containing debris (black arrow) and the canal lumen contains sloughed epithelial cells surrounded by connective tissue undergoing necrobiosis (*), while the disarrangement of connective tissue (**) can be seen in B

The formation of vesicles and basal cell layer dissociation from the basement membrane, possibly the pre-stage leading to epidermal sloughing, were observed in the severe lesions.

The epidermis and basement membrane remained intact during both mild and moderate stages of lesion formation, with the basal cells attaching firmly and forming an almost tombstone-like appearance. Occasionally didymozoan parasites were seen within the dermal connective tissue (**Fig. 4.12 Insert**), these having frequently been previously described to affect dusky grouper (**see Chapter 2.**).

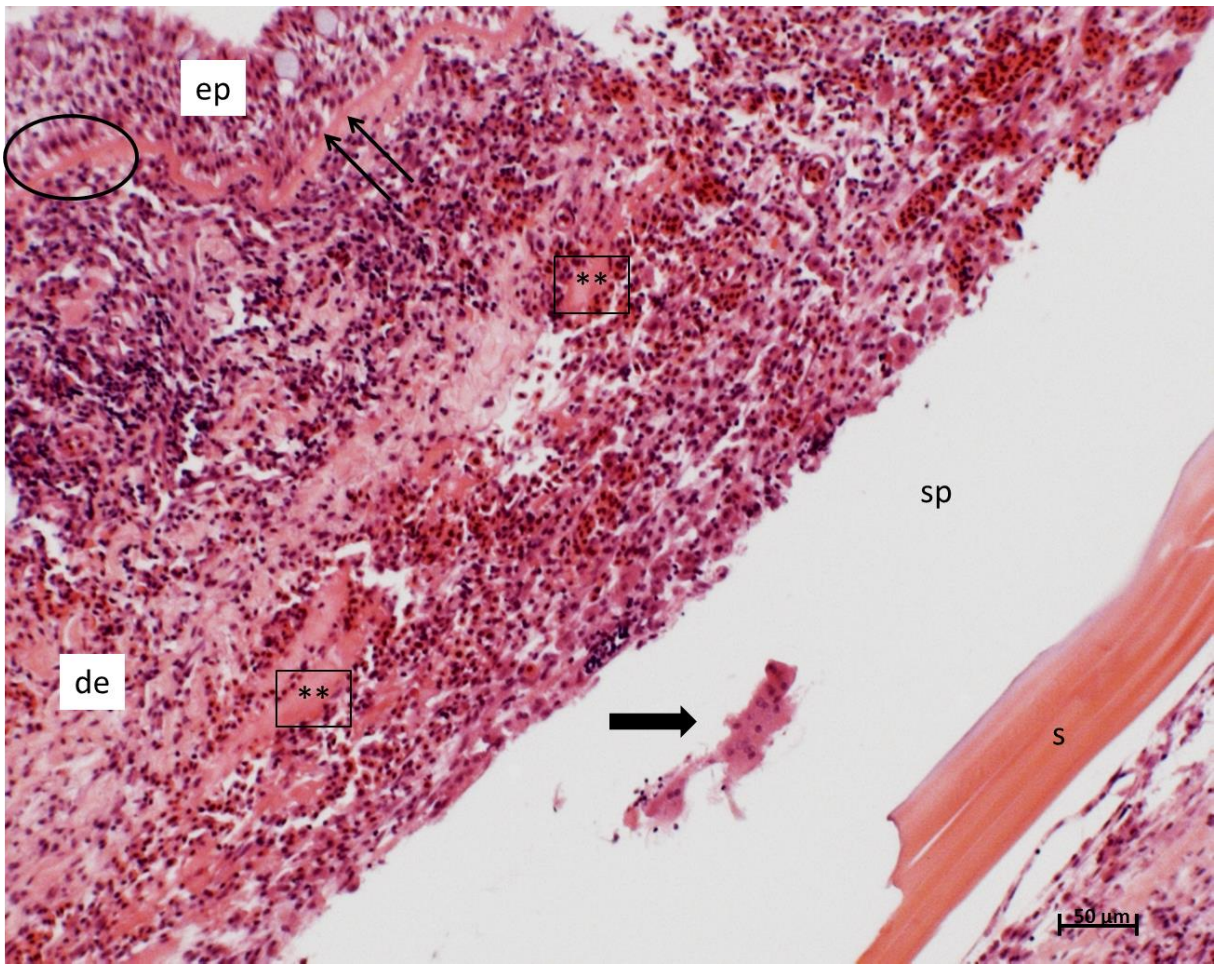

Figure 4.14. Superficial dermis showing fibrinoid necrosis (** in box), osteoclastic giant cell (black arrow), basal epidermal and loss of melanin granular cells and erythrocyte extravasation. *Abbreviations*: de=dermis; ep=epidermis; s=scale; sp=scale pocket (H&E)

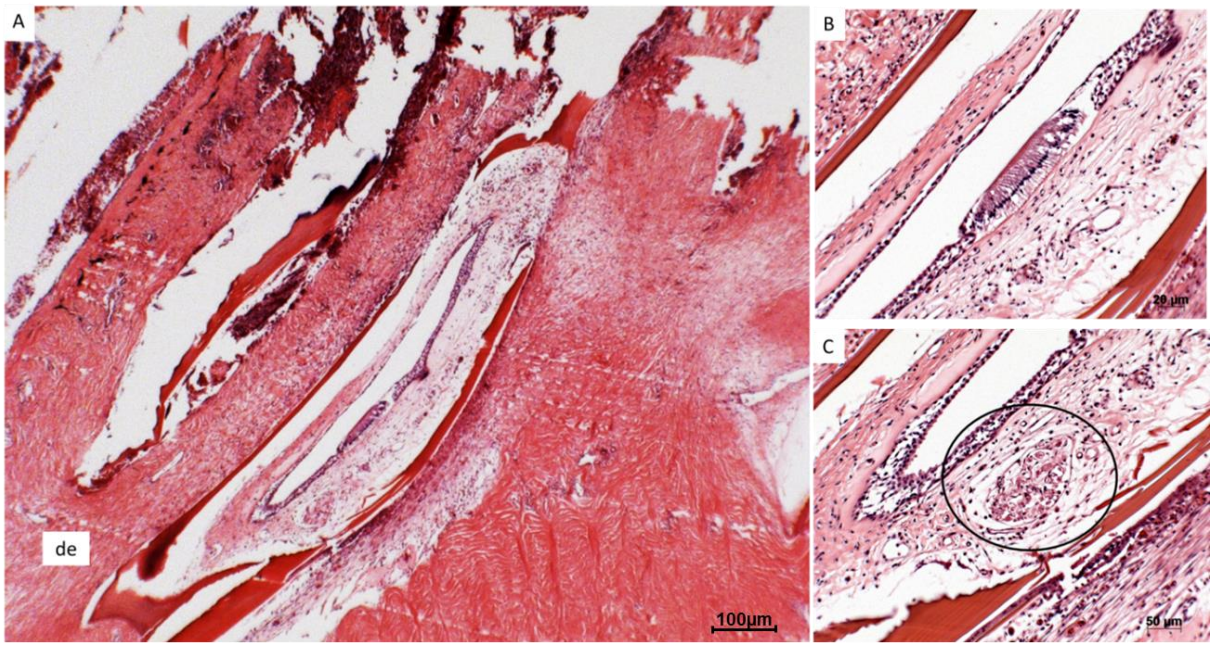

Figure 4.15. (**A**) Lesion at the lateral line area from skin lesions affecting the flank, showing increased cellular proliferation (**B**), scale resorption and (**C**) a few inflammatory cells infiltrating nerve bundles (black circle). *Abbreviations*: de=dermis (H&E)

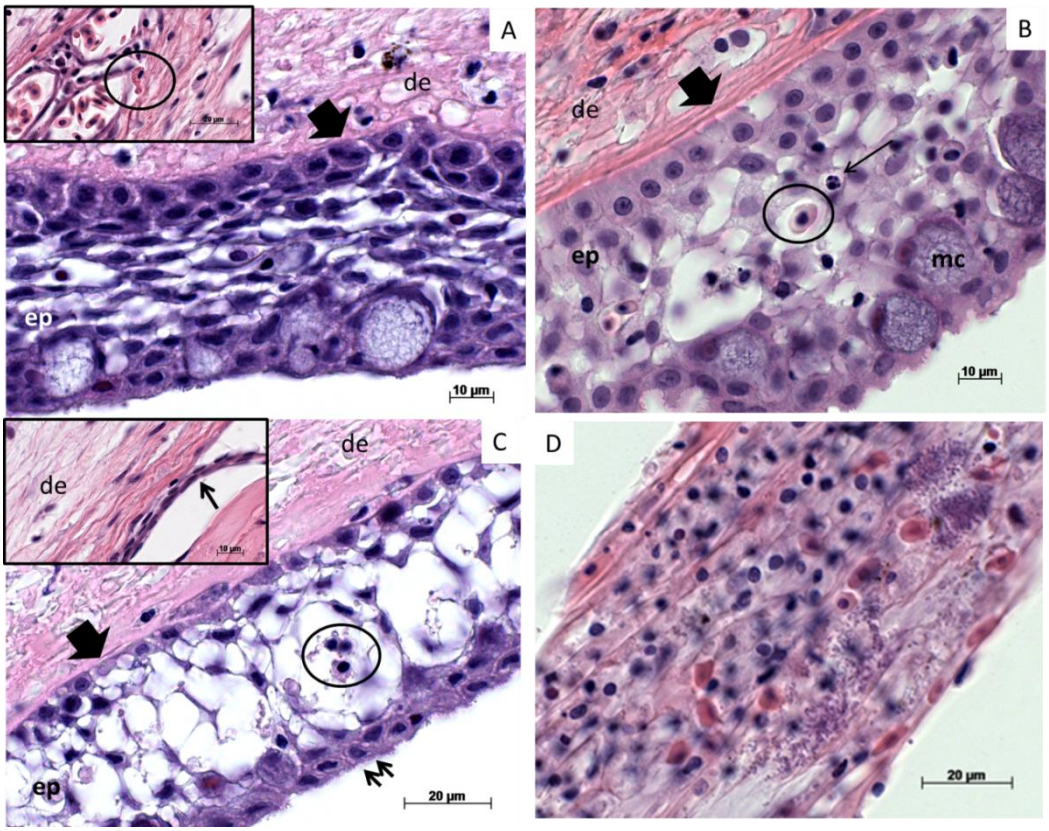

Figure 4.16. Moderate skin lesion, (**A**) epidermal spongiosis and the presence of few lymphocytes in mid epidermis, with degranulation eosinophilic granular cells (black circle) (insert) and absence of melanocytes (black arrow). (**B**) Rounded basal cells, some losing their attachment to the basal layer (thick arrow); a few inflammatory cells can be seen, RBC (black circle) and cells undergoing necrosis (long black arrow). (**C**) Hydropic degeneration of malpighian cells. The outermost cells, remaining intact, are flattened retaining their structure and continuity (double black arrow). the dermis contains sacles undergoing adsorption (insert). Few lymphocytes and one macrophage can be seen within the intra-epidermal vesicle (black circle). The basement membrane has lost its horizontal striation, showing persistent absence of melanocytes (thick black arrow). (**D**) Bacterial colonies are occasionally seen. *Abbreviations*: de=dermis, ep=epidermis, mc=mucous cells. (H&E)

4.3.2.4.3 Severe lesions

The centre of the lesions was characterised by a breach of the basement membrane (ulceration) (**Fig. 4.17**) and the inevitable consequential loss of osmotic integrity. Severe lesions lacked the epidermis, which had sloughed. The basement membrane was no longer

visible. In the dermis scales were resorbed, and scale pockets had disappeared, with occasional remnants of scale debris (**Fig. 4.18**).

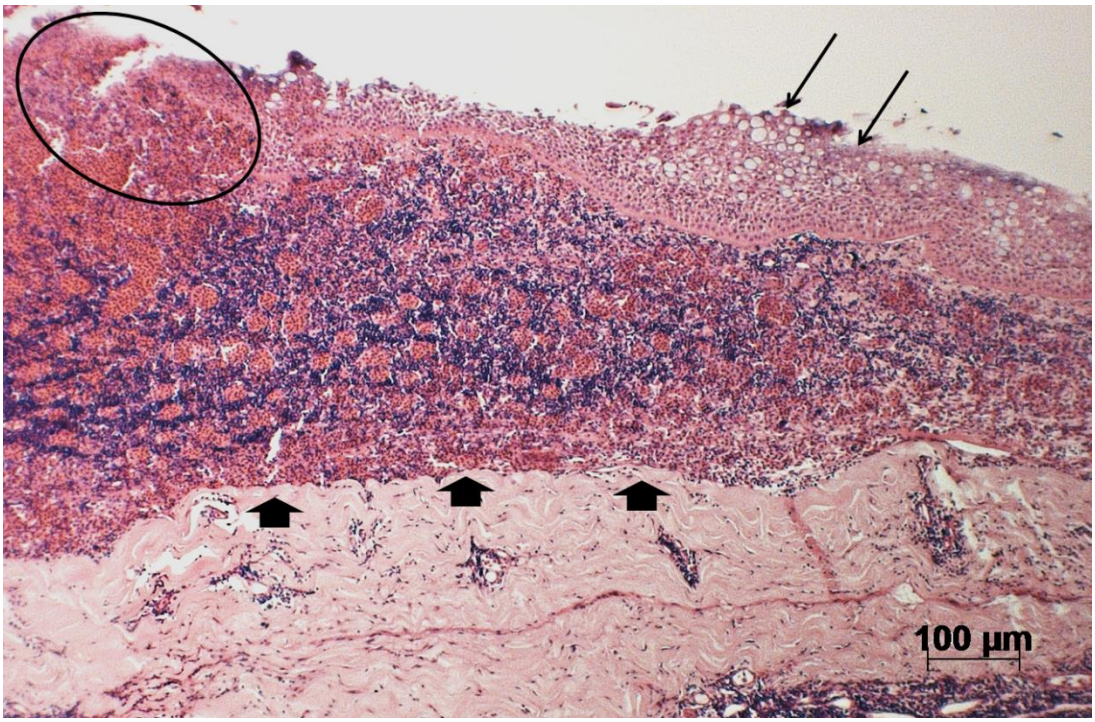

Figure 4.17. Epidermis is partially sloughed and infiltrated by extravasated RBC's originating from the dermis (circle), which is covered in the margins of the lesions by newly formed epidermis evident from the small mucous cells and disorganised epithelial cells (thin black arrow). Loss of scales and scale pockets and a clear demarcation zone between the severely affected and non-affected dermis (black arrow heads) are also evident. (H&E)

The dermis was markedly infiltrated with inflammatory cells comprising mainly eosinophilic and small basophilic mononuclear cells, situated between disorganized collagen bundles. Scattered cells undergoing necrosis were present, along with a few debris-laden macrophages, a general loss of normal architecture, as well as scattered (osteoclastic-type) multinucleated giant cells (**Fig. 4. 18 Insert & 4.19**).

The *stratum compactum* was, at this stage, often involved in the inflammatory reaction. A clear demarcation zone was observed between different inflammatory zones and normal

dermal tissue (**Fig. 4.17**). Lateral line scales involved in the inflammatory process were undergoing adsorption, and the lateral line lumen was often partially occluded by hyperplastic epithelial cells.

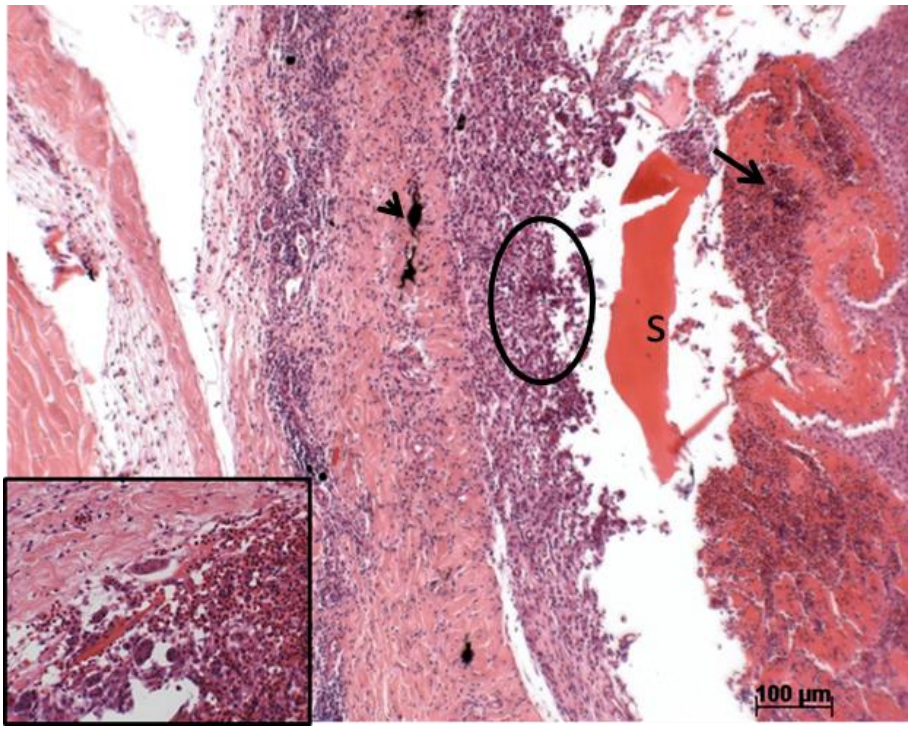

Figure 4.18. Histological section of the dermis from the centre of a severe lesion with osteoclastic-type multi nucleated giant cells, scale debris (insert), loss of normal architecture showing extensive haemorrhage (black arrow), inflammatory infiltrate (black circle), clustered melanin-containing cells (black arrow head), loss of scale pockets and residual scale debris (s). *Abbreviations*: de=dermis; ep=epidermis; s=scale (H&E)

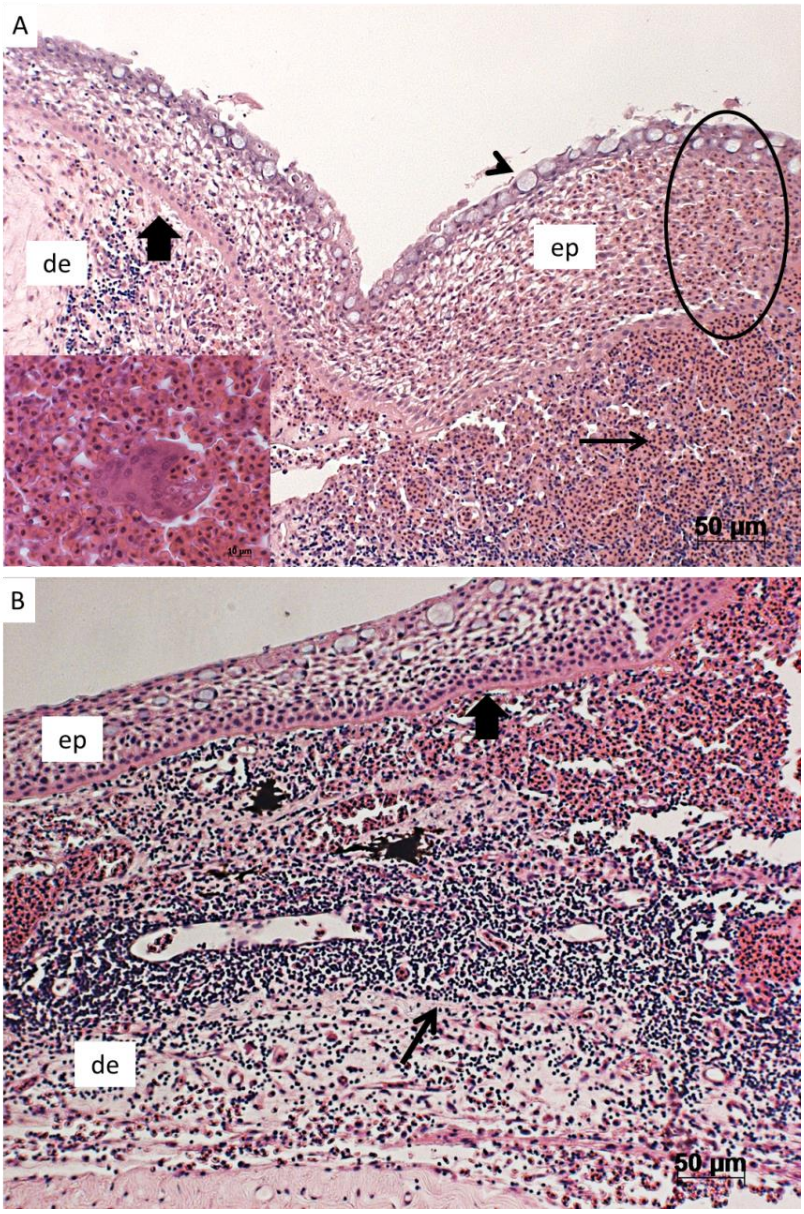

Figure 4.19. (**A**) Congestion of superficial dermal vessels from a cross section in the centre of a severe skin lesion, severe vascular injury, extravasated erythrocytes (thin black arrow) migrating to the epidermis (black circle), which is spongiotic. Giant cells were seen at this stage (insert) and lack of melanophores at the base of basement membrane (thick black arrow). The epidermis (ep) is severely spongiotic but mostly localised to the mid-section while the outer most layers retain its structure and mucous cells (black arrow head). (**B**) The absence of melanophores (thick black arrow) and the presence of basophilic inflammatory cells within disorganised dermis which has lost its normal architecture (thin black arrow) and small amount of melanin debris within the dermis (de), can all be seen. *Abbreviations*: de=dermis; ep=epidermis. *Abbreviations*: de=dermis; ep=epidermis; s=scale. (H&E)

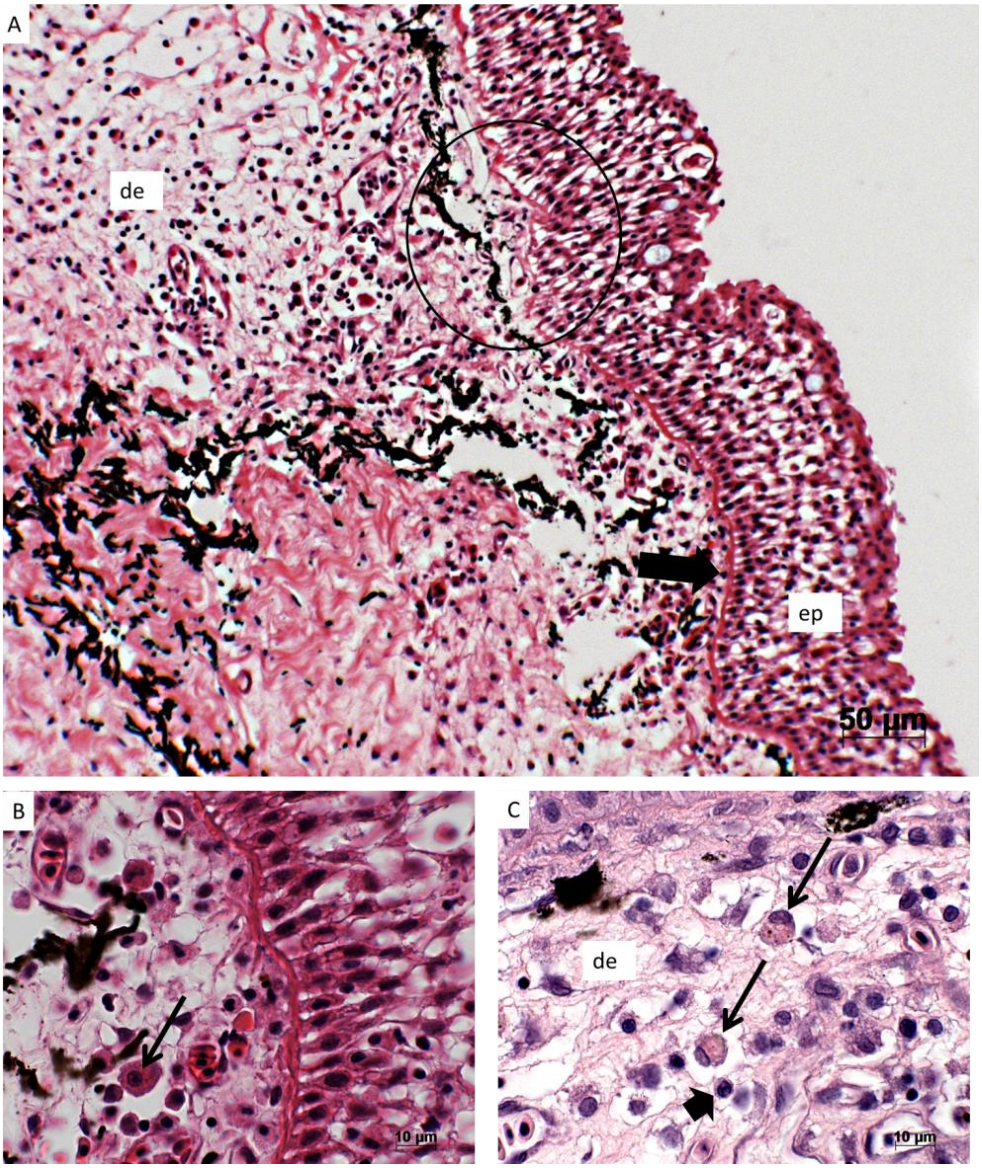

Figure 4.20. (**A**) Spongiosis showing loss of basal cell attachment to basal membrane (black circle). (**B**) One nucleated giant cell (black arrow). (**C**) Presence of macrophages (long black arrow) and lymphocyte (thick black arrow). *Abbreviations*: de=dermis; ep=epidermis (H&E)

Towards the margins of the haemorrhagic foci, new epidermal layers were seen covering parts of the ulcer, and immature mucous cells. Covering haemorrhagic foci were surrounded by mononuclear basophilic inflammatory cells. New epidermal cells were also seen undergoing spongiotic change, possible reflecting the tissue change in the dermis (**Fig. 19 & Fig. 20**).

In some severe lesions prominent RBC'S originating from the dermis migrated to the epidermis, occupying a large proportion of the epidermis (**Fig. 4.19A**).

4.3.2.5 Healing

4.3.2.5.1 Gross morphology

Healing seemed to take place from margin to centre, with the lesion margins becoming pale and losing their haemorrhagic appearance. In later stages this extended to the centre of the lesion which became almost white against the surrounding skin colour yet still kept its irregular margins (this was observed in lesions affecting the head, flanks and cauda peduncle) (**Fig. 4.7D&F**) (**Fig.4. 9E&F&G**). Some lesions posterior to the eye at the advanced healing stage appeared mottled (**Fig. 4.25D**). Moving from the centre inwards, the lesions showed increasing pallor, decreasing in size, leaving a persistent white centre and margins resuming normal skin colour (**Fig. 4.21B**).

The lesions affecting the isthmus of the jaw (which is normally yellow) retained a slightly darker discolouration against the normal surrounding skin (**Fig. 4.21A**). As previously mentioned, fish were affected by lesions at different stages of development, especially in multifocal lesions where healing lesions were also seen in fish with active lesions, such as moderate stages and severe stages (**Fig. 4.21C**).

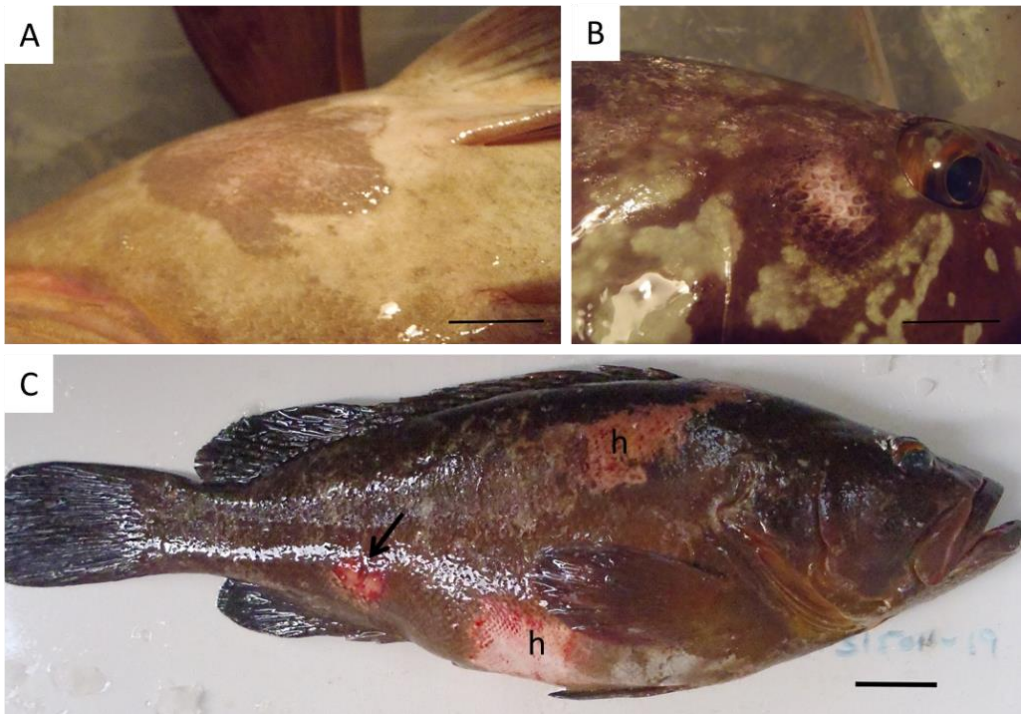

Figure 4.21. (**A**) Isthmus of the jaw; healing lesions are visible due to discoloration and darkening of the skin compared to pale surrounding tissue. (**B**) The same fish with a healed lesion posterior to the eye; in this the skin is white against the brown surrounding skin, plus late-stage healing lesion showing persistent loss of melanisation, with the margins returning to almost normal adjacent colouration. (**C**) Early healing lesions, at two stages of healing (h), and moderate lesion close to ventral fin (black arrow). Scale bar=5cm

4.3.2.5.2 Histopathology

Evidence for healing was seen at the margins of most stages, especially moderate and severe.

Healing lesions were characterised by the movement of malpighian cells from the margins of the ulcer, to form a four to five times thicker epidermis, compared to the adjacent ‘‘healthy’’ margins. Reflecting the process of restoration of tissue integrity, finger-like projections (**Fig. 4.22B**) were seen; these comprised piling-up epidermal cells, including un-differentiated epidermal cells and mucous cells (**Fig. 4.22A**).

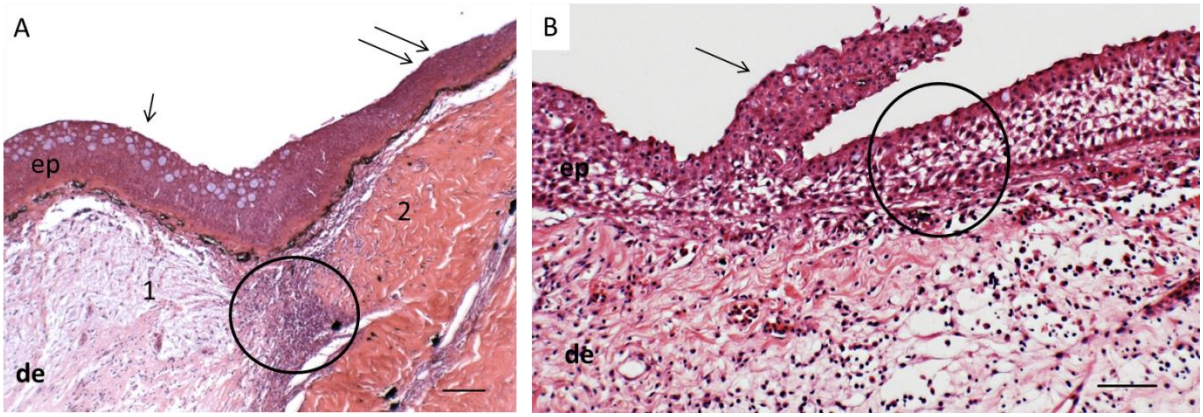

Figure 4.22. (**A**) Healing skin marked by loss of normal dermal architecture (1), covered by newly-formed epidermis originating from the adjacent Malpighian cells (two black arrows), with an increased number of unorganised mucous cells covering the active lesion (one black arrow). A band of inflammatory cells (black circle) separates the margin of the lesion from adjacent healthy skin (**B**) pilling up of Malpighian cells forming finger-like projections (black arrow) covering an early healing lesion. The new epidermis is often spongiotic (black circle). *Abbreviations*: de=dermis; ep=epidermis. Scale bar: (A)=100 µm; (B)=50 µm (H&E)

Epidermis covering a former ulcer was thin, and newly reformed scales were marked by their strong basophilic staining pattern. Epidermis lining the lateral line showed signs of recovery, but some were still seen with spongiotic changes. Throughout the dermis scattered lymphocytes were still seen within thin collagen bundles, and basement membrane was lined by clumped melanocytes (**Fig. 4.23**). During some of the healing stages, haemorrhagic foci were still present surrounded by basophilic inflammatory cells including lymphocytes, and covered by newly formed epidermis, showing an increased mucus secretion activity (**Fig. 4.23**). Grossly, these lesions give the skin a petechial appearance against a pale background.

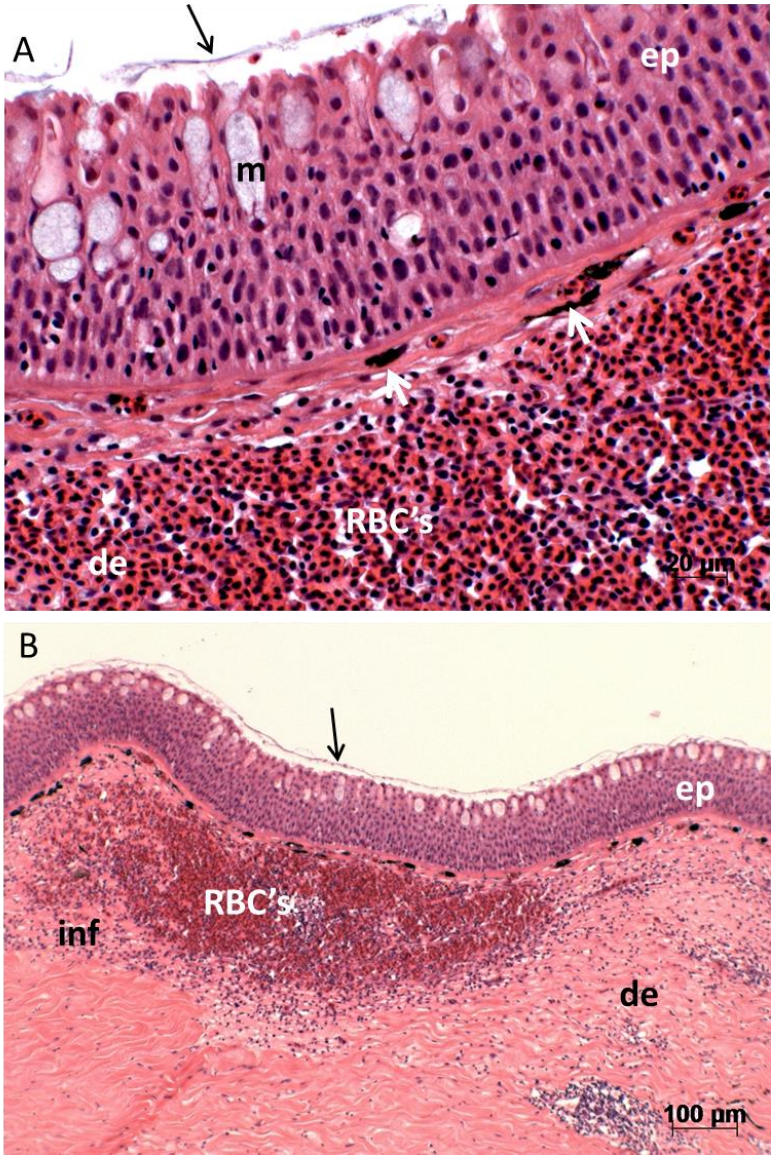

Figure 4.23. (**A**) Healing lesion covering an active dermal lesion. Newly formed epidermis is covered by a film of mucus (black arrow), secreted from the newly formed mucous cells (m). Newly formed melanocytes are adjacent to the basement membrane (white arrows). (**B**) Resolution of the inflammation in the dermis, which is divided into two zones, one haemorrhagic zone filled with extravasated red blood cells and next to it the inflammatory infiltrate zone. A thin mucous film can be seen covering the epidermis (black arrow) *Abbreviations*: de=dermis; ep=epidermis; inf=inflammatory infiltrate; RBC's=Red blood cells (H&E)

Healing was marked by re-organisation of dermis back to its normal architecture and regrowth of scales, which were often accompanied by lingering inflammatory cells (**Fig. 4.25B**). In the almost completely healed lesions these features were resolved, leaving largely

normal-appearing skin with a slightly mottled pale centre (**Fig. 4.25A**). Occasionally melanin aggregates were seen at the base of the dermis and within the epidermis (**Fig. 4.26**).

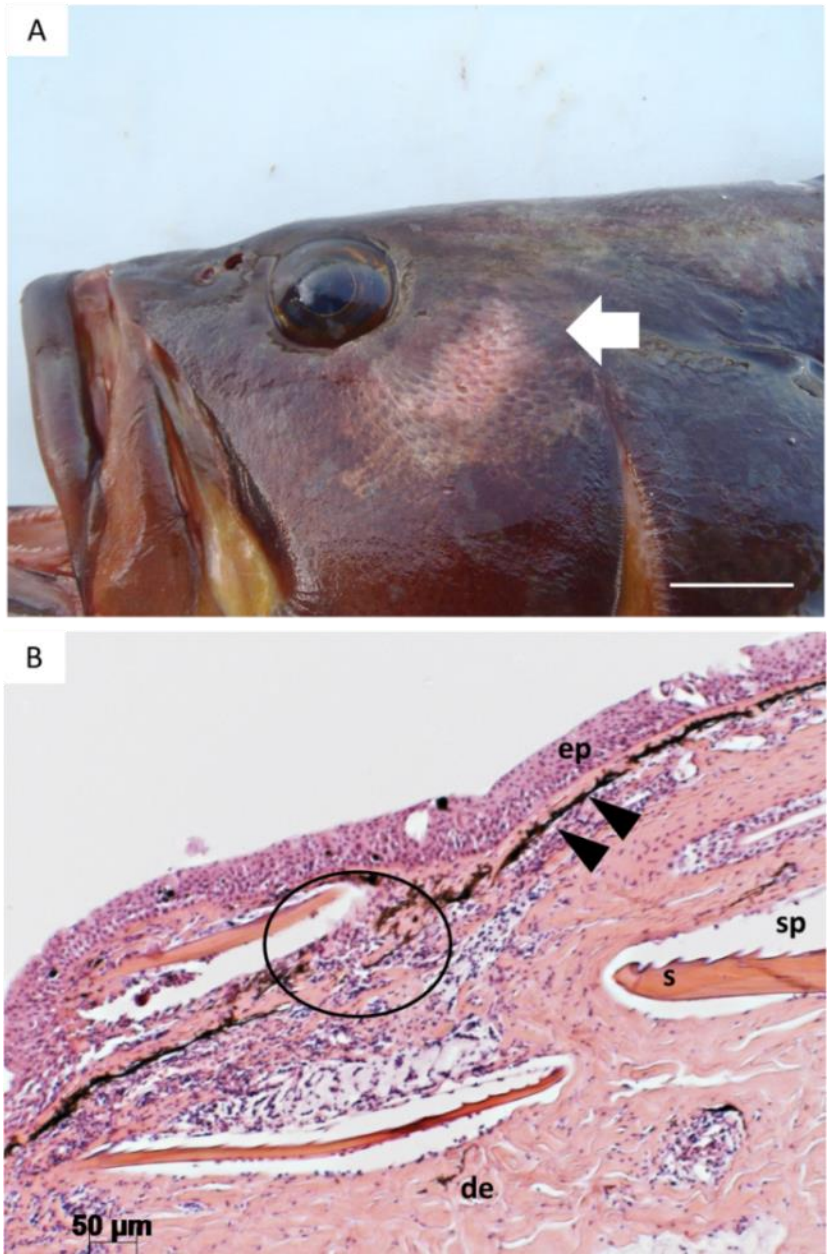

Figure 4.24 Dusky grouper caught in October 2014 and presenting a healing lesion. (**A**). With the gross appearance of the healing process characterized by pale areas visible against the dark surrounding skin in lesions posterior to the eye (black arrow). (**B**) Foci of inflammatory cells persist in dermis (black circle) but the continuity of melanocytes has been restored (double black arrow head) as has the epidermis (ep) and the restitution of normal dermal (de) architecture. *Abbreviations*: s=scale; sp=scale pocket. Scale bar=2 cm. (H&E)

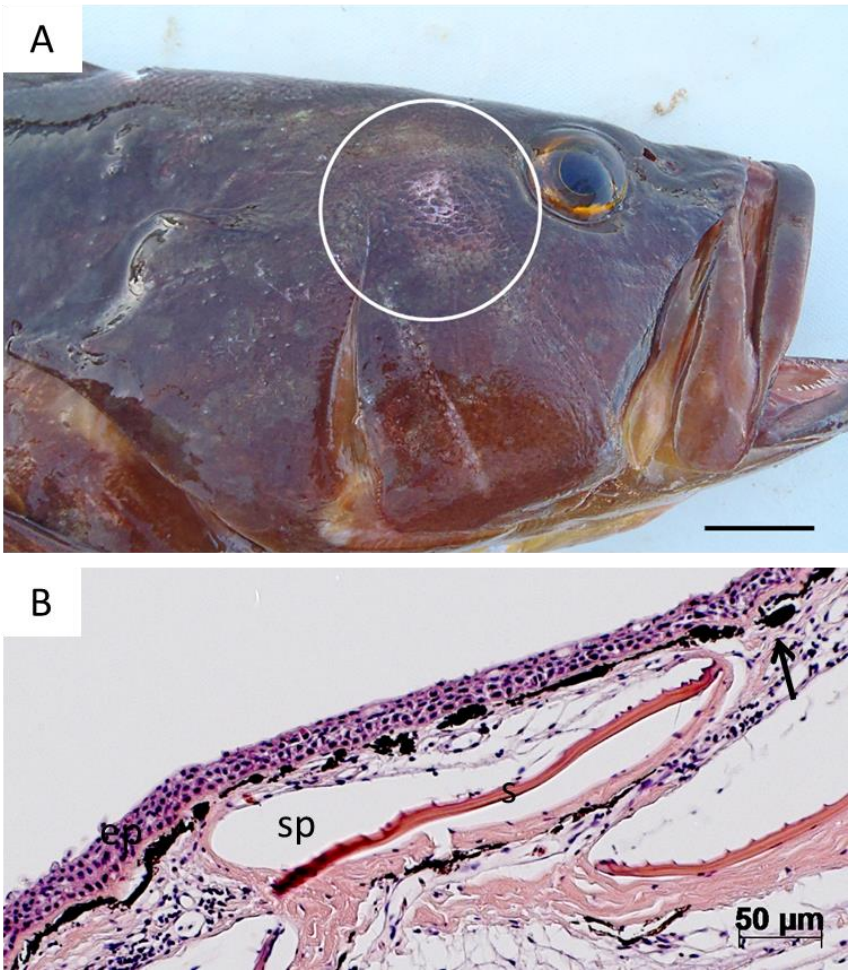

Figure 4.25. Dusky grouper caught in October 2014 with healing characterised by the (**A**) mottled gross appearance of healing skin taken from skin covering the operculum (white circle). (**B**) In the corresponding histological section, the 3-4 cell thick epidermis (ep) contains few mucous cells and the basement membrane is underlined by melanocytes (arrow). *Abbreviations*: sp=scale pocket. Scale bar=2 cm. (H&E)

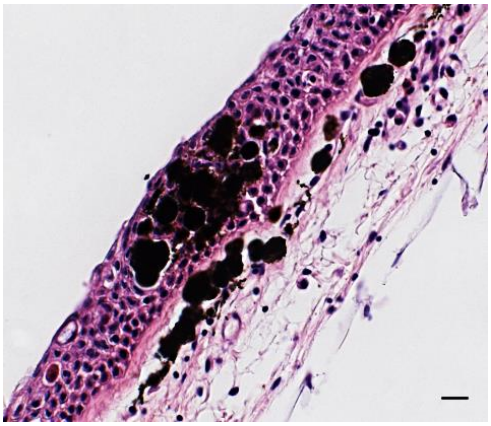

Figure 4.26. Healing lesions, late stage epidermis contains melanin aggregates within the epidermis probably originating from the dermis. Scale bar=20 µm. (H&E)

4.3.3 Histological change to the internal organs

4.3.3.1 Histopathological description

Although occasionally small colonies of bacteria or fungal hyphae were seen in severe lesions, there was no indication for a generalized infection. By and large, other organs were normal *e.g.* eyes, liver *etc.* except for the mild changes expected from increased demands for inflammatory cells, such as mild hyperplasia of the renal interstitium.

The heart of some fish was affected by multi focal myocarditis of the ventricle, with multi-focal lymphocytic aggregation, and few melano-macrophage centres (MMC's); however no blood flukes or eggs were seen in the heart, although further sampling trips are warranted for the future study of infected fish to confirm these findings. Myxozoans were frequently seen trapped in renal MMC, and myxozoan *Zschokkella* sp. often found within the lumen of bile ducts (**Fig. 4.28**). These findings are further mentioned in **Chapter 2**.

4.3.3.2 Parasitic infections

Dusky grouper are affected by a number of pathological changes including *Philometra* sp. infection of the gonads, encysted larval stages of digenetic parasites and encysted didymozoidae parasites attached to filaments of both gills and pseudobranchs; these findings were further mentioned in **Chapter 2**.

4.3.3.3 Case definition

All the above mentioned results are listed in a summarised case definition given in **Table. 4.3**. and adapted from that provided by Maddocks *et al* (2015) for the development of a case definition for the emerging skin condition 'Puffy Skin Disease'.

Table.4.3. Case definition for dusky grouper dermatitis (DGD), adapted from that of Maddocks *et al.*(2015) for "Puffy Skin Disease"

	Signalment	Dusky grouper, artisanal fisheries and spearfishing
Epidemiology		
	Aetiology	Unknown, suspected blood fluke needs further study (Rizgalla *et al*. 2016a)
	Age and size of fish affected	> 1000 g, > 420 cm TL
	Spread of disease	Not available
	Prevalence within fisheries	Approximately 15-20 % (Rizgalla *et al.* 2016 a&b)
	Geographic spread	Wild populations along the Libyan coastline (Rizgalla *et al* 2016b)
	Country and year first observed	Libya 2013, suspected dating to 2008 (Rizgalla 2016b)
	Temperature range	15-27 °C
	Season	All year, evidence for clinical resolution of the lesions
Clinical signs		
	Behaviour	Seem not affected by the lesions (Rizgalla *et al* 2016b). Still feeding
	Associated with mortality	No evidence
Gross clinical presentation		
	Lesion	Skin lesions only
	Location	Posterior to the eye, flanks, caudal peduncle and isthmus of the jaw. May occur unilaterally or bilaterally, usually one lesion per flank. Lesions may coalesce to give large area involvement
Gross pathology of lesion		
	Colour	Haemorrhagic, pale centre, ulcerated
	Size	Range from 5 to 50% total flank surface area
	Shape	Overall roughly oval, affecting the flanks Often in proximity to lateral line, poorly circumscribed when affecting the isthmus of the jaw and in severe stage
	Erosion	Erosion, scale loss in severe stage and ulceration
	Topography	Raised
	Severity index	Mild: Colour haemorrhagic, occasionally with petechiae, scales present Moderate: colour variable, pale centre and halo like haemorrhagic margins, raised, scales loose. Severe: colour haemorrhagic dramatically raised and oedematous, scale loss and focal or coalescing ulcers
	Healing	Pale and mottled
Histopathology of lesion		
	Epidermal	Spongiosis
	Dermis	Stratum spongiosum oedematous, scale pockets enlarged infiltrated by mixed inflammatory cells
	Hypodermis	Unaffected
	Inflammatory cell involvement	Mild, moderate and severe diffuse inflammatory cell infiltrate throughout skin layers. Cell types seen: polymorphonuclear leucocytes and monocytes, lymphocytes and eosinophilic granular cells
	Severity index	Mild, moderate or severe, graded individually based on grade of change from normal epidermal architecture
	Healing	Epidermis presence of low numbers of mucous cells, mild inflammatory cell in dermal stratum spongiosum. suggestive of clinical resolution

4.4 Discussion

A multifocal dermatitis, presenting as a continuum spanning mild, moderate and severe lesions is described for wild-caught dusky grouper from Libyan coastal waters. No other fish species captured at the same time and in the same locations displayed similar lesions. The described lesions appeared to have a well-defined profile of development, and frequently affected specific areas of the skin, particularly the flanks and the skin posterior to the eye. Moreover they were seen only on fish measuring more than 42 to 92 cm TL (1.4 to 18 kg). The lesions were seen all year round and at water temperatures ranging from 15-27°C, suggesting that no temperature threshold mediating the presence or absence of lesions was observable from the data.

Marino & Azzuro (2001), Vendramin *et al*. (2013) and Kara *et al*. (2014) reported that dusky grouper infected by nodavirus displayed skin lesions, with affected fish weighing 1.5 to 15 kg (Marino & Azzurro 2001) and 0.5 to 4 kg (Kara *et al*. 2014). None of the reported features of nodaviral infection were seen in the present study. In particular, skin lesions in nodavirus-infected fish differ from DGD,in that skin lesions of nodavirus-affected fish typically display lesions to the head and trunk which have been attributed to mechanical damage following the loss of equilibrium and sometimes prolonged recumbence due to the encephalitis seen in this disease. Lesions associated with nodavirus were seasonal, occurring between late September and October, and appeared to affect several grouper species (Kara *et al*. 2014). Furthermore, none of the other clinical signs and lesions that have been documented in association with nodavirus infections in grouper were seen; these include loss of appetite, emaciation, hyper-inflated swim bladder, head erosion, corneal opacity and vacuolation in the brain and retina, with accompanying mortalities (Al-Attar *et al.* 2009; Vendramin *et al*.

2013; Kara *et al*. 2014). In the current study the observation of partially digested prey / long-line bait within the stomachs of the sampled fish provides evidence for active feeding in affected individuals, something not observed in nodavirus-infected fish.

Al-Attar *et al*. (2009) reported lesions affecting dusky grouper and gold blotch grouper found in Libyan waters during a disease outbreak in 2008, and found that fish displayed a variety of symptoms including corneal opacity, a distended abdomen and systemic involvement. Al-Attar *et al*. (2009) and Al-Attar *et al*. (2013) suggested a viral aetiology, based on histological findings typical of viral encephalopathy and retinopathy, while Soliman *et al*. (2011) isolated *Pasteurella damsela* subsp*. piscicida (= P. piscicida)* from kidney and spleen, suggesting a bacterial aetiology.

Pasteurella damsela subsp. piscicida (= P. piscicida) and *M. marinum* were isolated during disease outbreaks in dusky grouper in Egypt (Marzouk *et al*. 2009, Eissa *et al*. 2011) and these were accompanied by clinical signs including skin lesions, inflammation in the internal organs, empty intestine, and corneal opacity.

Although only a few fish were classified as displaying mild lesions, *i.e.* 5/26 fish with sizes ranging from 57-64 cm TL, digenean trematode eggs and gravid adult digenean trematodes carrying similar eggs, were seen in lesions from all five. The egg seems to originate from the intra-vascular adult parasite containing eggs bearing similar morphological characteristics to the eggs found in dermal capillaries and interstitium as well as the epidermis.

Digenean parasites typically infecting the circulatory system of fish are generally members of the family Aporocotylidae (Smith 2002). While the adults usually do not elicit severe inflammatory responses from the host (Bullard *et al*. 2001), the eggs are more usually

pathogenic, with severe cases often leading to mortalities, which may reach up to 10% in pen-cultured southern bluefin tuna (SBT), *Thunnus maccoyii* (Castelnau, 1872), infected by *Cardicola forsteri* Cribb, Daintith *et* Munday, 2000 (Dennis *et al.* 2011). Padrós, Zarza & Crespo (2001) described eggs being released by the adults of an unidentified sanguinicolid trematode, present in the heart of gilthead seabream *Sparus aurata* (L.); eggs were also found in the gills, to which they had been transported via the blood circulation. The miracidium is the final stage of *Cardicola aurata* Holzer, Montero, Repullés, Sitjà-Bobadilla, Alvarez-Pellitero, Zarza *et* Raga, 2008 and hatches from eggs within the final host, into the aquatic environment (Holzer *et al*. 2008). The eggs of *Cardicola forsteri* elicit a granulomatous reaction involving mixed inflammatory cells, basophilic and eosinophilic granular cells. The sheer number of eggs occluding gill filaments is responsible for the reduced capacity of gills for gas exchange, often leading to the fish dying by asphyxiation (Dennis *et al*. 2011). The gills of the fish in the current study, however, had no eggs within the gills, and no fresh eggs were collected from the skin during the present study.

Some of the eggs underwent necrotic changes, with inflammatory cells attached to the external egg shell, which is further described in **Chapter 5**. Although limited, this evidence suggests the possible involvement of these parasites in the initiation and subsequent progression of the lesions. A more extensive sampling programme is therefore warranted to investigate this observation further.

Spongiosis is one of the main tissue changes seen in the epidermis during lesion pathogenesis (Ferguson 2006) and has been described for a number of skin lesions affecting fish, such as in puffy skin disease affecting rainbow trout (Maddocks *et al*. 2015) and UDN (Roberts *et al*. 1970) affecting salmon *Salmo salar* (Linnaeus, 1758), which could, in more

severe cases fuse to form intra-epidermal vesicles and acantholysis (Maddocks *et al*. 2015). In the present study spongiosis was observed, with intra-epidermal vesicles formed only during the moderate stage of infection.

No parasites were seen in skin scrapes, although monogeneans were seen infecting gills and isopods infecting the oral cavity, skin and gills. Rodlet cells were not seen in any of stages of skin lesions in the present study. (Bullard *et al.* 2015) described rodlet cells in skin lesions of yellowfin tuna *Thunnus albacares* caused by *Capsala* cf*. biparasiticum* (Goto, 1894) Price, 1938 (Monogenoidea: Capsalidae) infections, located at the outer most layer of epidermis.

Malpighian cells have been observed to migrate from the margins of lesions to cover ulcers, and re-epithelialise lesions (Richard *et al*. 1971). Similar observations were made during the healing process for lesions in the current study. The breach of the basement membrane was observed in the later moderate and early severe lesions, while the constant re-epithelialisation occurred throughout the various stages, being evident from newly-formed epidermis affected by spongiosis covering often active lesions.

Roberts *et al*. (1970) described the absence of leukocytes in the early stages of ulcerative dermal necrosis (UDN) affecting Atlantic salmon, while after the breaching of the basement membrane, leukocytes, mainly neutrophils, were observed, and were related to a response to secondary invaders. Indeed, secondary invaders in skin lesions can often obscure the primary insult (Ferguson 2006). In severe lesions bacteria as well as fungal hyphae were seen in tissue sections in the current study, which might be the reason for the frequent variation in histological presentation, with some lesions being more haemorrhagic while others contained more leukocytes. Several inflammatory cells were observed in the mild, moderate

and severe lesions, with predominantly lymphocytes and eosinophilic granulocytes associated with the observed digenean infection, and mixed inflammatory infiltrate in the severe lesions. Macrophages with melanin inclusions in the cytoplasm were found in mild and moderate stages, which might be an indication of the host's efforts to rid itself of injury.

The healing of skin starts with the reinstatement of the lost osmoregulatory barrier (Ferguson 2006), which serves to help in restoring internal homeostasis (Bereiter-Hahn 1986) and restores the protective role of the fish skin (Elliott 2000). This process can be divided into two stages; the first of which involves re-epithelialisation of the lesion (Bereiter-Hahn 1986). Roberts *et al*. (1971) observed during healing of UDN lesions that Malpighian cells migrate from the margins of the lesions to cover the ulcerative lesion. This process could occur in a matter of hours and was temperature-dependent, with the healing process increased from 7 to 10 days during winter. In the present study, histological and gross observations confirmed the eventual resolution of the observed skin lesions, even though the size of some of the healed lesions suggested the presence of a relatively severe preceding lesion. The time taken for these lesions to heal could not be established from these wild-caught fish due to the great number of uncharacterised variables involved and this aspect of the pathology therefore requires further research using appropriately controlled conditions. The presence of melanin-containing cells in the re-instated epidermis is evidence for healing in progress, as described by Roberts & Bullock (1976).

The second stage of wound repair takes longer (Roberts & Bullock 1976) and involves reorganisation of the dermal connective tissue (Bereiter-Hahn 1986). In UDN, dermal disorganisation was observed in healed lesions a year after the initial skin lesion (Roberts & Bullock 1976). In the present study the dermis of lesions categorized as later severe to early

and later healing stages, were observed to be covered by newly-formed epidermis, evident from the latter's disorganisation and the finger-like projections of piled-up epidermal cells. While the dermis was often disorganised, having lost its normal architecture, the activated fibroblasts create newly formed fibrous tissue that may often be irregular in its morphology (Roberts *et al.* 1971). The presence of newly-formed scales also provides an indication of healing in progress. Described as one of the early stages of epidermal damage by Roberts & Bullock (1976), in the present study the newly formed epidermis was often observed to show newly occurring spongiotic changes, despite the fact that healing was in progress. Thus it is possible that the epidermis might be subjected to several sloughing and re-instating cycles during skin ulcer repair, which might extend the period taken for the healing process of the skin. It is also important to note the presence of invading bacterial colonies in severe lesions, which might also have had an effect on the development of spongiosis. As bacterial infections to the skin are often accompanied by spongiosis (Roberts & Bullock 1976; Ferguson 2006) this might explain the spongiosis in the newly formed epidermis covering active dermal lesions during the early stages of the healing process.

No indications were present for other factors such as habitat and diet that could conceivably affect the aetiology or prevalence of lesions although a shift in water depth for dusky grouper is generally correlated with fish size / age, with smaller individuals most frequently found in shallower waters. Some juveniles were nevertheless caught at depths of 12 m and 20 m (Harmelin & Hermeline-Vivien 1999). A correlation between the presence of skin lesions and habitat / diet could not therefore be discerned in the present study.

Size and age related infection has been observed in some skin lesions infecting cultured fish, such as ulcerative dermal necrosis (UDN) (Roberts *et al*. 1970) and strawberry disease (SD)

(Ferguson *et al*. 2006). In the present study, a specific size range from 42 to 92 cm TL seemed to be affected. The significance of the occurrence of lesions only in the sexually mature females, and in developing and mature males is not yet understood. The occurrence in the sampled population of milt-releasing males and spawning / post-spawning females, suggests that the observed lesions do not disrupt reproductive activities. Although it was not possible to assess the spawning behaviour, sexual selection in fish can depend, at least in part, upon the visual appearance of prospective partners, as it does in three-spined stickleback (*Gasterosteus aculeatus*) (Gardner 2010). The presence of skin lesions on prospective partners could similarly affect mating behaviour and thus successful recruitment in dusky grouper. While in the current study, all affected dusky grouper were sexually mature, according to the images posted on Facebook (FB) (see Chapter 3), the possibility that smaller and (or) larger fish were also affected is not excluded. The data provided from FB suggest that fish of 40 cm TL provide a size threshold for lesion occurrence, however, given the wide variation of size at maturity in dusky grouper, with smallest mature females measuring 38 cm TL and largest immature females measuring 54 cm TL (Reñones *et al.* 2007), size cannot be considered a reliable indicator of sexual maturity.

Several skin lesions of unknown aetiology are known to affect freshwater and marine fish. Skin lesions showing characteristic patterns can be seen in ulcerative dermal necrosis, UDN, affecting Atlantic salmon *Salmo salar* L., with lesions mostly affecting the skin of the head (Roberts 1993). Red mark syndrome (RMS) (Verner-Jeffreys *et al*. 2008), strawberry disease (SD) (Ferguson *et al* 2006) and puffy skin disease (PSD) (Maddocks *et al.* 2015) are a number of conditions that display skin lesions which seem to largely affect the skin of the trunk of rainbow trout, *Oncorhynchus mykiss* (Walbaum). Attempts have been made to build case definitions for these skin lesions as in puffy skin disease (PSD) (Maddocks *et al*. 2015). To

avoid confusion researchers have resorted to the use of pictures as a visual aid in the assessment of lesions observed in cultured fish (Oidtmann *et al*. 2013).

Skin lesions often affect fish and are easily seen (Ferguson 2006) and most stages of the dusky grouper skin lesions are easily differentiated, especially the moderate and severe. In the current study only few moderate lesions were sampled, giving a less secure understanding and description of this phase. Further research is therefore required in order to provide a better understanding of the aetiology, pathogenesis and diagnostic criteria for these lesions.

In conclusion this study reports upon an apparently emerging skin condition frequently affecting an endangered species, the dusky grouper, in Libyan coastal waters. An appropriate term for this condition, in view of the observed pathology, is “dusky grouper dermatitis” (DGD). Further studies are needed to fully characterise the pathological changes, the aetiology of the condition, its possible impacts upon wild fish–populations, and its current distribution throughout the Mediterranean and other waters where this species is endemic. Future research needs to employ tools that can provide greater in-depth understanding *e.g*. TEM and molecular-based methods for elucidating the aetiology of pathology as well as rational studies of the morphological development and healing of these lesions undertaken in a controlled enclosed environment

Chapter 5

Morphology and pathogenesis of the aporocotylid blood fluke infecting the skin of dusky grouper

5.1 Introduction

In the period between 2013 and 2015, skin lesions were frequently seen on larger specimens of wild dusky grouper, *Epinephelus marginatus* (Lowe, 1834), that had been caught in Libyan waters and were being offered for sale at local fish markets (**Chapter 2**). The lesions were subsequently described as a condition referred to as dusky grouper dermatitis (DGD), a pattern of lesions for which adult metazoan parasites and their embryonated eggs were described from mild and early moderate lesions (**Chapter 4**). The research reported in this chapter employs histology to investigate the morphology of the adult metazoans and their eggs and also to characterise the host inflammatory response. The study uses the new knowledge arising from this study, supplemented by relevant information from the existing literature, to identify the parasite and begin to reconstruct its probable life-cycle.

5.1.1 Blood flukes affecting fish

A number of fresh and seawater fish are hosts to blood flukes belonging to the Family Aporocotylidae Odhner, 1912 (Platyhelminthes: Trematoda) [syn: Sanguinicolidae von Graff, 1907] (see **Table. 5.1**). The first description of a blood fluke from a piscine host was *Aporocotyle simplex* Odhner, 1912 (see Bullard *et al.* 2009) made by Odhner (1900) and since then further species have been described and synominised and these are discussed in Smith (2002). In the present study, unless otherwise stated, the nomination Aporocotylidae Odhner, 1912 is applied.

Table 5.1. A summary of the 33 valid genera within the Aporocotylidae. The current list includes *Kritsky* Orélis -Ribeiro *et* Bullard, 2016 although it should be noted that this description has yet to be formally accepted and is currently under peer-review.

Abbreviations: ***n.d.*** = Not described

Genus	Spines	Body shape	Eggs	Uterus	Testis
Acipensericola Bullard, Snyder, Jensen *et* Overstreet, 2008	Robust, spike-like body spines arranged in ventro-lateral transverse rows	Flat, ventrally concave, elongated, <4 times longer than wide with anterior and posterior ends tapering	Uterine eggs oblong	Lobed, near ovarian level	Two
Adelomyllos Nolan et Cribb, 2004	Tegumental spines, ventro-lateral transverse rows	Elliptical, flattened	Elongate	Post-ovarian	Two
Ankistromeces Nolan *et* Cribb, 2004	Tegumental spines forming incomplete transverse rows	Elongated, cylindrical, with strong dorsal curve posteriorly, distinctly notched at male genital pore	May be highly compressed 23–39 (27) × 7–23 (15)	Post-ovarian, thin walled, contains eggs and sperm	Single
Aporocotyle Odhner, 1900	Clusters of 13-50 spines on ventro-lateral body margins	Spindle-shaped, flattened	Fusiform, no size	Pre-ovarian	28-203 round or irregularly lobed positioned between the posterior caeca

Table 5.1. Continued

Genus	Ovary	Vitellarium	Host	Country	Ref
Acipensericola Bullard, Snyder, Jensen *et* Overstreet, 2008	Inter-testicular ovary	Extensive network of narrow, interconnecting branching bands, situated both dorsal and ventral to gonads and caeca	Infects the heart of the American paddlefish *Polyodon spathula* (Walbaum, 1792)	USA	Bullard, Snyder, Jensen & Overstreet, 2008
Adelomyllos Nolan *et* Cribb, 2004	Lying between anterior, testis and cirrus-sac	Follicular, either side of lateral nerve chords extending anteriorly past nerve chord commissure	*Epinephelus coioides* (Hamilton , 1822)	From Moreton Bay, southeast Queensland.	Nolan & Cribb, 2004
Ankistromeces Nolan *et* Cribb, 2004	Spherical to ovoid, posterior to, abutting or slightly overlapping posterior margin of testis.	Dorsal to testis	Ventricle (heart) of monacanthid fish	Tasmania	Nolan & Cribb, 2004
Aporocotyle Odhner, 1900	Ovoid median	Follicular, from near anterior end to ovary	Gill blood vessels and heart	Cooler seas of northern and southern hemispheres	Smith, 2002

Table 5.1. Continued

Genus	Spines	Body shape	Eggs	Uterus	Testis
Braya Nolan *et* Cribb, 2006	Body spines distributed along lateral body surface from level of mouth to posterior body end, distributed in rows wrapping dorso-ventrally around body margin	Elliptical to lanceolate, flattened, may curve ventrally marginally	Spherical to ovoid	Post-ovarian, convoluted	Single roughly square to rectangular
Cardicola Short, 1953	Ventrally recurved body margins armed with transverse rows of small spines with curved tips	Small, flattened, elongate-oval	Pyriform with spiniform terminal filament, no size	Long lateral or/and post-ovarian	Single
Chaulioleptos Nolan *et* Cribb, 2005	Complete ventro-marginal transverse rows, continuous along length of body	8.5 times longer than wide	Ovoid	Post-ovarian	Two
Chimaeribemecus Van der Land, 1967	Ventro-lateral body margins bear curved spines	Small, flattened, mainly uniform in breadth. Anterior and posterior ends slightly tapering	Thin-shelled intra-uterine eggs, n.d	Winding ascending and straight descending limb	Single, large from intestinal bifurcation to level of genital pore
Cruoricola Herbert, Shaharom-Harrison et Overstreet, 1994	Single row of sub-marginal spines except at anterior extremity	Lanceolate, flattend	Ovoid	Post-ovarian	Single, extensive

Table 5.1. Continued

Genus	Ovary	Vitellarium	Host	Country	Ref
Braya Nolan *et* Cribb, 2006	Single, posterior to, abutting or slightly overlapping posterior margin of testis	Follicular, symmetrical from about level of anterior nerve commissure to ovary	From the atrium of *Scarus ghobban* (Forsskål)	Heron Island, southern Great Barrier Reef	Nolan & Cribb, 2006
Cardicola Short, 1953	Bilobed quadrangular or sub-triangular with irregular margins	Extensive nerve ring to ovary	9 families	Atlantic, Pacific South China sea and Hawaiian	Smith, 2002
Chaulioleptos Nolan *et* Cribb, 2005	Anterior testis and cirrus sac	Follicular; solid branching mass, occupying area anterior to intestinal bifurcation to termination of right posterior caecum	Parasites of circulatory system of marine fish (Polynemidae)	Sandgate, Moreton Bay (southeast Queensland, Australia).	Nolan & Cribb, 2005
Chimaeribemecus Van der Land, 1967	Bilobed between caeca, post testicular	Follicular	Blood system of holocephalans (Chimaera)	Northern north-east Atlantic Ocean	Smith, 2002
Cladocaecum Orélis-Ribeiro *et* Bullard, 2016			Catfishes	Peruvian Amazon	Orélis-Ribeiro & Bullard, 2016
Cruoricola Herbert, Shaharom-Harrison et Overstreet, 1994	Bilobed, post-testicular	Follicular, extensive and pre-ovarian	Mesenteric venules of *Lates calcarifer*	Malaysian, Thai and Australian marine waters	Smith, 2002

Table 5.1. Continued

Genus	Spines	Body shape	Eggs	Uterus	Testis
Deontacylix Kinton, 1910	Entirely covered with transverse rows of small spines	Elongate fusiform	*n.d.*	Winding, between testis and lobed ovary	Single, pre -uterine
Elaphrobates Bullard *et* Overstreet 2003	Ventro-lateral tegumental rows of spines, each consisting of 4-7 spines	Elongate, less than 8 times longer than wide, dorso-ventrally flattened, ventrally concave	Spheroid to elongate depending on the stage of miracidial development	Not extending anterior to ovary (post-ovarian)	Single intercaecal
Elopicola Bullard, 2014	Tegumental body spines of juvenile specimens minute, straight, delicate, barely discernible with light microscopy, lacking recurved tip, not distributing in ventrolateral transverse rows, enveloped by tegument	Flat, oval, ventrally concave, lacking postero-lateral body protuberance	Uterine eggs having a tetrahedral body, with elongate polar filaments; Eggs lodged in gill epithelium of ladyfish bearing elongate polar filaments with distally recurved ends, enveloping ciliated miracidium	Compact post-testicular, pre-ovarian	Single

Table 5.1. Continued

Genus	Ovary	Vitellarium	Host	Country	Ref
Deontacylix Kinton, 1910	Multilobed, near posterior end	Follicular	Flukes were recovered from the coelom and gills washings from *Kyphosus* spp.	Florida and Hawaii marine waters	Smith, 2002
Elaphrobates Bullard *et* Overstreet 2003	Post-testicular or slightly overlapping posterior testicular border	*n.d*	Heart and branchial vessels of the red snapper (*Lutjanus campechanus*) and the heart of the gray snapper (*Lutjanus griseus*)	Gulf of Mexico	Bullard & Overstreet, 2003
Elopicola Bullard, 2014	Occupying posterior body extremity, post-testicular	Extensive network of narrow, interconnecting, branching bands extending lateral beyond ventro-lateral nerve cords	Ladyfish, *Elops saurus*, (Elopiformes: Elopidae)	North-central Gulf of Mexico	Bullard, 2014

Table 5.1. Continued

Genus	Spines	Body shape	Eggs	Uterus	Testis
Hyperandrotrema Millard *et* Ktari, 1978	Three to four rows of hook-shaped spines ventro-laterally	Elongate, flattened	*n.d.*	Post ovarian	Single, large between caeca
Kritsky Orélis-Ribeiro et Bullard, 2016	Interim unpublished	Lanceolate body	Minute, spheroid uterine eggs	*n.d*	*n.d*
Littorellicola Bullard, 2010	Lateral spine rows, slightly curved distal tip spined; tegumental body spines minute	Extremely elongated, approximately 10–30× longer than wide, bearing sinistral postero-lateral body protuberance	*n.d*	Post-testicular, primarily post-ovarian	20–60
Metaplebniella Lebedev *et* Parukhin, 1972	Marginal paired spines	Foliate, flattened	Ovoid, *no size*	Post-ovarian with a few coils	Small, deeply lobed and posterior to caeca
Myliobaticola Bullard *et* Jensen, 2008	Aspinous	Minute, flat, ventrally concave	Uterine eggs are oblong and empty having a membranous shell	Postgonadal flanking the internal seminal vesicle	Single extensively looped

Table 5.1. Continued

Genus	Ovary	Vitellarium	Host	Country	Ref
Hyperandrotrema Millard *et* Ktari, 1978	Bilobed	Follicular	Heart and blood-vessels of sharks (*Cetorhinus*)	Mediterranean Sea and northern north-east Atlantic Ocean	Smith, 2002
Kritsky Orélis-Ribeiro *et* Bullard, 2016	Finger-like lateral projections	*n.d*	Catfishes	South America , Peruvian Amazon	Orélis-Ribeiro & Bullard, 2016
Littorellicola Bullard, 2010	Single, post-testicular	Follicular	Myocardial lacunae of the ventricle and atrium of Florida pompano, *Trachinotus carolinus*	Northern Gulf of Mexico	Bullard, 2010
Metaplebniella Lebedev *et* Parukhin, 1972	Lobed, posterior to testis	Anterior end to mid-level of cirrus-sac	Blood vessels of the stomach wall (?) of *Lethrinus miniatus*	Indian Ocean off the coast of India	Smith, 2002
Myliobaticola Bullard *et* Jensen, 2008	Post-testicular	Dendritic, diffuse and extensive	Between the cardiac trabeculae of Atlantic stingrays *Dasyatis sabina* (Lesueur, 1824)	Mississippi Sound (type locality), Mississippi, and Apalachicola Bay, Florida.	Bullard & Jensen, 2008

Table 5.1. Continued

Genus	Spines	Body shape	Eggs	Uterus	Testis
Neoparacardicola Yamaguti, 1970	Fine ventro-marginal spines	Elongate, flattened, with deep notch on left posterior margin	*n.d.*	Winds forward, beginning posterior to ejaculatory duct and then right of cirrus-sac	Two, the posterior testis is largely posterior to ovary elongated and irregularly lobed
Orchispirium Madhavi *et* Hanumantha Rao, 1970	Spination status unknown - spines may have been lost during preparation	Spatulate, flattened with tubercles laterally	Ovoid	Post-testicular	Single transversely coiled tube
Parasanguinicola Herbert *et* Shaharom, 1995	Single row of spines ventro-laterally	Spatulate, flattened	Small, thin shelled triangular	Single, post-testicular dendritic	Single
Paracardicola Martin, 1960	Laterally spined along most of its length	Spatulate, flattened	Ovoid, terminal spine	Post-ovarian, loops, long pre-ovarian	Two, posterior to uterus immediately anterior to ovary
Paracardicoloides Martin, 1974	Spined; tegumental body spines robust, spike-like, lacking recurved tip, distributed in a narrow field along the ventro-lateral body margins	Small, flattened and lanceolate, oval, ventrally concave, lacking posterolateral body protuberance	Thin-shelled, no size	Pre-ovarian	Two

Table 5.1. Continued

Genus	Ovary	Vitellarium	Host	Country	Ref
Neoparacardicola Yamaguti, 1970	Unequal lobes	Follicular	Blood vessels of teleosts (*e.g. Naso* spp.)	Hawaiian marine waters	Smith, 2002
Orchispirium Madhavi *et* Hanumantha Rao, 1970	Lobed	Follicular	Mesenteric blood vessels of rays	North-east Indian Ocean	Smith, 2002
Parasanguinicola Herbert *et* Shaharom, 1995	Single, dendritic, post-testicular	Follicular	Blood vessels of *Lates calcarifer*	Malaysia	Smith, 2002
Paracardicola Martin, 1960	One compact	Small follicles	Mesenteric veins of marine fish (*e.g. Arothron* spp.)	Hawaiian waters	Smith, 2002
Paracardicoloides Martin, 1974	Deeply indented, situated between the testes	Follicular	Blood vessels of eels (*Anguilla*)	Australia	Smith, 2002 & Bullard, 2014

Table 5.1. Continued

Genus	Spines	Body shape	Eggs	Uterus	Testis
Paradeontacylix McIntosh, 1934	Ventro-lateral transverse rows of rose thorn-shaped spines	Small, flattened and slender	Ovoid, no size	Coiled, posterior to ovary	19-71
Pearsonellum Overstreet *et* Koie, 1989	Spined; tegumental spines minute, lacking peduncle, with recurved tip, distributing in equally spaced ventrolateral transverse rows along entire length of adult body	Spatulate, flattened, 5-7 X longer than wide	Operculated, ovoid, no size	Anterior to ovary	Single, lobed
Phthinomita Nolan *et* Cribb, 2006	Tegumental spines in incomplete lateral transverse rows, along the entire length of the body	Thread-like body	*n.d*	Uterus extending anteriorly from oötype, to posterior abutting or overlapping posterior margin of ovary	Two
Plethorchis Martin, 1975	Clusters of laterally positioned minute spines	Slender, flattened, elongate	Fusiform with terminal spine, no size	Pre-ovarian	Numerous

Table 5.1. Continued

Genus	Ovary	Vitellarium	Host	Country	Ref
Paradeontacylix McIntosh, 1934	Posterior to caeca& testis	Follicular, extensive to anterior margin of ovary	Heart and gill-arteries of marine fish	Pacific and Atlantic ocean	Smith, 2002
Pearsonellum Overstreet *et* Koie, 1989	Single, medial or slightly dextral, post-cecal, post-testicular, narrower than testis, occupying posterior 1/4-1/3 of body	Follicular	Ventricle and *bulbus arteriosus* of heart *Plectropomus leopardus* (type host) and *Epinephelus* spp.	Great Barrier Reef, Australia	Smith, 2002 & Bullard, 2012
Phthinomita Nolan *et* Cribb, 2006	Abutting or slightly overlapping posterior margin of anterior testis	Follicular; extensive	Ventricle (heart), from Siganidae, Labridae and Mullidae (Teleostei: Perciformes)	Indo-west Pacific region	Nolan & Cribb, 2006
Plethorchis Martin, 1975	Dextro-laterally to posterior part of uterus and near posterior end of body	Follicular	Blood vessels of mesenteries, intestine and liver of mugilids	Australia	Smith, 2002

Table 5.1. Continued

Genus	Spines	Body shape	Eggs	Uterus	Testis
Primisanguis Bullard, Williams *et* Bunkley-Williams, 2012	Tegumental body spines distributed along lateral body surface from the level of the mouth to posterior body end, distributed in rows wrapping dorso-ventrally around body margin (not entirely ventro-lateral), spines are straight and not recurved	Flat, approximately 5–10 times longer than wide, bearing Sinistral postero-lateral body protuberance in posterior 1/5 of the body	*n.d*	Proximal extensively convoluted and having dense aggregation of sperm and functioning as uterine seminal receptacle	Single
Psettarium Goto *et* Pzaki, 1930	Lateral margins with transverse rows of spines	Narrow, elongate and flattened with symmetrical lobe at posterior end	Ovoid, no size	Lateral and post-ovarian	Single
Pseudocardicola Parukhin, 1985	With ventro-lateral transverse rows of rose-thorn-shaped spines	Small, flattened, lanceolate	*n.d*	Anterior and posterior to ovary	Five

Table 5.1. Continued

Genus	Ovary	Vitellarium	Host	Country	Ref
Primisanguis Bullard, Williams *et* Bunkley-Williams, 2012	Abutting the posterior margin of the testis	Asymmetrical	Stoplight parrotfish, *Sparisoma viride* (Bonnaterre, 1788), (Labridae: Scaridae)	Caribbean Sea off La Parguera Puerto Rico	Bullard, Williams & Bunkley-Williams, 2012
Psettarium Goto *et* Pzaki, 1930	Tubular mass between testis and uterus	Tubular acini	Intestine? *Sphoeroides pardalis* heart ventral aorta of *Sebastes* spp.	North Pacific Ocean	Smith, 2002
Pseudocardicola Parukhin, 1985	Weakly lobed	Follicular	Gill blood vessels of marine fish (*Emmelichthys*)	Indian Ocean	Smith, 2002

Table 5.1. Continued

Genus	Spines	Body shape	Eggs	Uterus	Testis
Rhaphidotrema Yong *et* Cribb, 2011	Tegumental spines in marginal transverse rows, along the length of the body	Flattened, lanceolate, with dextral bend towards posterior end in region of genital pores	*n.d*	Coiled and post-ovarian	18–19
Sanguinicola Plehn, 1905	Marginal spines or unarmed	Small, flattened, slender	Triangular and bearing spine, no size	Reduced post-ovarian	Single
Selacbobemecus Short, 1954	C-shaped spines on ventro-lateral body margins	Small, flattened, slender	Spherical to ovoid, no size	Entirely post-ovarian long	Single
Skoulekia Alama-Bermejo, Montero, Raga *et* Holzer, 2011	Ventro-lateral transverse rows of spines	Flat, curved, long, 3–6 times longer than wide. Sinistral dorso-lateral protuberance at male genital pore level	Uterine irregular, spheroid, flexible, thin-shelled eggs, to regular ellipsoid, elongated eggs with a well-defined shell	Lateral and posterior to ovary	Single and diffuse

Table 5.1. Continued

Genus	Ovary	Vitellarium	Host	Country	Ref
Rhaphidotrema Yong *et* Cribb, 2011	Median, entire, posterior to testes	Follicular	Heart of the stars-and-stripes pufferfish, *Arothron hispidus* (L.) (Tetraodontidae)	Great Barrier Reef, Australia	Yong & Cribb, 2011
Sanguinicola Plehn, 1905	Bilobed, post-testicular	Follicular, present throughout most of the body	Blood vascular system of cyprinids and other families of freshwater teleosts	Europe, north-east Africa, Israel, Asia and North America	Smith, 2002
Selacbobemecus Short, 1954	Slightly lobed	Follicular	Sharks (*Rhizoprionodon*)	Gulf of Mexico	Smith, 2002
Skoulekia Alama-Bermejo, Montero, Raga *et* Holzer, 2011	Post-testicular	Follicular extending anteriorly, from nerve commissure to dextral posterior end of ovary and to anterior end of uterus, and laterally to body margins	Mediterranean common two-banded seabream *Diplodus vulgaris* (Geoffroy Saint-Hilaire, 1817) blood vessels surrounding the optic lobes of the brain	Mediterranean	Alama-Bermejo, Montero, Raga *et* Holzer, 2011

5.1.2 General description

Blood flukes from fish are hermaphrodite, *i.e.* having both ovaries and testes within the same parasite (see **Table. 5.1**). Over 37 genera of blood flukes have been described to date (Palacios-Abella *et al.* 2015) and these are summarised in **Table 5.1**; most species infect the vascular system of marine and freshwater fish (Smith 2002) and though they are commonly found within the heart, gills and visceral mesenteries (Nolan & Cribb 2004), they are not limited to these (Nolan *et al.* 2014) and have been described from other body locations. *Skoulekia meningialis* Alama-Bermejo, Montero, Raga *et* Holzer 2011, for example, has been described from the ectomeningeal veins surrounding the optic lobes of the brain of common two-band seabream, *Diplodus vulgaris* (Saint-Hilaire, 1817) (see Alama-Bermejo *et al.* 2011), while Padrós *et al.* (2001) described blood flukes from outside the circulatory system with specimens found encysted in the head kidney of the sea bream, *Sparus aurata* L.

5.1.3 General external morphology

Blood flukes, which are important pathogens of several species of captive bred fish, are principally dorso-ventrally compressed to leaf-like-shaped parasites (Smith 2002) or have a long and thread-like body, *e.g. Phthinomita* Nolan *et* Cribb, 2006 (see Nolan & Cribb 2006). Specimens can also be long and slender (Palacios-Abella *et al.* 2015), while in cross section species such as *Cardicola* Short, 1953 are typically round to oval (Padros *et al.* 2001) see **Table 5.1.** The tegument can be adorned by spines which may either partially or entirely cover the surface (Smith 2002). Some species possess minute spines or rows of lateral tegumental spines, all of which facilitate attachment and movement along the vascular system (Bullard, Overstreet, Snyder & Bruch 2001). When alive, the flukes can be opaque or nearly transparent (Bullard *et al.* 2001; Bullard & Overstreet 2008). Grooves can be seen on

some specimens and a simple, non-ridged, non-annulated genital pore is always present; there maybe two genital pores in some species and their position may vary between genera.

5.1.4 General internal morphology and reproductive system

At the anterior extremity of aporocotylid flukes are an oesophagus which leads into a bifurcated intestine, which in turn ends in two blind-ended caeca. Oral and ventral suckers are absent, with other classical digenean structures such as the Mehlis gland *etc*. being present or absent depending on the species in question. A full discussion of the taxonomy of genera and species belonging to the Aporocotylidae [*sic* Sanguinicolidae] is presented in Smith (2002).

Blood flukes are hermaphrodites and both the female and male organs are joined together. The uterus can be a short or long, straight, U-shaped or coiled organ that is located in the mid to distal part of the parasite (**Table.5.1**). The uterus opens onto the external surface at a genital pore. The ovaries can vary in shape between genera, for example in *Cardicola* they are bilobed and quadrangular (Smith 2002) while in *Braya* Nolan *et* Cribb, 2006 there is just a single ovary (Nolan & Cribb 2006). The position of the ovaries can vary; the ovary is, for example, inter-testicular in *Acipensericola* (see Bullard *et al.* 2008) and post-testicular in *Braya* (see Nolan & Cribb 2006).

There may be a single, paired or multiple testes, the position of which varies from one parasite genus to the next. The mature testis is surrounded with a basal lamina and a thick extracellular matrix with muscle cells extending into and attached to it.

5.1.5 Eggs, shape and size

Blood flukes produce eggs which vary in shape and size depending on the genus, but the eggs, in general terms, are oval or pyriform, un-operculated and thin shelled with some species having a spiniform terminal filament (Smith 2002). The distribution of egg types across the family are summarised in **Table 5.1.** The sizes of eggs range from 25-54 µm with these continuing to develop on their release from the adult and as they enter the host's blood circulatory system.

5.1.6 Blood flukes general life-cycle

Apart from a few exceptions, the life-cycles of marine aporocotylids are poorly known. The life-cycle of fish blood flukes lack a second intermediate host and do not have encysted or encapsulated metacercaria (Bullard *et al.* 2001). In general, the life-cycle follows the basic digenean dixenous life-cycle (*i.e.* two-host strategy) in which there are sexual and asexual stages of parasite development (**Fig 5.1**).

Adult blood flukes are found in the vascular system, often in the heart. Once the eggs are released via the parasite's genital pore into the blood circulation, the eggs start their passive migration towards the gills, and in *Cardicola aurata* Holzer, Montero, Repullés, Sitjà-Bobadilla, Alvarez-Pellitero, Zarza *et* Raga 2008, the eggs accumulate in the afferent vessels of primary and secondary gill filaments (Holzer *et al.* 2008). The embryo within the egg continues developing until it becomes a miracidium, at which point the larval blood fluke is sufficiently developed to exit the egg and to seek out an appropriate host to infect. Commonly within the *Aporocotylidae*, the eggs become lodged within the gill filaments of its host. The miracidia then hatch and are released into the external aquatic environment as the free-living larval phase (Galaktionov & Dobrovolskij 2003).

The miracidia then seek their intermediate host, which could be a bivalve, gastropod or a polychaete (Cribb *et al.* 2011). Within this intermediate host, the miracidia go through several stages of asexual reproduction and development culminating in the development and release of cercariae into the aquatic environment. The cercariae penetrate their fish host via the gills and other epithelial areas, showing a preference for skin with less scales (Sommerville & Iqbal 1991) including the eyes, fins or digestive tract. The cercariae then undergo further development to become a schistosomule, a juvenile stage that migrates to its final position within its host's circulatory system. Once it reaches its final position, which is frequently the heart, the parasites matures into the adult, it then copulates and the cycle repeats (Bullard *et al.* 2001). While this is the generalised pattern of events, it may not always be the case as the presence of blood flukes in parts of the body other than the heart suggests that certain species of blood fluke may use alternative routes of infection and these require further investigation (Bullard *et al.* 2001).

5.1.6.1 Life-cycle models

One of the few life-cycles that has been described in detail is that of the blood fluke *Cardicola forsteri* Cribb, Daintith and Munday, 2000 (Trematoda: Aporocotylididae), which commonly infects ranched southern bluefin tuna, *Thunnus maccoyi* (Castelnau, 1872). The adults are found in the heart of the bluefin tuna and the eggs are trapped in the gill filaments, which allow the miracidia to hatch into the aquatic environment. A polychaete serves as the intermediate host (Cribb *et al.* 2011). For *Cardicola opisthorchis* Ogawa, Ishimaru, Shirakashi, Takami *et* Grabner, 2011 that infects the Pacific bluefin tuna, *Thunnus orientalis* (Temminck *et* Schlegel, 1844) (see Sugihara *et al.* 2014), the polychaete *Terebella* (Polychaeta: Terebellidae), serves as the intermediate host.

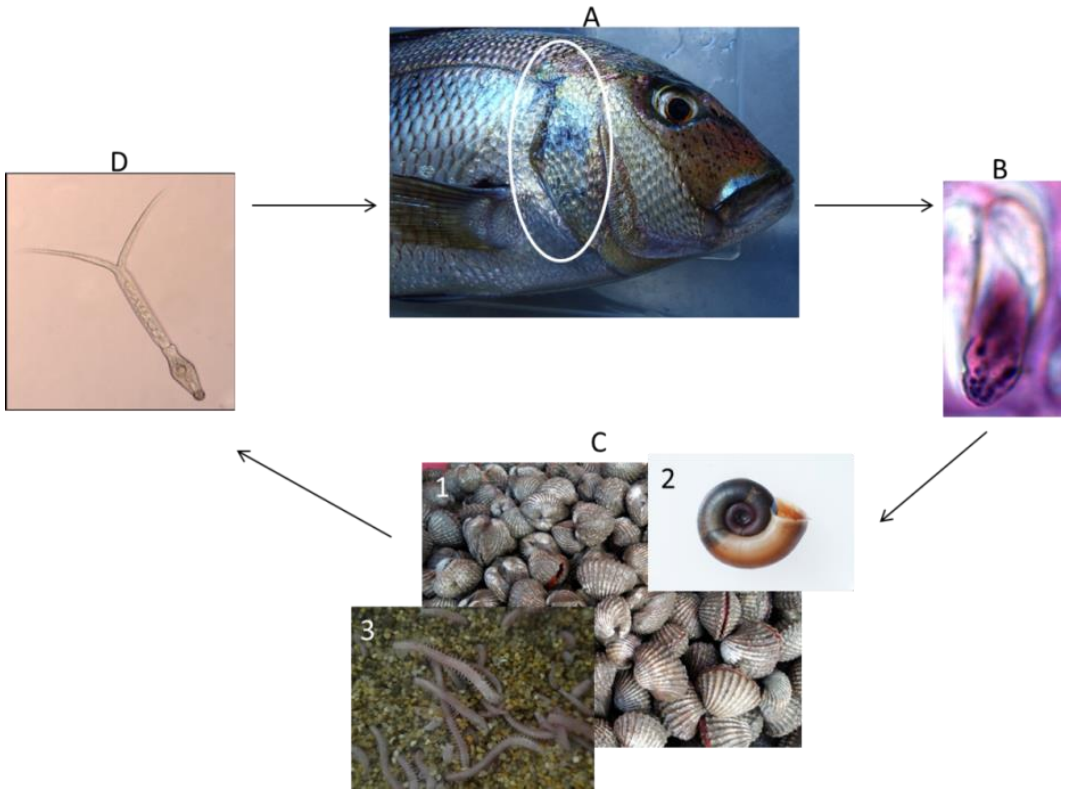

Figure 5.1. Generalised life-cycle of a blood fluke. **A**=final host (fish) where the miracidium hatches from its host's gill filaments (circled); **B**=miracidium (exiting in its egg shell); **C**=first intermediate host, which could be a bivalve (1), mollusc (2) or polychaete (3); **D**=cercariae (general not aporocotylid). (Image C &D curtesy Dr. A. Shinn)

5.1.7 Blood flukes from the Mediterranean Sea

According to Pérez-del-Olmo *et al.* (2016) in the Mediterranean Sea thus far only five genera have been documented infecting ten species of fish species.

5.1.8 Serranidae blood flukes

Only three aporocotylids from two genera are described within 11 Epinepheline (Nolan & Cribb 2004; Bullard 2012), most of which are described from the Indo Pacific region; none are documented from fish hosts within the Mediterranean Sea (**Table 5.2**).

Table 5.2. Aporocotylid blood flukes described from the family Epinephelinae.

Type host	Parasite	Site in host	Ref
Cromileptes altivelis	*Pearsonellum pygmaeus*	Ventricle (heart)	Nolan & Cribb, 2004
Epinephelus coioides	*Adelomyllos teenae*	Unknown	Nolan & Cribb, 2004
Epinephelus merra (Bloch, 1793)	*Pearsonellum corventum*	Ventricle & bulbus arteriosus	Overstreet & Køie, 1989
Epinephelus ongus (Bloch, 1790)	*P. corventum*	Ventricle & bulbus arteriosus	Overstreet & Køie, 1989
Epinephelus quoyanus (Valenciennes, 1830)	*P. corventum*	Ventricle & bulbus arteriosus	Overstreet & Køie, 1989
Plectropomus leopardus (Lacepe`de, 1802)	*P. corventum*	Ventricle & bulbus arteriosus	Overstreet & Køie, 1989

5.1.9 Fish inflammatory responses to helminth parasites

The immune defence mechanisms of fish, with respect to adult blood flukes and eggs within the hosts' vascular system, involves a battery of host inflammatory cells, which are used to combat the invasive organism.

The life span of adult blood flukes can range from a few years as is the case with some blood flukes such as *Schistosoma* (Pearce & MacDonald 2002), to a few months as is seen in *Sanguinicola inermis* Plehn, 1905, which die after 70 days post-infection (Kirk & Lewis 1992). The most severe reactions are triggered by eggs, which may become encapsulated, forming granulomas, by host tissue (Padrós *et al.* 2001; Holzer *et al.* 2008; Dennis *et al.* 2011; Horák *et al.* 2014).

In fish, several leukocytes play a role in the inflammatory process. During helminth infection a range of inflammatory cells are recruited to the site of infection and these include macrophages, neutrophils, eosinophilic granular cells and lymphocytes (Secombes &

Chappell 1996). Eosinophilic granular cells (EGCs) are inflammatory cells containing granules that stain red with H&E. In the skin, resident EGCs are described from the epidermis and dermis (Ferguson 2006) and have a role in the initiation and propagation of different inflammatory responses (Hogan *et al.* 2008). In fish they can be found as part of the inflammatory response to infections by bacteria (Reite & Evensen 2006), viruses (Dezfuli *et al.* 2012), helminths (Balla *et al.* 2010) and copepods (Dezfuli *et al.* 2011). They are also found in increased numbers in the skin of salmonids irritated by formalin baths.

Eosinophilic cells react differently depending on the stage of the inflammatory process. During acute stages they degranulate, while they increase in numbers in the chronic stage (Reite & Evensen 2006).

Lymphocytes are small cells showing strongly basophilic staining with H&E. They are part of the immune response and are recruited to the site of infection following EGC infiltration.

Macrophages and neutrophils are inflammatory cells which, through their receptor-driven and phagocytic activities, recognise and eliminate invading organisms and damaged tissue (Secombes & Fletcher 1992).

5.1.10 Pathology attributable to blood fluke infection

Aporocotylids are one of the few trematodes associated with severe pathologies in fish culture (Paperna & Dzikowski 2006). Blood flukes in cultured fish can cause severe mortalities, for example as has been seen in southern bluefin tuna (Dennis *et al.* 2011) and *S. aurata* culture (Padrós *et al.* 2001).

5.1.10.1 Gross lesions

Given the life-cycle of this parasite, which relies on the eggs reaching an appropriate exit site so that their miracidia can be released into the aquatic environment, it is at these miracidia release sites where the most severe tissue damage is seen. Grossly, the gill filaments of southern Bluefin tuna infected by *Cardicola forsteri* are streaked with pallor and segmentally covered in a heavy mucous secretion (Dennis *et al.* 2011). In heavy infections, blood fluke eggs can be seen in the heart of butterflyfish *Chaetodon trifascialis* (Quoy *et* Gaimard, 1825), giving a distinct flecked appearance to the muscle (Yong *et al.* 2013). Similarly, large aggregations of *Plethorchis acanthus* Martin, 1975 eggs in flathead grey mullet *Mugil cephalus* L. can be observed through a brownish discoloration of the intestine (Lester *et al.* 2009).

5.1.10.2 Histopathological lesions to the gills, heart and in other organs

Histopathological lesions are mostly seen in the gills of infected fish, in the Mediterranean a blood fluke sp. suspected to belonging to *Cardicola* sp. is known to cause a severe necrotic reaction to the gills resulting in the mortality of sea pen reared *S. aurata* (see Padrós *et al.* 2001) and also of southern bluefish tuna (Dennis *et al.* 2011). After their release from the adult, some eggs are caught in the circulatory system (Holzer *et al.* 2008) and are passively transported to a range of body organs including the liver, spleen, heart and kidney where they are sequestered resulting in a localised inflammatory response and the formation of encapsulating granulomas (Holzer *et al.* 2008).

The tissue inflammatory response to the eggs and migrating miracidium in the gills varies from mild, moderate to severe. Padrós *et al.* (2001) described the inflammatory response in the gills of *S. aurata* to the eggs of an unidentified *aporocotylid* to be a mild to moderate

diffuse inflammatory response with lymphocyte and granulocyte infiltration and occasionally aggregates of eosinophilic granular cells surrounding the eggs with no striking epithelial hyperplasia. Holzer *et al.* (2008) described granulomatous formations surrounding trapped eggs *of C. aurata* in the gill tissue of *S. aurata* causing gill fusion and clubbing of gill filaments.

In heavy *Cardicola orientalis* Ogawa, Tanaka, Sugihara *et* Takami, 2010 infections in Pacific bluefin tuna, up to 6400 blood fluke eggs can be found infecting a single gill filament. This level of infection can account for observed levels of mortality, and impacts on growth rate (Shirakashi *et al.* 2012).

While adult *Cardicola* found in the heart cause little inflammation, granulomas that had formed around degenerating eggs were seen scattered throughout the spongiosis layer of the myocardium (Dennis *et al.* 2011). Padrós *et al.* (2001) described a moderate chronic inflammatory response, mainly melanomacrophages, in the kidney of *S. aurata* where adult blood flukes (species not recognised) were found embedded in the renal parenchyma surrounded by a thin fibrocytic capsule.

5.1.10.3 Parasite-induced inflammation of the skin

Dermatitis is the inflammation of the skin. In fish, lesions involving the skin can be divided into superficial, involving the epidermis and deep involving the dermis (Ferguson 2006). Dusky grouper skin ~~in brief~~ is constructed of three layers: the outer most layer is the epidermis which is connected via the basement membrane to the mid-section; the dermis (the vascular part of the skin); and the inner section, the hypodermis (the source of blood

supply and nerves to the skin. For a more detailed description **see Chapters 2 and 4**, while for a more general skin description see Elliot (2000) and Ferguson (2006).

5.1.11 Aims and objectives

This study aims to:

1) Describe the parasite associated with dusky grouper dermatitis (DGD) lesions that are detailed in **Chapter** 4. In the previous chapter, blood flukes were found at lesion sites sampled from five fish characterised as having mild DGD. Blood flukes were found within the dermal blood vessels; the study set out to identify the blood fluke and to describe its morphology by reconstructing it from histological sections. The eggs of the parasite and their development as they pass through the parasite's uterus and, following their release from the parasite, their passive migration through the host's tissue culminating in the release of the miracidium from the egg. The information gathered will be used to reconstruct the life-cycle of this blood fluke.
2) A second objective of the study was to review the position of this blood fluke within the Aporocotylidae by comparing its morphological features with congeners., to investigate the host-parasite relationship and to identify the parasite by using a detailed histology-based evaluation of infected host material.
3) Finally, the study investigates the host inflammatory response to the blood fluke and its eggs and the significance in the DGD pathology.

5.2 Materials and Methods

Five dusky groupers presenting mild and early moderate lesions that had been caught in Libyan coastal waters were post-mortemed and samples of tissue excised. From the findings presented in **Chapters 2** and **4,** a metazoan parasite was found associated with the lesions.

5.2.1 Serial slide reconstruction of the parasite

Serial 5 micrometre thick sections through skin lesions were taken and then photographed at the same magnification. The pictures were then printed and anatomical landmarks (*e.g.* eggs, uterine tubes, seminal vesicles *etc*), were used to align the serial images and to reconstruct the blood fluke. The anatomy of type species belonging to genera within the Aporocotylids were used as guides. Specifically, the images were laid out and then placed into pre-arranged slots, to better understand the 3D dimensional structure of the parasite. The structure of each body organ / feature was then followed and described in an anterior to posterior direction. Drawings of the parasite were then made from the composite images and scanned. **Figure 5.2** shows the sequential steps in reconstructing the parasite.

5.2.2 Identifying the blood fluke eggs

Digenean eggs that were recovered from several different locations within the host epithelium were examined under an oil immersion (×100) lens using an Olympus BX51, measured and photographed. The development of the embryos within the eggs at each location was mapped and used to plot their movement through the host's tissues.

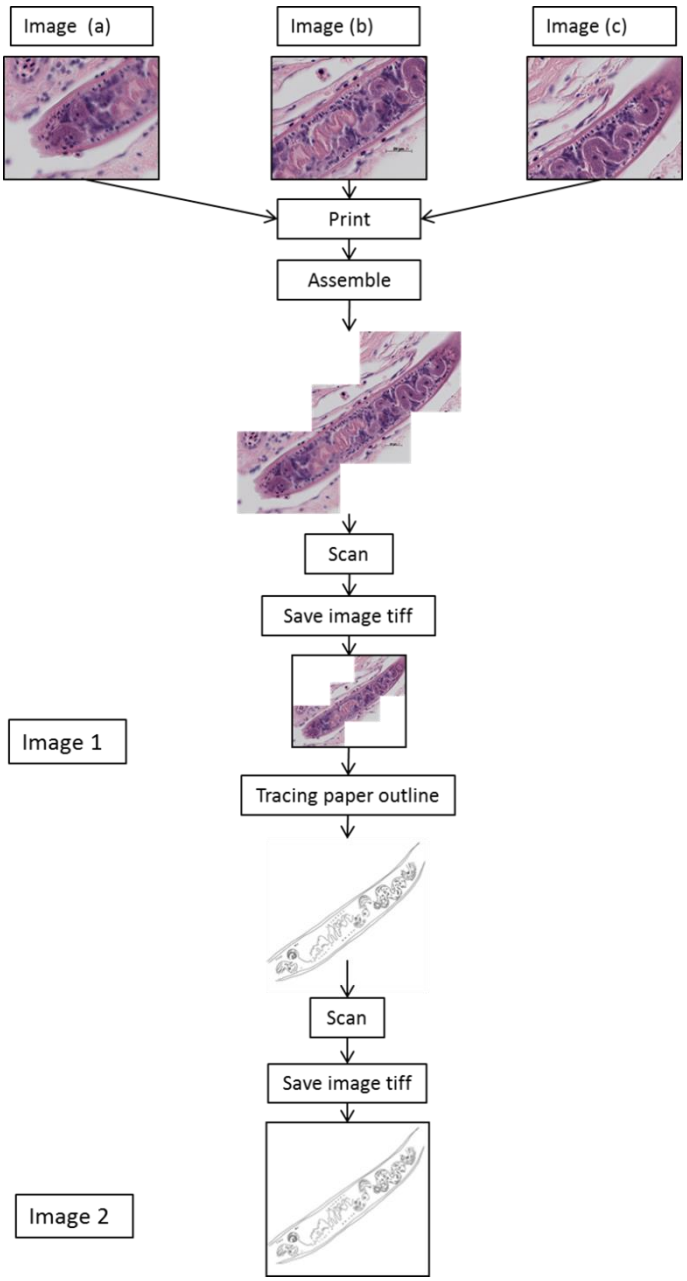

Figure 5.2. The methodological steps in reconstructing the digenean parasite found infecting the dermal blood vessels and skin epithelium of the dusky grouper, *Epinephalus marginatus*

5.3 Results

From the collected tissues isolated from dusky grouper skin lesions, several adult gravid digeneans were found lodged within dermal blood vessels, in the *stratum compactum* and in the *stratum spongiosum*; one parasite was found within a blood vessel between the hypodermis and the dermis. Eggs were also commonly seen in close proximity to adults. By reconstructing the parasite from consecutive longitudinal and cross sections, it was possible to confirm that the parasite was a digenean trematode. This was supported by the descriptions detailed by Chitwood & Lichtenfels (1972) and (Gardiner & Poynton 1999).

5.3.1 Description of the adult parasite

5.3.1.1 Morphology of adult gravid parasite

In cross section, the parasite is round to oval. The longest section of the parasite that could be measured was 1500 µm, while the total length of the parasite is estimated at 1500-2000 µm (**Fig. 5.4 A**) and 20-80 µm (narrowest/widest) in width. The parasite is covered by a tegument which varied in thickness from 1-2.6 µm; it appeared to be thicker towards the distal and proximal ends.

Tinny spines can occasionally be seen covering the tegument (**Figs. 5.3 A & 5.5 A**), while the tegument covering the distal portion of what could be the male auxiliary reproductive organ was found to be discontinuous, interrupted by pits or grooves (**Fig. 5.12 C&D)**. Beneath the tegument are parenchymal cells with very little cytoplasm, the nuclei of these cells give an intense basophilic staining response.

The morphology of the parasite will be described in an anterior to posterior direction; key body organs are used as landmarks in reconstructing the layout of the internal anatomy, a reconstruction of the parasite is shown in **Figure 5.13**.

5.3.1.1.1 Oesophagus

The oesophagus is seen as a pale acidophilic structure, with a narrow acentric lumen that is surrounded by a dense aggregation of glandular cells, the lumen measuring 56.55 × 57.69 in circumference while the total width of the parasite at that section is 58 x 60 µm (**Fig. 5.3 B**). Posterior to this, the oesophageal tube is surrounded by nervous tissue (**Fig. 5.3 D**).

5.3.1.1.2 Female reproductive organ

In most histology sections, some part of the female reproductive apparatus was seen and from which, one or two and occasionally three uterine tubes were seen. The lumen of these tubes were filled with eggs at various stages of development, as were the slightly basophilic filaments identified as spermatozoa (**Figs. 5.4-5.7**).

The ovary is positioned medially and immediately posterior to the testicular column, and distally located to the descending uterus. Within the ovary several detached oocysts could be seen (**Fig. 5.6 C & Fig. 5.7 A**).

5.3.1.1.3 Ovary

5.3.1.1.4 Uterus

The uterus, as seen in cross section, can be divided into the ascending and the descending (upper, mid, and distal) uterus. At some stage, the uterus narrows such that it allows the passage of only one egg at a time (**Figs. 5.7 & 5.10 C**). From the histology sections, the diameter of the uterine tubes varies from 20 µm to 92.37 µm along its length. The most

distal part of the descending part of the tube measures 20 µm while the widest part of the tube is at the mid-point of the descending uterus where it measures 92.37 µm. It is interesting to note that the uterine tube at this point occupies almost the entire cavity of the parasite, which has a diameter of 95.92 µm.

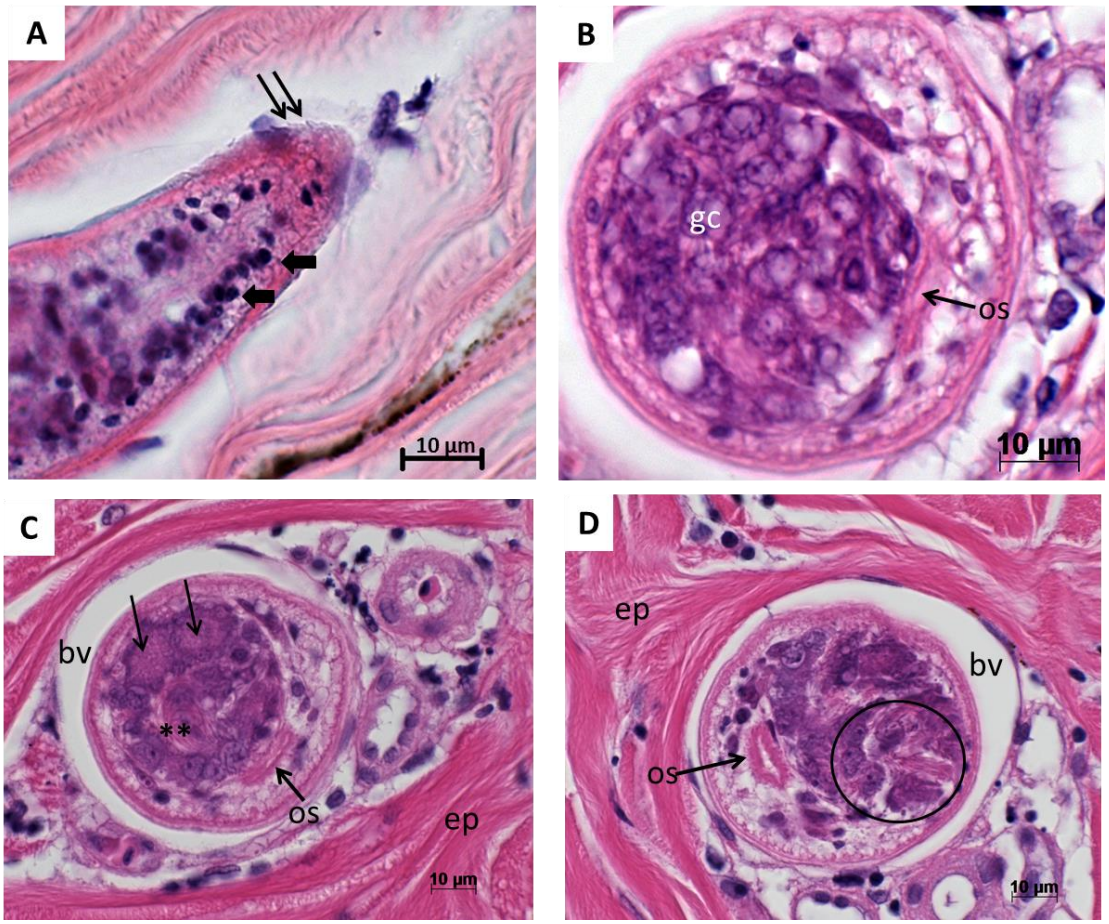

Figure 5.3. (**A-D**) Serial H&E stained sections through a blood fluke situated within a blood vessel in the epithelium of a dusky grouper, *Epinephalus marginatus*. (**A**) Longitudinal section of the proximal end of the adult blood fluke, showing parenchymal cells (thick arrow) and short spines on the tegument (thin arrows).(**B & C**) Serial sections through the same blood fluke within a blood vessel (bv) cut at different depths, where **B** is a section through the oesophagus (os) which is surrounded by glandular cells (gc); and,(**C**) is a second section through the oesophagus that shows the presence of unknown cells and structures denoted by the two thin arrows and also by a "**" which are seen distal to the oesophagus (os).(**D**) Aurther lower section through the oesophagus suggests that the oesophagus is surrounded by nerve tissue (circled). *Abbreviations:* bv = blood vessel; ep=epidermis; gc=glandular cells; os=oesophagus (H&E)

The ovary containing oocytes appears to be present in islands along sections of the uterus; these ovarian pockets are seen as aggregates of basophilic cells containing one strongly basophilic nucleus with a paler basophilic cytoplasm (**Fig. 5.7 A**).

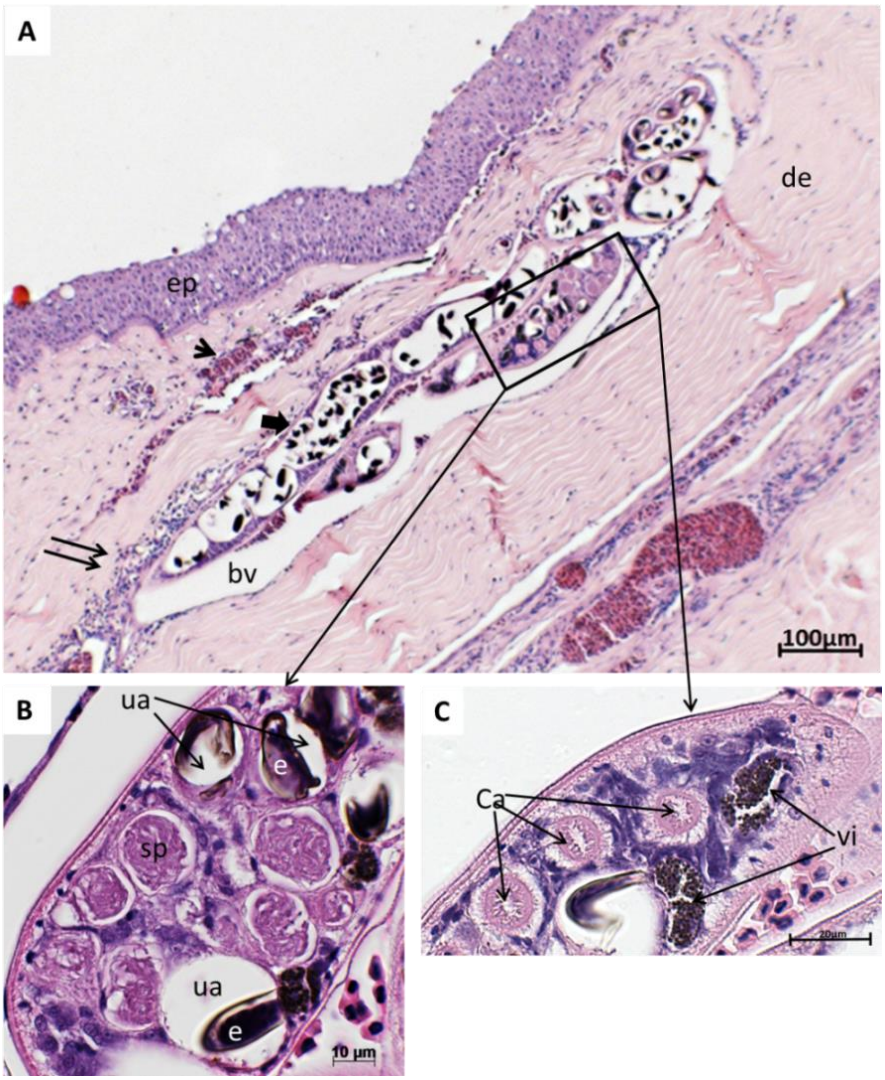

Figure 5.4. (**A**) An adult blood fluke lodged within a dermal blood vessel (bv) that contains numerous eggs within its uterine tube (thick arrow) eliciting a minor perivascular inflammatory response (double arrow) and congestion (arrow head). (**B**) Cross section through the vas deferens (?) showing the presence of sperm (sp); the tubes appear to be spiral-shaped intersecting with sections of the ascending uterus (ua) within which several eggs (e) can be seen. The morphology of the uterine histological suggests a similar loop-like structure. (**C**) Cross section through the same parasite presented in image (C) showing a cross-section through the parasite's intestinal caeca (ca) and vitellarium (vi). *Abbreviations:* bv=blood vessel; ca=caecum; de=dermis; e=egg; ep=epidermis; sp=sperm; ua=ascending uterus; vd=vas deferens; vi=vitellarium. (H&E)

Vitelline cells were seen in aggregates located to one side of the ascending and upper descending portions of the uterus (**Fig. 5.6 A&B**). In only a few sections, a number of narrow tubes containing villi-like structures, sitting adjacent to the uterus were identified as intestinal caeca (**Fig. 5.4 A&C**). To the side of this, there were several cross sections through a sperm-filled tube identified by its basophilic response when stained with H&E (**Fig. 5.4 B**).

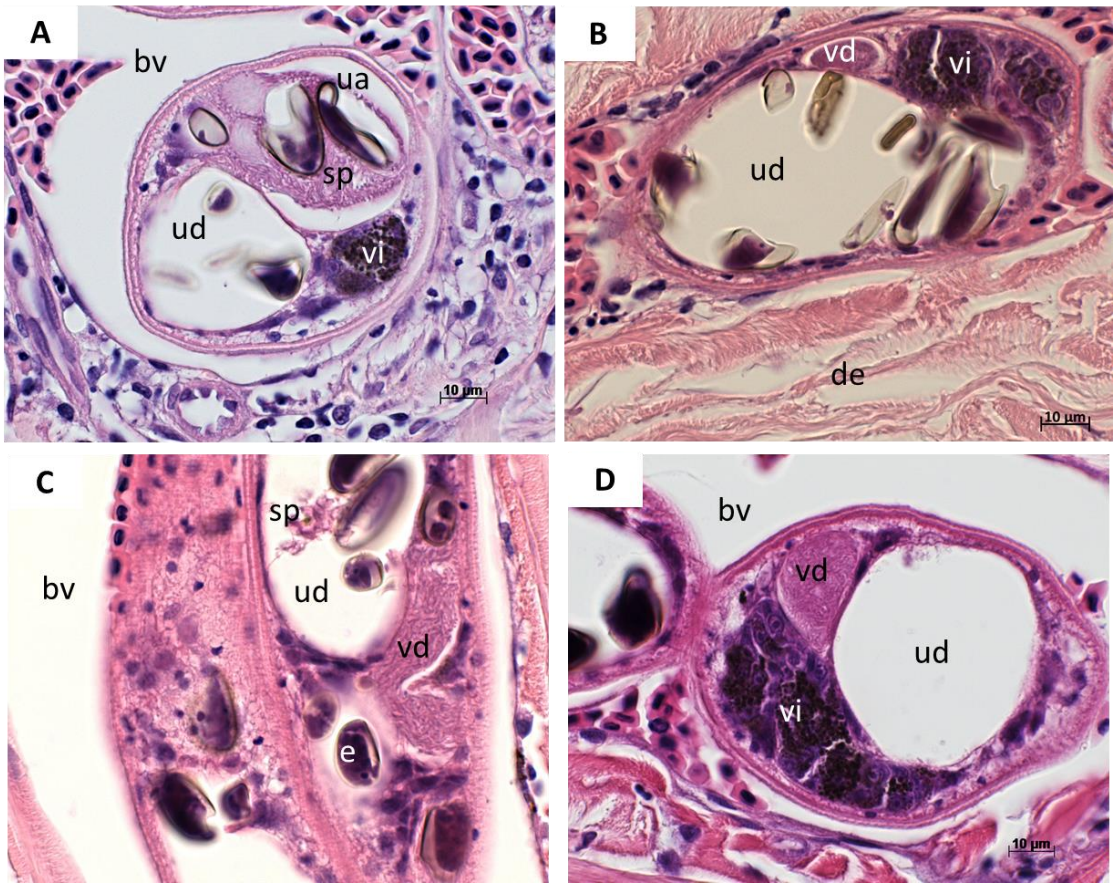

Figure 5.5. Cross section through an adult gravid blood fluke within a blood vessel. (**A**) Cross section through the uterus which is bordered by vitellarium (vi). (**B**)**. (C)** Vas deferens (vd) subsequently narrows; seen in longitudinal section, were the vas deferens has a loop-like appearance. Vitellaria is seen along the entire length of the (A) ascending and (D) descending uterus. *Abbreviations:* bv=blood vessel; de=dermis; e=egg; sp=sperm; ua=ascending uterus; ud=descending uterus; vd=vas deferens; vi=vitellarium (H&E)

A structure resembling the Oötype borders the descending uterus which was seen to contain only a few spermatozoa (**Fig. 5.6 D**). The ascending uterus is situated next to the mid-portion of the descending uterus which contains eggs with a homogenously formed embryo that are principally basophilic when stained with H&E and are surrounded by a yellow egg shell. The ascending uterus carries eggs at the earliest phase of development and is possibly the zone where fertilisation occurs. This is supported by the presence of vitelline cells and spermatozoa evident by the presence of vitelline cells and spermatozoa (**Fig. 5.6 A&B**).

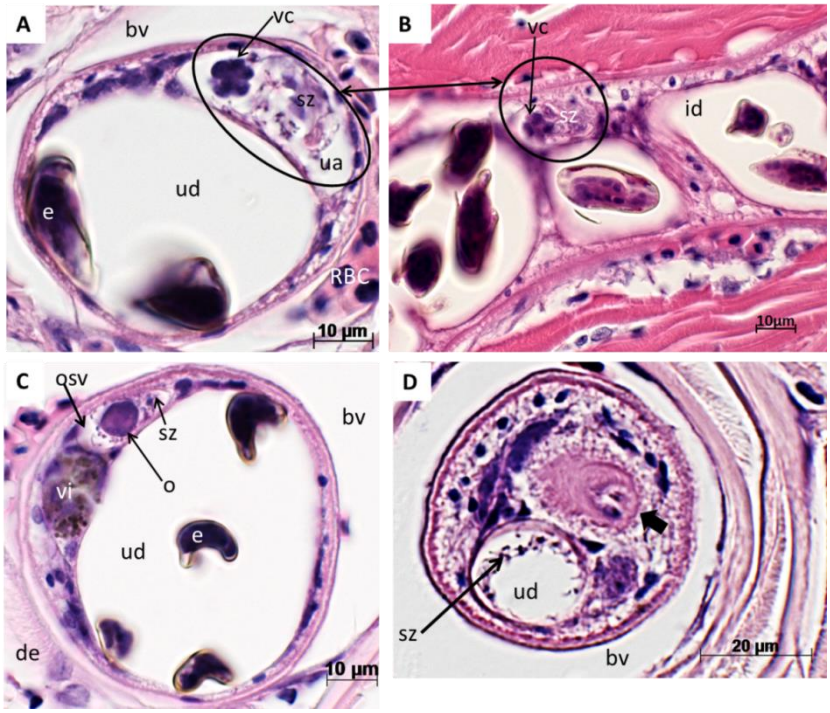

Figure 5.6. (**A**) Cross section and (**B**) longitudinal section through two ascending loops of the uterine tube taken from a similar position – both tissue sections can be seen to contain the ascending uterine loop (ua) which contains spermatozoa (sz) and vitelline cells (vc; circled). The ascending uterus borders the descending uterus (ud). (**C**) Cross section through the ascending (ua) and descending (ud) loops of the uterus, possibly the uterus at its post- Oötype position. (**D**) A structure morphologically resembling the Oötype (short arrow) is adjacent to a descending loop of the uterine tube (ud) which contains a few spermatozoa (sz) which can be seen close to its inner margins. *Abbreviations:* bv=blood vessel; de=dermis; e=egg; osv=oviduct seminal vesicle; sz=spermatozoa; ua=ascending uterine loop, ud=descending uterine loop, vc=vitelline cells; vi=vitellarium (H&E)

The above suggests that at some point, the uterine tube descends and travels parallel to an ascending uterine tube; the ascending and descending uterine tubes can be differentiated by the development and maturity status of their eggs within.

5.3.1.1.4.1 The first loop of the uterine tube

Several sections contain one descending uterine tube. When viewed in cross section at the level of what is identified as the mid to distal section of the descending uterus, this has a lumen that measures approximately 80 µm in diameter whole parasite at that section is 84 µm and was found to contain more up to 7 eggs in certain sections, this is bordered by testicular tissue (**Fig. 5.10 D**).

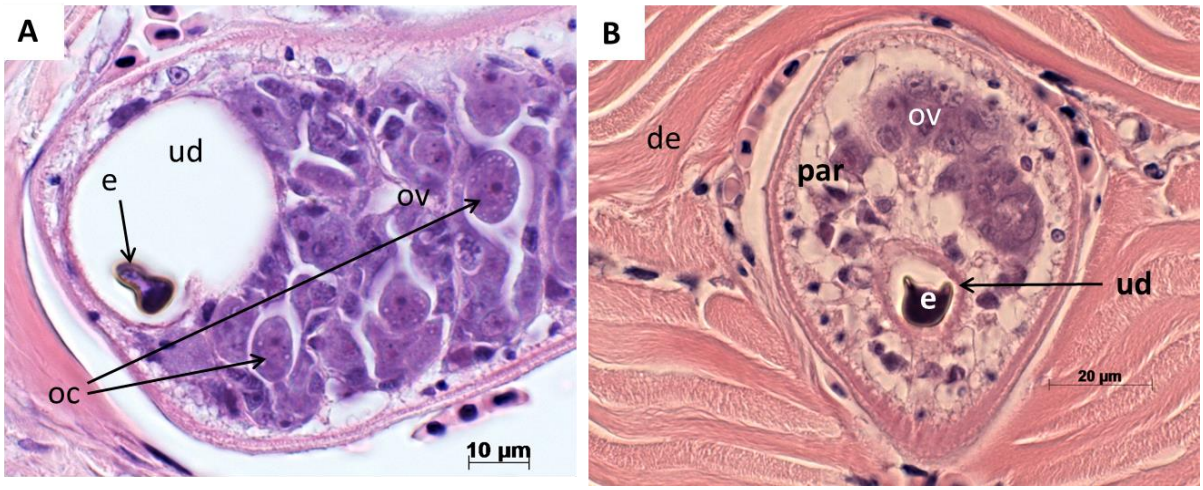

Figure 5.7. (**A**) Longitudinal section through a blood fluke within a dermal blood vessel. Within the parasite the descending uterine loop (ud), which contains an egg fragment (e), can be seen bordered by the ovary (ov) which is evident by the presence of oocytes (oc) within. (**B**) Narrow descending uterine tube (ud) containing one egg (e). *Abbreviations:* bv=blood vessels; de=dermis; e=egg; oc=oocytes; ov=ovary; par=parenchyma; ud=descending uterine loop (H&E)

The eggs at this point have features resembling those seen within the dermal blood vessels, dermal tissue and epidermis of its host. At some point the uterine canal narrowness down which allows the passage of only a single egg at a time is most likely situated in the most

distal portion of the uterus and is sided at some point by ovarian and testicular tissue in longitudinal section (**Fig. 5.10 B**), while in cross section only testicular tissue (**Fig. 5.10 C**) or only ovarian tissue (**Fig. 5.7)** can be seen at any one time. It is possible that the ovaries intersect with the testes.

5.3.1.1.4.2 The second and third loops of the uterine tube

The eggs in the second and third loops of the uterine canal ranged from very early stages to more developed eggs. In the early stages, the eggs may contain a vitelline mass (**Fig. 5.8 D**) and have undifferentiated embryos, while in the second uterine loop, the eggs were at a more advanced stage of development (**Fig. 5.8 C)**. These findings (*i.e.* the sequential progressive development of eggs in the different sections / loops of the uterine tube) suggested that there was only a single tube that not only had a clear U-bend, but most likely consisted of an intricate U-bend with loops as commented on above. The two juxtaposed uterine canals, each contained eggs at different stages of maturation. This therefore provided compelling evidence for the existence of a single, coiled tube. Eggs at a late stage of maturation were also seen within uterine canal adjacent to a tube containing spermatozoa (**Fig. 5.10**).

In **Figure 5.11 C**, the appearance of a cone-like structure (body protuberance), in histological sections is most likely the genital pore. A cross section through the most distal portion of the uterus, which lies adjacent to which is most probably the cirrus sac, or a seminal receptacle, can be seen to contain eggs with fully developed embryos within (**Fig. 5.9**). The embryos at this point begin to resemble the miracidia found in the eggs within the dermis and epidermis. Several vesicles between the egg shell and embryo can also be seen (**Fig. 5.9 A**), while the seminal receptacle or cirrus sac is observed to contain spermatozoa (**Fig. 5.9**).

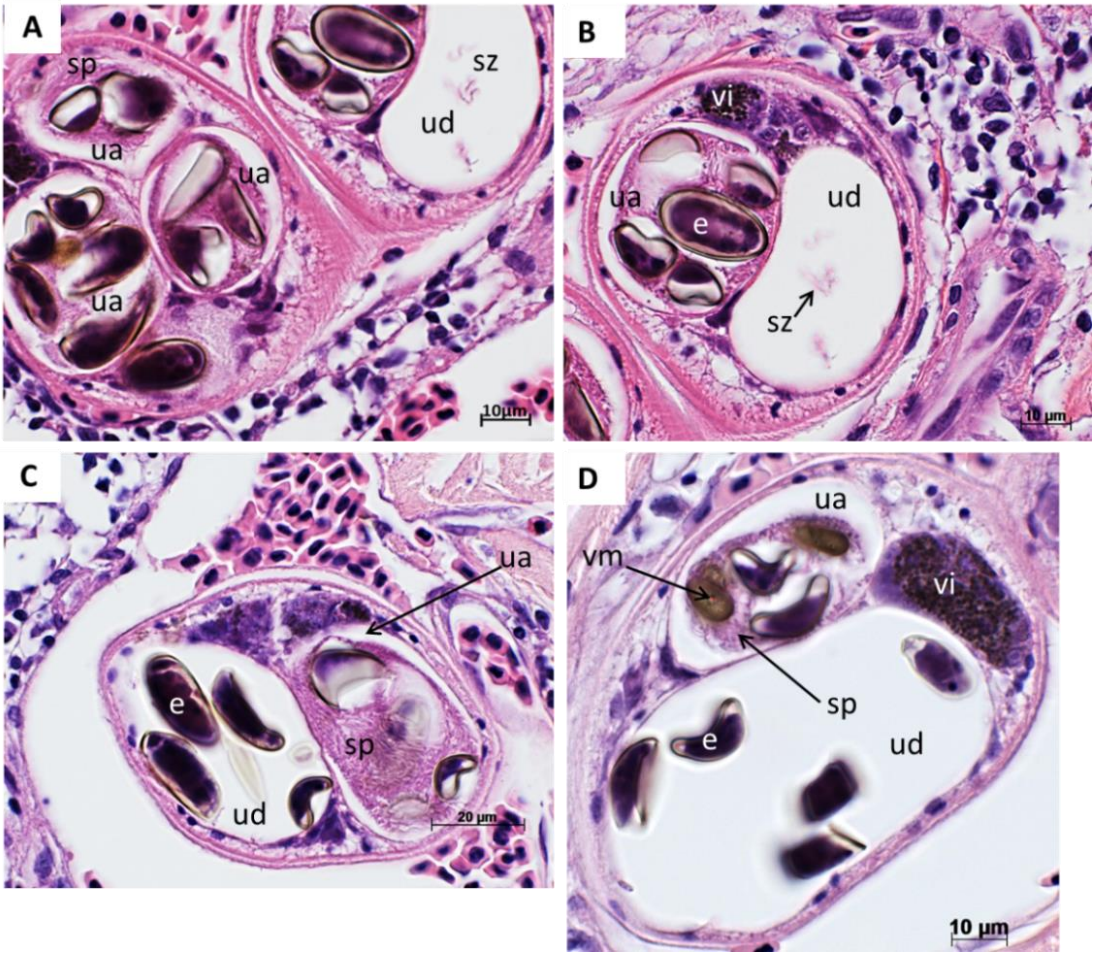

Figure 5.8. Cross section through a blood fluke showing the uterine tube at different points along its length. (**A**) Dense concentration of sperm (sp) and several eggs are seen in cross section through the ascending uterus (ua) suggesting the uterus is looped. While the cross-section through two uterine tubes are shown, one is the descending uterus (ud), the other an ascending uterus (au). (**B**) Section is a continuation of the image presented in (**A**) showing two descending loops of the uterine tube (ud), these are almost devoid of sperm and only a few spermatozoa (sz) and vitellarium (vi) can be seen. (**C**) Cross section through two similar-sized loops of the uterine tube (ua, ud) with a large amount of sperm (sp) and a few eggs within the ascending uterine loop (ua) and four eggs within the descending uterine loop (ud). (**D**) Cross section through two loops of the uterine tube (ua) containing sperm (sp), misshaped eggs and a vitelline mass (vm).The wide lumen of the descending uterine loop (ud) can be seen to contain five egg fragments. Both the ascending and descending loops (ua, ud) are bordered by vitellarium (vi). *Abbreviations:* e=egg; sp=sperm; sz=spermatozoa; ua=ascending uterus; ud=descending uterus; vi=vitellarium; vm=vitelline mass (H&E)

5.3.1.1.5 Testis

The testis is possibly located pre-ovarian (**Fig. 5.10**) within a thin connective tissue. In histological section it presented as not being lobulated (**Fig. 5.10 B**), while in longitudinal and oblique sections it appeared to have a more ~~sprawling~~ spiral morphology (**Fig. 5.10 C&D**). An additional stucture, which could be a distal auxiliary seminal receptacle/ seminal receptacle extending in parallel with lateral body margin, was also seen in several sections (**Figure 5.12**). The spiral-shaped, possibly auxiliary external seminal vesicle is bordered by a tubular structure that extends in parallel with the lateral margin of the body. The spiral auxiliary external seminal vesicle is packed with spermatozoa while the straight tube appears to be empty.

5.3.2 Description of juvenile parasite

No juvenile blood flukes were found in any of the 26 dusky grouper that were examined for the purposes of this study, which might be due to these stages being present at a different site to adults.

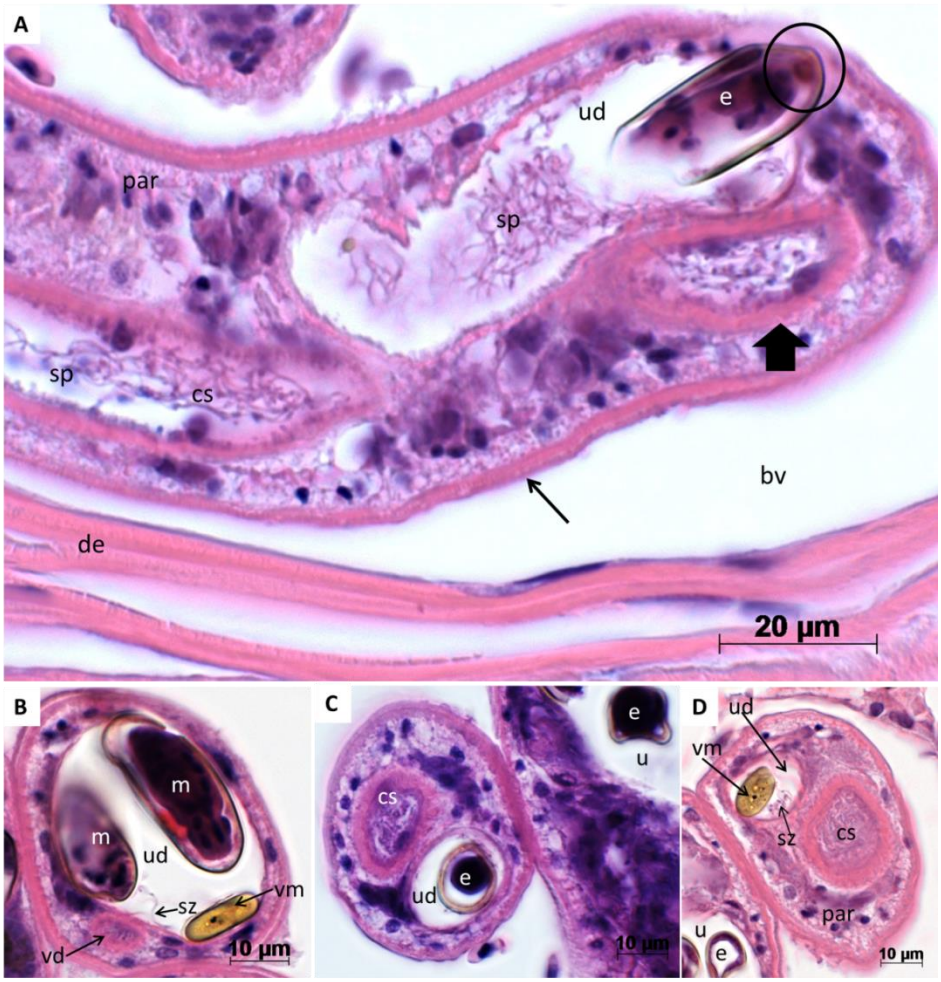

Figure 5.9. (**A**) Longitudinal to oblique section through the uterus and the cirrus sac (thick arrow) of the blood fluke - both structures contain sperm (sp) while the uterus contains an egg (e) which contains vesicles (circled). The parasite is covered by a smooth cuticle (arrow), and is lodged in a dermal (de) blood vessel (bv). (**B**) A cross section through one end of the cirrus sac (cs), which might be the vas deferens (vd). The descending uterus (ud) contains an egg with a miracidium at an advanced stage of development and with a few spermatozoa (sz). (**C**) The cirrus sac and narrow descending uterus (ud) which contains one egg (e). (**D**) A large cirrus sac (cs) and a narrowing descending loop of the uterus with a few spermatozoa (sz) and vitelline mass (vm) within. *Abbreviations:* bv=blood vessels; cs=cirrus sac; de=dermis; e=egg; par=parenchyma; sp=sperm, sz=spermatozoa; u=uterus, ud=descending uterine loop; vd=vas deferens; vm=vitelline mass (H&E)

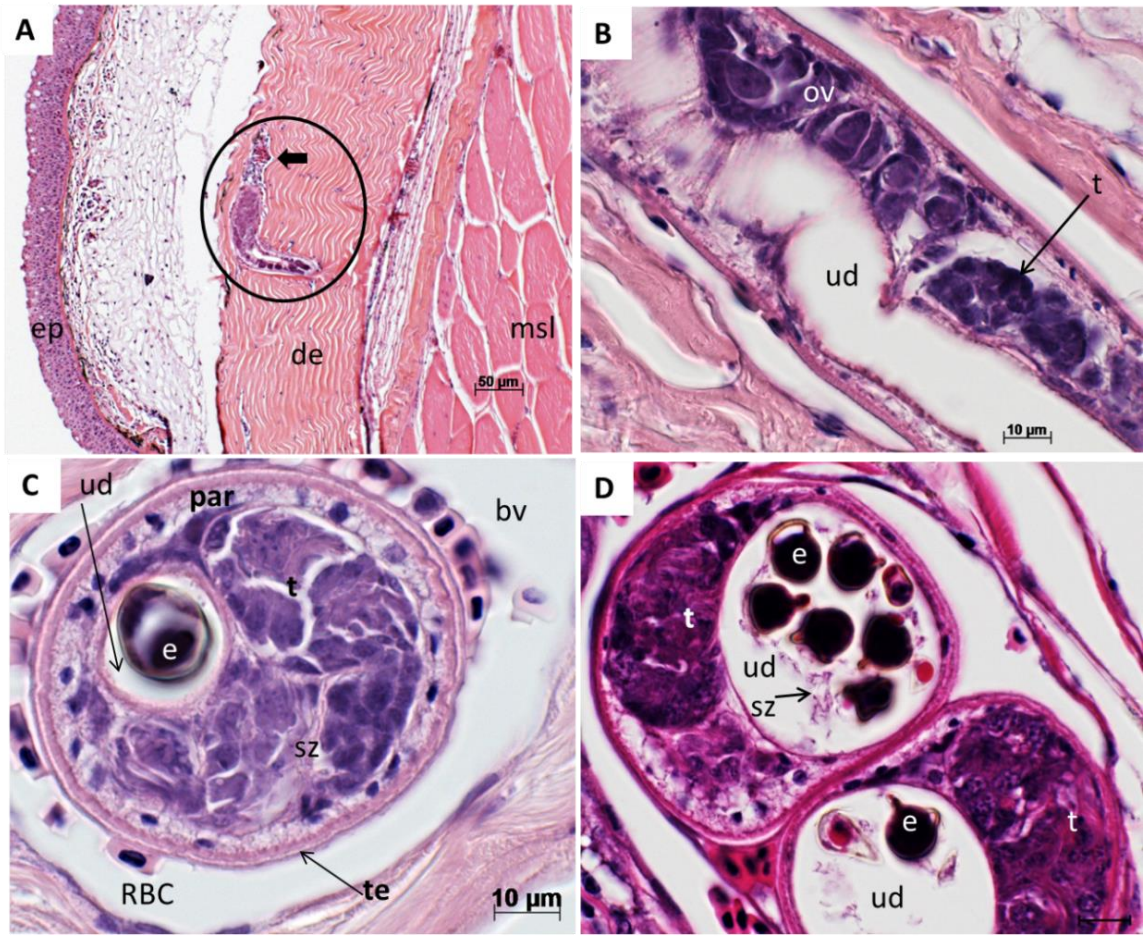

Figure 5.10. (**A**) Longitudinal section through a blood fluke lodged within a dermal blood vessel (circled), eliciting a minor inflammatory response (arrow). (**B**) A longitudinal section through the descending loop of the uterus bordered by the ovary and testis. (**C**) Narrow uterus containing one egg (e) and bordered by the testis (t) within which spermatozoa (sz) can be seen, and ovary (ov). **(D)** The descending uterus (ud) is this section is wider and contains several eggs as well as spermatozoa (sz). *Abbreviations:* bv=blood vessel; de=dermis; e=egg; msl=host muscle; ov=ovary; par=parenchyma; RBC=red blood cells; sz=spermatozoa; t=testis; te=tegument; ud=descending loop of the uterus (H&E)

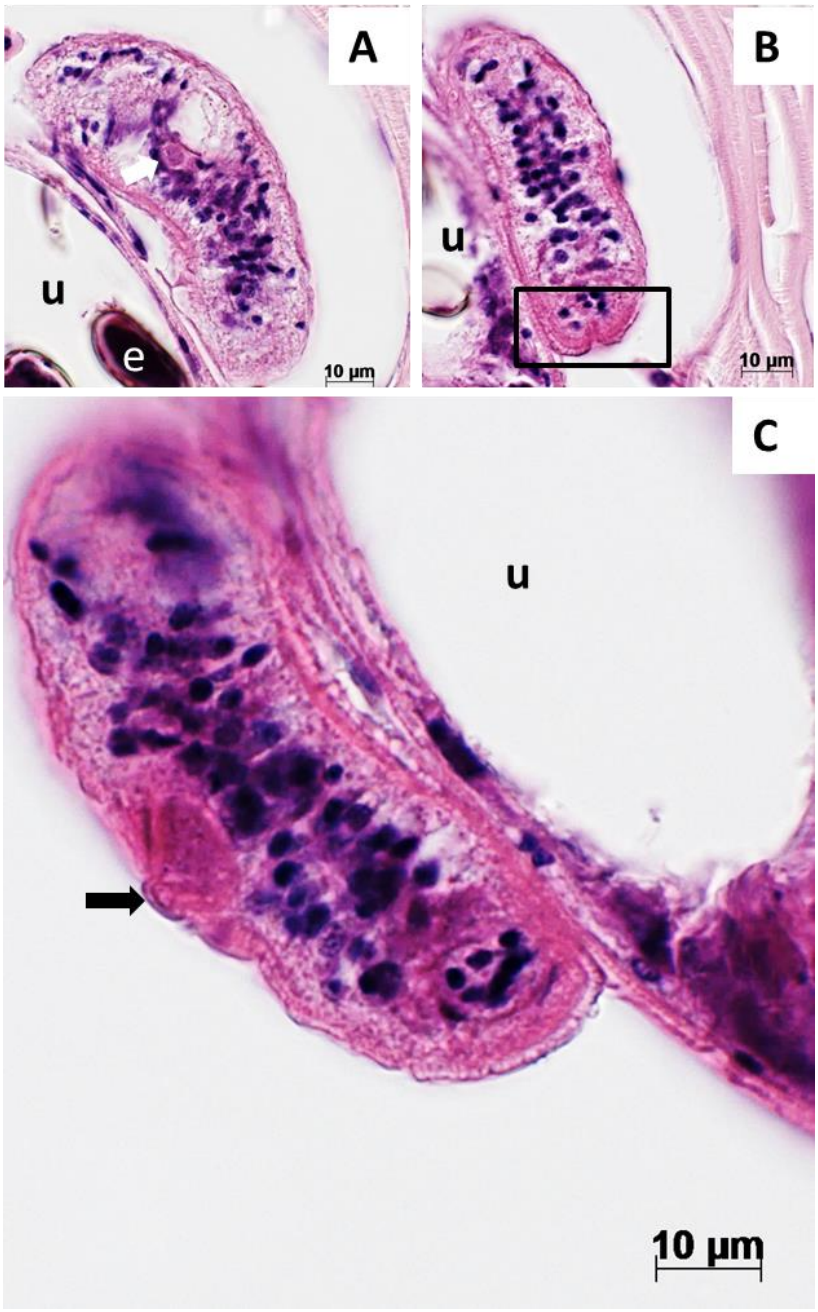

Figure 5.11. A, B & C. Serial sections through the posterior end of the parasite. (**A**) An unknown structure (black arrow). (**B**) An infolding or groove within the posterior end of the parasite (highlighted by the boxed area). (**C**) Arrow is pointing at what might be the genital pore or an excretory pore. *Abbreviations:* e=egg; u=uterus (H&E)

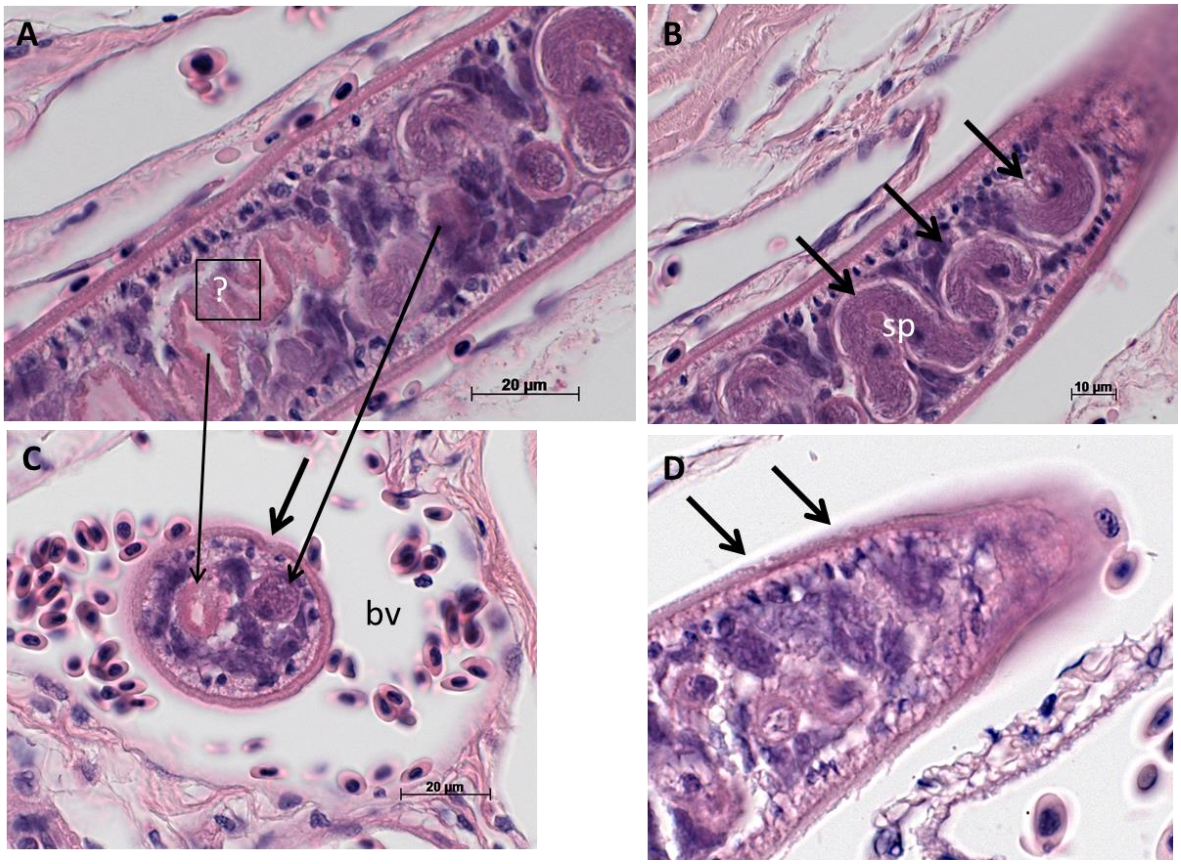

Figure 5.12. The presence of an auxiliary external seminal vesicle (**A, B & C**) seen in the longitudinal section as spiral shaped canal (denoted by the three arrows in image B) that contains a dense aggregation of sperm (sp). (**B**) The possible auxiliary external seminal vesicles appear spherical in cross section and contain at their margin, a dense structure resembling a nucleus (see two arrow that extends from image A to C). The adjacent canal marked as (?) in plate (**A**) has a thick margin and an empty lumen is indicated by an arrow that stretches from image A to C. (**C**) The presence of a groove in the tegument is also highlighted by a thick arrow. (**D**) Shows the presence of two grooves in the tegument (arrowed). *Abbreviations:* bv=blood vessel; sp=sperm (H&E)

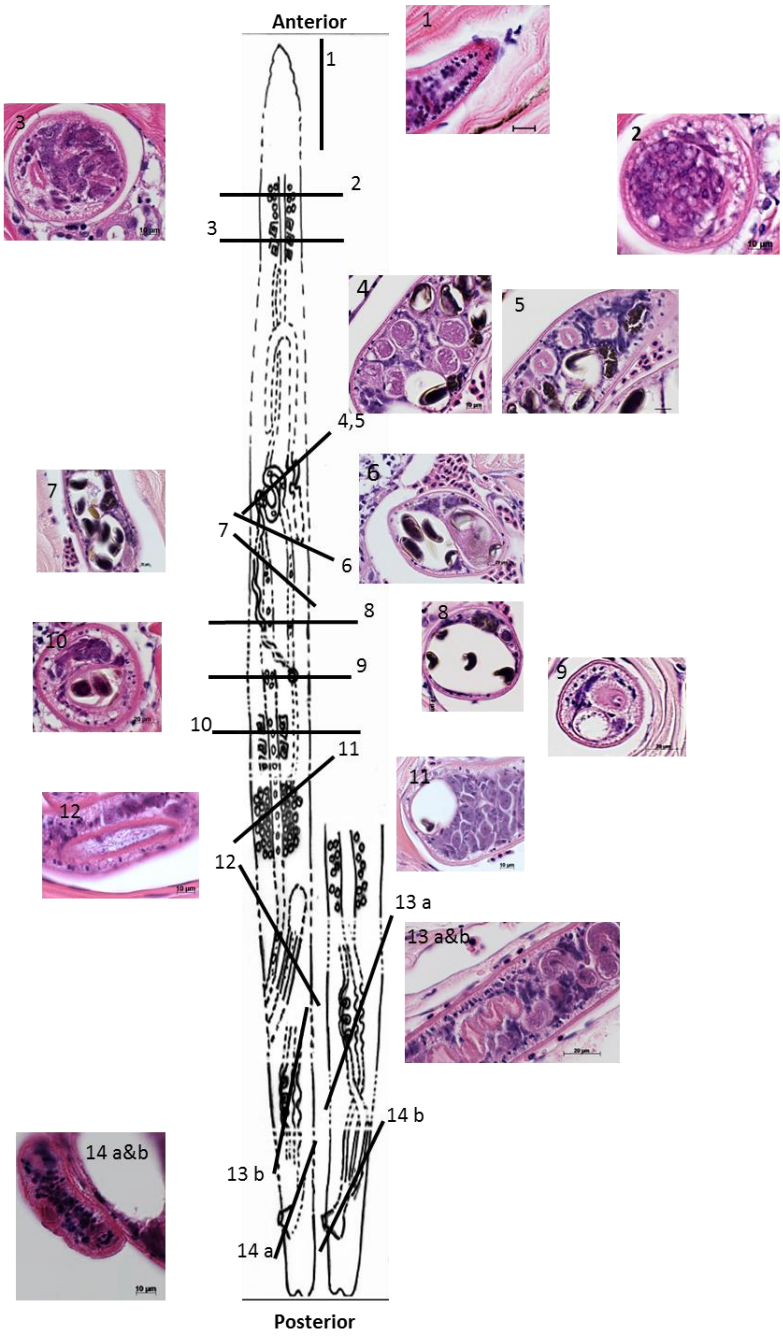

Figure 5.13. A reconstruction of the aporocotylid digenean parasitising the blood vessels in the dermal tissue of dusky grouper, from serial histology sections. The diagram focuses on the reproductive apparatus of the blood fluke which presents the passage of the looped uterine tube and the position of the seminal vesicle. The following collection of histology sections provide anatomical landmarks in the parasite's reconstruction. (**1-3)** Anterior end containing the oesophagus and associated cellular structures. **(4-5)** Ascending uterus. **(6-9)** Descending mid-uterus, ascending mid-uterus, and the descending uterus and Oötype. **(10-13)** Auxiliary externa seminal vesicle. **(Fig. 14 a & b)** Execratory pore or distal genital pore **(H)**. No histological sections covering the areas are marked by the discontinuous line

5.3.3 Description embryonated metazoan egg

5.3.3.1 Morphology

The digenetic trematode egg within the dermis when cut longitudinally has an almost oval shape with a smooth wall, rounded at both ends (anterior and posterior) and with no visible operculum (**Fig. 5.14 & Fig. 5.15 & 5.16**).

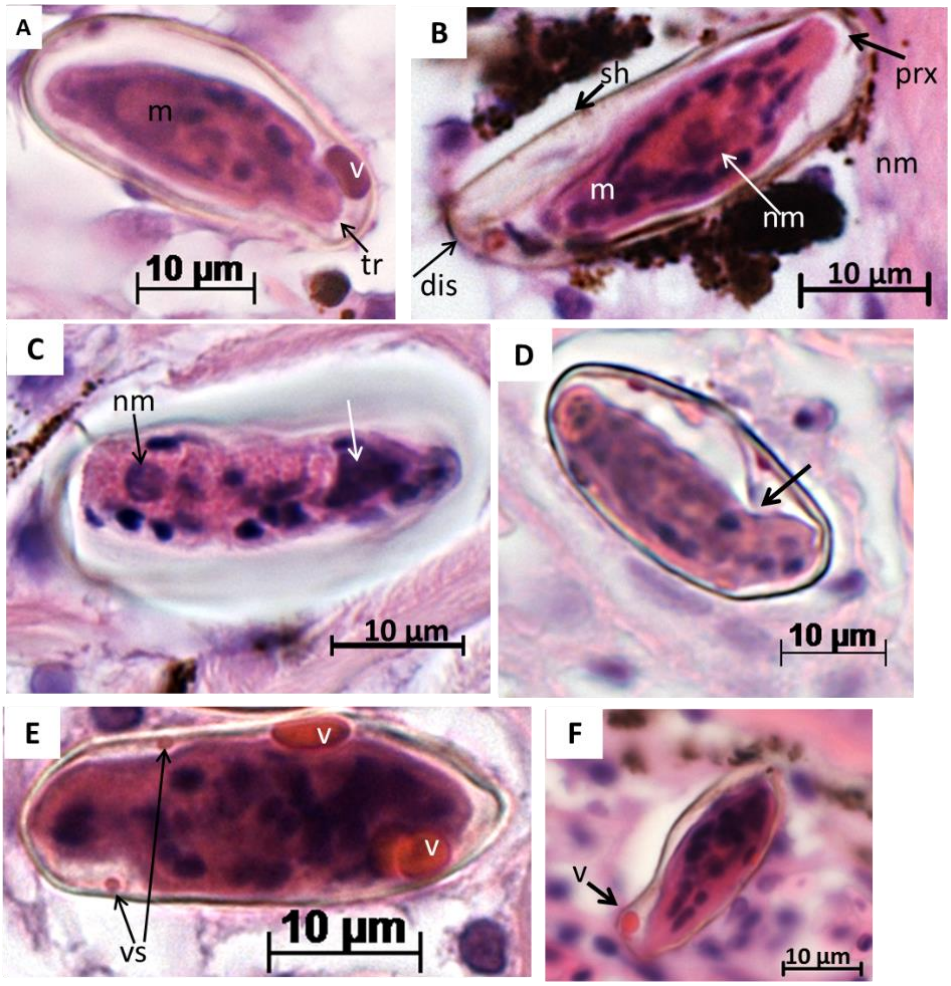

Figure 5.14 (**A-F**) Blood fluke eggs as seen at various stages containing developing embryos and miracidia, often with a visible terebratorium (tr); all were found within the dermal connective tissue. (**D**) The embryo has a visible indentation (arrow) though this is most likely an artefact. *Abbreviations:* dis=anterior; e=egg, m=miracidium; n=nucleus; nm=neural mass; o=ova; prx= posterior; sh=shell; sp=sperm; tr=terebratorium; vc=vitelline cells; vs/v=vesicles; u=uterus (H&E)

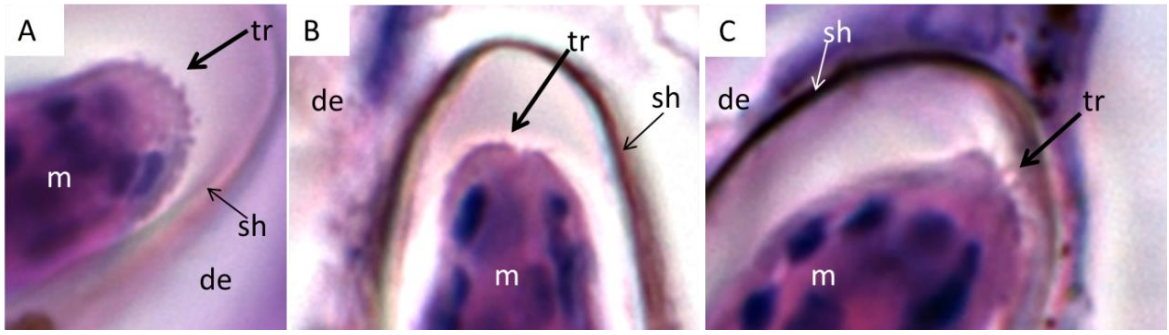

Figure 5.15. (A, B & C.) The proximal end of a longitudinal section through an egg containing a miracidium (m) that was located within the dermis (de) of its host. The terebratorium (tr) is visible on the proximal end of the miracidium. *Abbreviations:* de=dermis; m=miracidium; sh=egg shell; tr=terebratorium. (H&E)

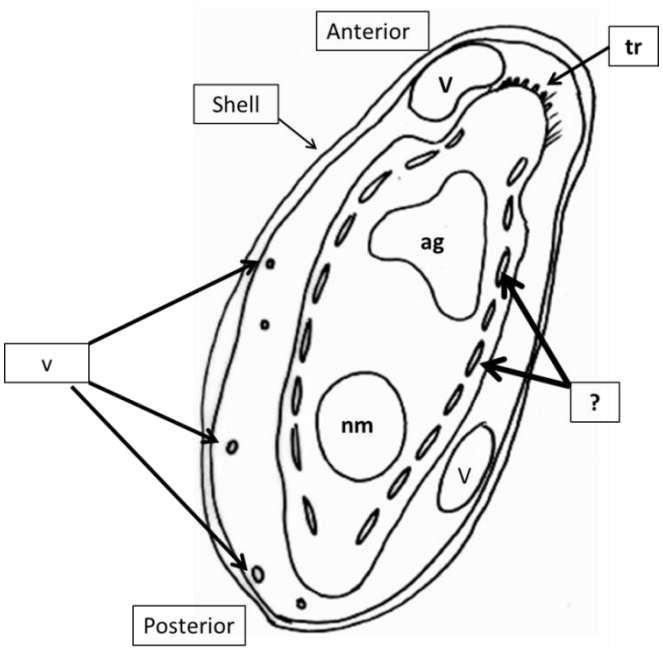

Figure 5.16. Diagram showing some of the features of the egg and the embryo. *Abbreviations:* ag=apical gland; m=miracidium; m=miracidium; nm=neural mass; tr=terebratorium; v=vesicles

The eggs ranged in size from 23.5 to 37.52 µm long. The egg shell consists of two layers, an internal layer and an external layer measuring 0.98-1.02 µm in width. The eggs appear to

have a yellowish colour in H&E stained sections and stain positively with PAS, *i.e.* has a light pink colour (**Fig. 5.18)**.

In longitudinal section, an elongated slightly curved embryo with slightly pointed ends was found lodged within the egg but not touching the egg wall (**Fig. 5.16**). The miracidium converges to a blunt or slightly pointed papilla at the anterior end, which may represent a "terebratorium", which is the organ used to penetrate the host. Moving posteriorly from the terebratorium the miracidium widens to give a narrow anterior bulb which extends for approximately 1/6th of the miracidium's length. After this point the miracidium widens to the full width of the egg and maintains a consistent width until it finally narrows in the final 1/4 of the miracidium's length as it approaches the posterior end (**Fig. 5.14**). Occasionally, a neural mass can be seen in some sections (**Fig. 5.14 B&C**). The ends of the embryo appear more pointed but this may be due to the angle of the section through the developing embryo. The embryo stains eosinophilic with dark basophilic granular inclusions, forming two rows at either side of the embryo (**Fig. 5.14 B**).

The fully developed miracidium has cilia on the posterior margins of its proximal end (**Fig. 5.14 A&B**), just distal to and almost lodged between the terebratorium and internal egg wall, where there appears to be one or two oval homogeneous vesicles staining eosinophilic and varying in size, (**Fig. 5.14 A, E&F**). The vesicles are commonly seen within the egg and these may exert pressure on the embryo, and result in the walls of the egg bulging out 3.27-6.77 µm. The chronological development of the blood fluke is shown in **Fig. 5.19**.

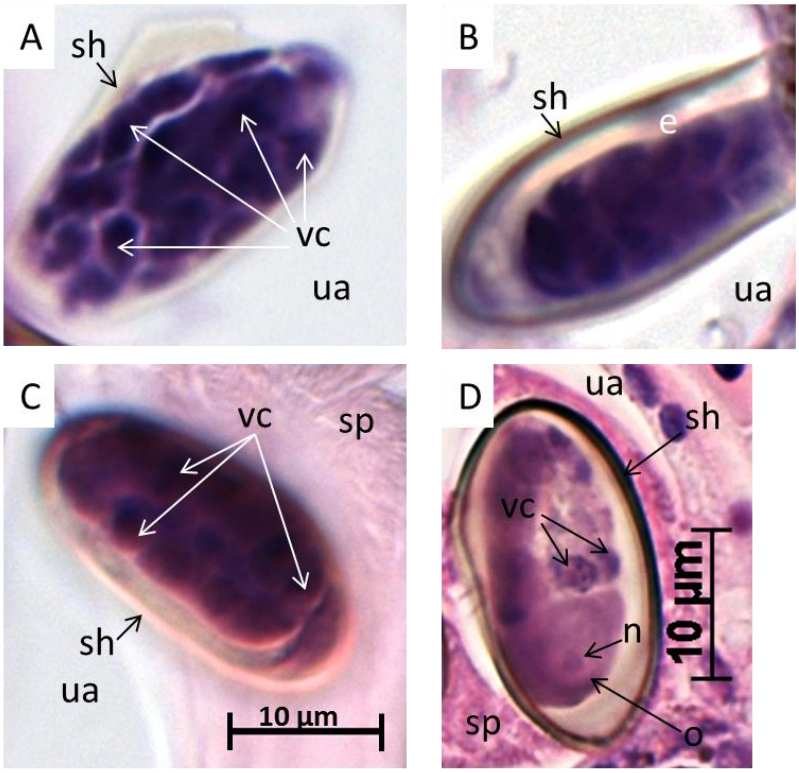

Figure 5.17. (**A-D**) Development of the egg and the embryonic miracidium within the ascending loop of the uterus. *Abbreviations:* e=egg; n=nucleus; o=ova; sh=shell; sp=sperm; ua=ascending uterus; vc=vitelline cells. (C: phase contrast image) (H&E)

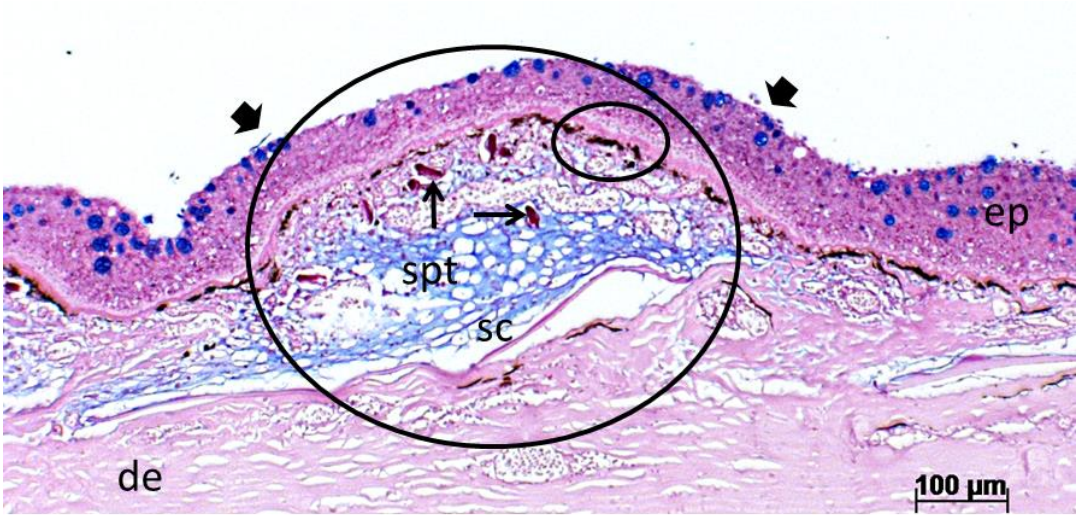

Figure 5.18 Blood fluke eggs found in the dermis close to the basement membrane, located above a scale pocket (spt) (within the large black circle) giving a strongly positive reaction to PAS (black arrow). The mucous cells (thick black arrow) in the epidermis (ep), however, are negative to PAS. A disrupted band of melanin can also be seen (in small black circle). *Abbreviations:* de=dermis; epi=epidermis;sc=scale; spt=scale pocket (PAS)

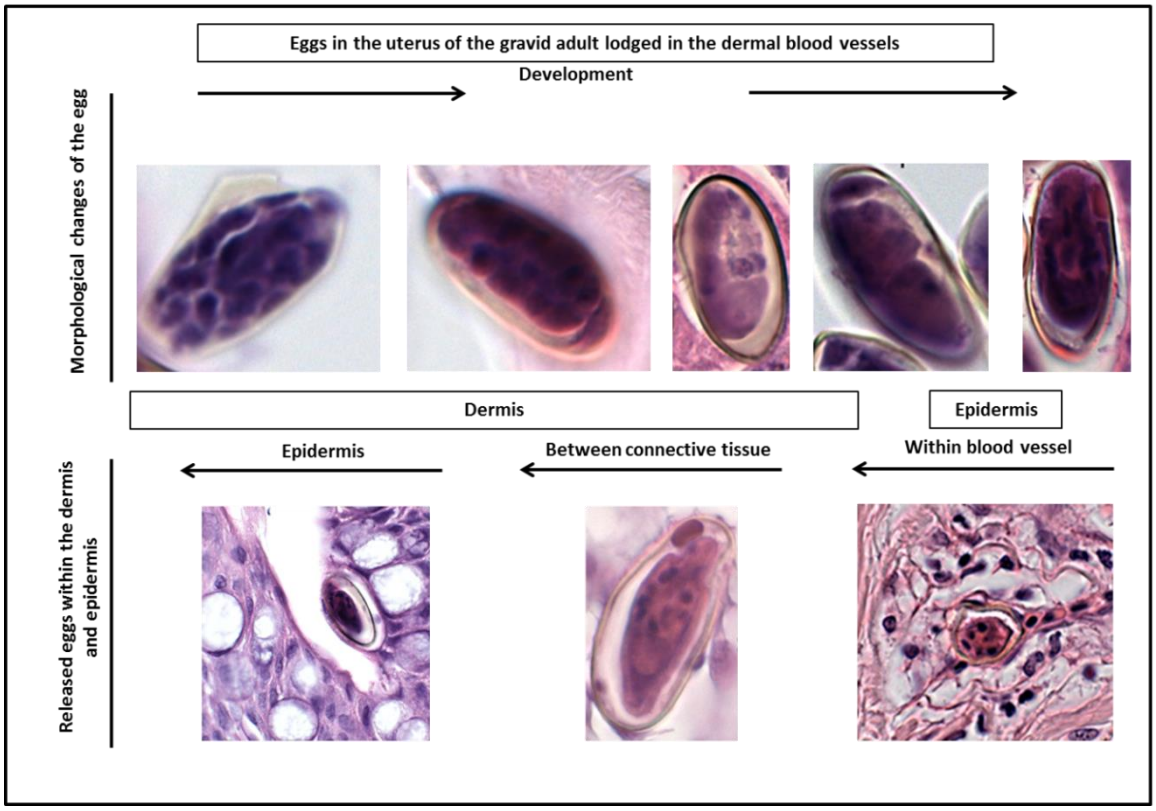

Figure 5.19. Chronological development of the blood fluke egg as determined from histological sections. The development of the parasite's eggs and embryos are followed through its passage along the uterus until its release and then its progression through the dermal tissues of its dusky grouper host until its release and hatching at the epidermis. (H&E)

Almost all the eggs that contained vesicle-like structures were seen within the descending distal uterus, in blood vessels, in the dermis and the epidermis. By comparison, the eggs in the descending uterus appeared to have fewer vesicles (**Fig. 5.9 A&B**). Whilst the eggs in within the ascending loop of the uterus contain embryonic miracidium at an early stage of development visible by the presence of vitelline cells, there seem to be no vesicle at this stage of egg development (**Fig. 5.17**). Eggs situated in the dermis were found to contain one and more vesicles and several vesicles. The vesicle-like structures that were seen were found lining the inner wall of the egg either as an aggregation or scattered over the internal lining (**Fig. 5.14 E)**; in one egg more than 5-8 vesicles were counted.

5.3.4 Life-cycle

The egg containing the miracidium appears to leave its fish host via the epidermis. The sexual cycle of the parasite takes place in the fish host, making the dusky grouper its definitive host (**Fig. 5.20**). What is currently unknown is the manner in which the fish host is infected and whether this is by ingestion, penetration of the skin and/or gills, the intermediate host(s) involved and the development time between infection until the appearance of the adult at the infection site and the production of eggs.

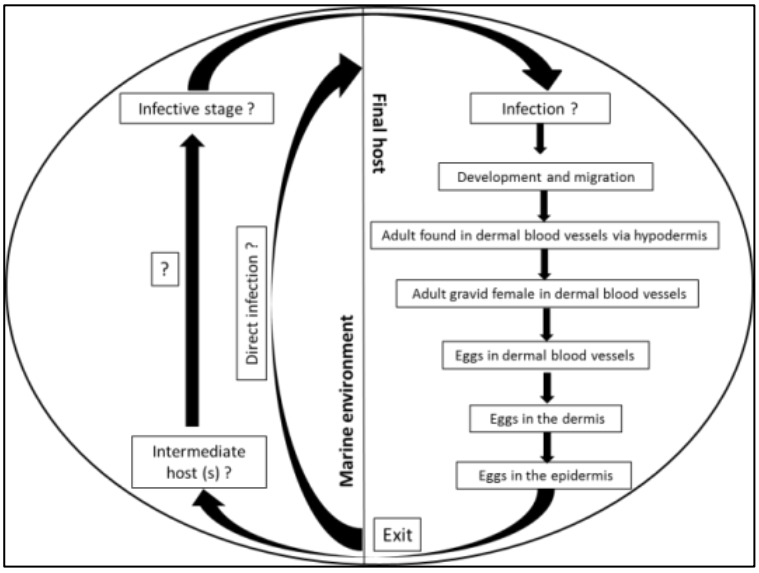

Figure 5.20 A diagram of the life-cycle of the blood fluke as determined from the analysed material

5.3.5 Summary of main morphological features

- Elongated, measuring approximately 1500-2000 µm long and 20-80 µm (narrowest/widest) in width.
- Tegument is smooth, with some spines present at the mid-distal end, at the approximate level of the descending uterine tube. Occasionally tegumental folds can be seen in cross sections within the mid to proximal sections, however, it is not clear if this is a fixation artefact or a genuine structural peculiarity.

- The presence of pits in the tegument within the distal portion of the fluke.
- The oesophagus is thin walled and surrounded by a semi-circle of gland cells.
- The ovary is multi-lobed that surrounds the uterus in the mid-section. Ovarian tissue, however, appears to be absent in the vicinity of the cirrus pouch situated at the terminus of the uterine canal.
- It is possible that there are separate female and male genital pores.
- The vitellaria is extensive and extends along the first ascending section of the uterine tube and its first loop, and then adjacent to the first section of the descending uterus.
- Oviseminal duct filled with a high concentration of spermatozoa, which can be seen surrounding ova.
- Uterus can be regarded as pre-ovarian, ovarian and post-ovarian.
- The uterus is long and folded into loops.
- Seminal vesicle is present, bordering the mid-uterus.
- Vas deferens runs alongside the mid and early sections of the uterus
- Possible posterior auxiliary seminal vesicle present; spiralled.
- Single tubular testis.
- The cecum intercepts the uterine tube.
- The appearance of there being only a single intestinal caecum may be misleading, given that in other members of the Aporocotylidae, the caecum bifurcates to give two blind ending sacs. The true condition may therefore be one where there are two intestinal caeca of unequal length, with only the longer one visible in the cross section. Presence of vitelline cells, within what might be the vitelline canal.
- Oötype, suspected to be present but not possible to confirm.
- Eggs in the proximal uterus are seen surrounded by spermatozoa.

- Embryonated eggs, at various stages of maturation and measuring 20-30 µm in length were seen along the entire length of the uterus.
- Spermatozoa observed within the vas deferens, the cirrus pouch and the uterus from the post-oviduct position almost until its distal terminus.
- A spiral auxiliary external seminal vesicle packed with spermatozoa, bordered by an empty, tubular structure vesicle.
- Laid eggs measure from 23.5-37.52 µm long and 5 - 10 µm wide.
- The presence or absence of the following structures cannot be stated:
    - Separate or joined genital pore
    - Female genital pore
    - Male genital pore
    - A comprehensive map of body spination
    - The precise shape of the intestine could not be confirmed, although it may consist of two blind ended caeca, one longer than the other, extending down each side of the body.
    - Excretory pore.

5.3.6 The position of the current parasite in the blood fluke genera

The parasite described in the current study is a trematode and shares a number of key features with blood flukes belonging to the family Aporocotylidae. These features include: location of the adult within the host (vascular system), ovoid un-operculated eggs containing miracidia, body spines, similarly positioned and configuration of the ovaries, testis and uterus. The position of and extent of the uterus, however, being pre-ovarian, ovarian, and post ovarian, does not correspond to the structure described for other recognised blood fluke genera (see **Table. 5.2**).

Table 5.2 A summary of the key morphological features determined on the blood fluke recovered from the dermal tissue of dusky grouper and their comparison with the features seen in the other 33 genera within the Aporocotylidae.

Genus	Spines	Body shape	Eggs	Uterus	Testis	Ovary	Ref
Current parasite	**4**	**1;10;11**	**2**	**1;3;4**	**maybe 1**	**3;6**	
Acipensericola Bullard, Snyder, Jensen *et* Overstreet, 2008	1	21	6	2	2	1	Bullard, Snyder, Jensen & Overstreet, 2008
Adelomyllos Nolan *et* Cribb, 2004	2	3;4	1	1	2	2	Nolan &Cribb, 2004
Ankistromeces Nolan *et* Cribb, 2004	2	1;6	*n.d*	1	1	3;4;5	Nolan &Cribb, 2004
Aporocotyle Odhner, 1900	2	3;7	7	3	5	5	Smith, 2002
Braya Nolan *et* Cribb, 2006	2	5;3	2;8	1	1	3;7	Nolan & Cribb, 2006
Cardicola Short, 1953	2	8;3;9;10	9;10	1	1	6;8	Smith, 2002
Chaulioleptos Nolan *et* Cribb, 2005	2	1	3*2	1	2	2	Nolan & Cribb, 2005
Chimaeribemecus Van der Land, 1967	2	8;3	*n.d*	*n.d*	5*1	2;6	Smith, 2002
Cladocaecum Orélis-Ribeiro *et* Bullard, 2016	Requested						Orélis-Ribeiro & Bullard, 2016
Cruoricola Herbert, Shaharom-Harrison *et* Overstreet, 1994	2	11;3	2	1	1	2;6	Smith, 2002
Deontacylix Kinton, 1910	4	9;12	*n.d.*	3	1	9	Smith, 2002
Elaphrobates Bullard *et* Overstreet 2003	2	9;3	1;8	1	1	2;10	Bullard & Overstreet 2003
Elopicola Bullard, 2014	4	3;10	Uterine eggs 5/ external 11	3;4	1	2	Bullard, 2014
Hyperandrotrema Millard *et* Ktari, 1978	1	3;9	*n.d.*	1	1	6	Smith, 2002
Kritsky Orélis-Ribeiro et Bullard, 2016	Interim unpublished	11	8			11	Bullard, 2016
Littorellicola Bullard, 2010	4	9	*n.d.*	1;4	5	7;2	Bullard, 2010
Metaplebniella Lebedev *et* Parukhin, 1972	4	12;3	2	1	*n.d*	2;9	Smith, 2002
Myliobaticola Bullard *et* Jensen, 2008	3	3;8	Uterine eggs 11	1;4	1	2	Bullard & Jensen, 2008

Genus	Spines	Body shape	Eggs	Uterus	Testis	Ovary	Ref
Neoparacardicola Yamaguti, 1970	3	3;9	*n.d.*	*n.d*	2	9	Smith, 2002
Orchispirium Madhavi *et* Hanumantha Rao, 1970	unknown	3;14	2	4	1	9	Smith, 2002
Parasanguinicola Herbert *et* Shaharom, 1995	4	3;14	5	4	1	2;7	Smith, 2002
Paracardicola Martin, 1960	2	3;14	2	4;3	2	7	Smith, 2002
Paracardicoloides Martin, 1974	1	3;8;10; 11	*n.d.*	3	2	2	Smith, 2002 ; Bullard, 2014
Paradeontacylix McIntosh, 1934	2	3;4;8	2	1	5	2	Smith, 2002
Pearsonellum Overstreet *et* Koie, 1989	4	3;14	2;4	3	1	2;7	Smith, 2002 & Bullard, 2012
Phthinomita Nolan *et* Cribb, 2006	2	15	*n.d*	1;3	2	3	Nolan & Cribb, 2006
Plethorchis Martin, 1975	4	3;4;9	3;7	1	5	12	Smith, 2002
Primisanguis Bullard, Williams *et* Bunkley-Williams, 2012	2	3	*n.d*	*n.d*	1	13	Bullard, Williams & Bunkley-Williams, 2012
Psettarium Goto *et* Pzaki, 1930	2	3;4;9	2	1	1	2;14	Smith, 2002
Pseudocardicola Parukhin, 1985	1	3;8;11	*n.d*	1	4	*n.d*	Smith, 2002
Rhaphidotrema Yong *et* Cribb, 2011	2	8;11	*n.d*	1	5	2	Yong & Cribb, 2011
Sanguinicola Plehn, 1905	2	3;4;8	3;5	1	1	4;6	Smith, 2002
Selacbobemecus Short, 1954	1	3;4;8	2;8	1	1	6	Smith, 2002
Skoulekia Alama-Bermejo, Montero, Raga *et* Holzer, 2011	2	1;3;16	Uterine 8;13 ;1	1	1	2	Alama-Bermejo, Montero, Raga &Holzer, 2011

Key to table (current species underlined)

Body shape: Long = **1**; Short = **2**; Flat = **3**; Thin = **4**; Elliptical = **5**; Cylindrical = **6**; Spindle-shaped = **7**; Small = **8**; Elongate = **9**; Oval = **10**; Lanceolate = **11**; Fusiform = **12**; Foliate = **13**; Spatulate = **14**; Thread-like = **15**; Curved = **16**

Spines: Robust, Spike-like body, Large spin = **1;** Spins = **2;** Spineless **3;** Small spines = **4**

Eggs: Elongate = **1**; Oval = **2**; Spines = **3**; Operculum = **4**; Triangular = **5**; Oblong = **6**; Fusiform = **7**; Spherical = **8**; Pyriform = **9**; Spiniform terminal filament = **10**; Polar filament = **11**; Oblong = **12**; Irregular = **13**

Uterus: Post-ovarian = **1**; Near ovarian level = **2**; Pre-ovarian = **3;** Post-testicular = **4;** Testicular = **5**

Testis: Single = **1**; Two = **2**; Five = **3**; Numerous = **4**

Ovary: Inter-testicular ovary = **1**; Post testicular = **2**; Posterior to, abutting or slightly overlapping posterior margin of testis = **3**; Spherical = **4;** Ovoid = **5;** Bilobed = **6;** Single = **7;** Triangular = **8;**

Multi-lobed= **9;** Post testicular border abatting = **10**; Finger like lateral projections = **11**; Dextro-laterally to posterior part of uterus = **12**; Abutting the posterior margin of the testis = **13**; Pre-ovarian = **14**

The parasite described in the present study, despite resembling a number of other genera, has features that preclude it from belonging to any of the previously described genera. The following list of established blood fluke genera explicitly highlights the features that distinguish them from this parasite:

1. *Acipensericola* possesses robust large, spike-like body spines
2. *Adelomyllos* possesses robust, spike-like body/ large spines
3. *Ankistromeces* is typified by having a post-ovarian uterus
4. *Aporocotyle* has fusiform eggs
5. *Braya* species have a post-ovarian uterus
6. *Cardicola* has pyriform eggs with a spiniform terminal filament
7. *Chaulioleptos* possesses spines that are arranged in ventro-marginal transverse rows, continuous along length of body; the uterus is post-ovarian
8. *Chimaeribemecus* has a large single testis, extending from the intestinal bifurcation to the level of the genital pore
9. *Cladocaecum* - no data currently available
10. *Cruoricola* species have a post-ovarian uterus
11. *Deontacylix* species are entirely covered with transverse rows of small spines; the ovary is positioned close to the posterior end of the fluke
12. *Elaphrobates* species have a post-ovarian uterus and a single, intercaecally positioned testis
13. *Elopicola* species produce triangular eggs with a polar filament
14. *Hyperandrotrema* species have large, robust, spike-like body spines
15. *Kritsky* produces minute, spheroid eggs; species possess a highly branched intestine terminating anterior to the ovary

16. *Littorellicola* species have a post-testicular, primarily post-ovarian uterus
17. *Metaplebniella* have a flattened, foliate-shaped body; the uterus is post-ovarian
18. *Myliobaticola* produce eggs with a polar filament; the body does not possess spines
19. *Neoparacardicola* produces eggs with a polar filament
20. *Orchispirium* species have a post-testicular uterus
21. *Parasanguinicola* species produce triangular eggs
22. *Paracardicola* have ovoid eggs ovoid with a terminal spine; testis is positioned posterior to the uterus and immediately anterior to the ovary
23. *Paracardicoloides* produce ovoid eggs with a terminal spine
24. *Paradeontacylix* possess ventro-lateral transverse rows of rose thorn-shaped spines; body shape is small, flattened and lanceolate; uterus is pre-ovarian
25. *Pearsonellum* produce operculated eggs
26. *Phthinomita* produce triangular eggs each bearing a spine
27. *Plethorchis* species produce fusiform eggs with a terminal spine
28. *Primisanguis* the uterus in these species is post-ovarian
29. *Psettarium* the uterus is lateral and post-ovarian
30. *Pseudocardicola* have ventro-lateral transverse rows of rose-thorn-shaped spines
31. *Rhaphidotrema* have a uterus that is coiled and post-ovarian
32. *Sanguinicola* produce triangular eggs bearing a spine
33. *Selacbobemecus* species have a long uterus that is entirely post-ovarian
34. *Skoulekia* have a lateral uterus that is positioned posterior to the ovary

In conclusion, from the above, it seems likely that the aporocotylid infecting dusky grouper belongs to a new genus. Further sampling and analysis are needed, however, to confirm this finding.

5.3.7 Inflammatory process

5.3.7.1 To adult blood flukes

Most adults found lodged in the dermal blood vessels elicit no inflammatory response from the host. When an inflammatory response was seen, it was in sections where there were aggregates of eggs both inside and outside the dermal blood vessels that were in close proximity to adult flukes within blood vessels. At this point, perivascular inflammatory infiltrates were observed.

The adult blood flukes were commonly found in the deep and superficial dermal blood vessels. Infected blood vessels are often enlarged and the parasite occupies almost the entire lumen. No extravagated adult parasites were seen in any histological sections.

5.3.7.2 To migrating eggs

The digenean eggs within the lumen of blood vessels, appear to either pass unnoticed (not surrounded by an inflammatory reaction), or are involved in an inflammatory process. Occasionally, one or more eosinophilic granular cells in the process or degranulation were seen attached to the surface of a parasite egg (**Fig. 5.20)**. The presence of flukes caused congestion of the very small blood vessels, which became engorged with RBCs.

In one instance, an egg could be seen occluding a small blood vessel; RBCs could be seen piled up behind the egg, which was three times larger than the diameter of the blood vessel lumen (**Fig. 5.20 B)**.

Eggs situated within the dermal connective tissue were found surrounded by melanin containing inflammatory cells (**Fig. 21 C**), several eosinophilic granulocytes were seen in the vicinity of migrating eggs, as well as lymphocytes, macrophages (**Fig.21 B**) and RBCs.

5.3.7.3 Other organs

Mild inflammatory responses and thrombi were occasionally observed in the endocardium and ventricular cavity of infected and non-infected fish. Occasionally, melanomacrophage centres (MMCs) were seen between in the endocardium. No eggs or adult parasites were seen in the heart of any of the fish that were examined. Apart from congested spleens and increased renal interstitium (**see Chapter. 4**), no obvious pathological changes in relation to the parasite were observed in any other host organs.

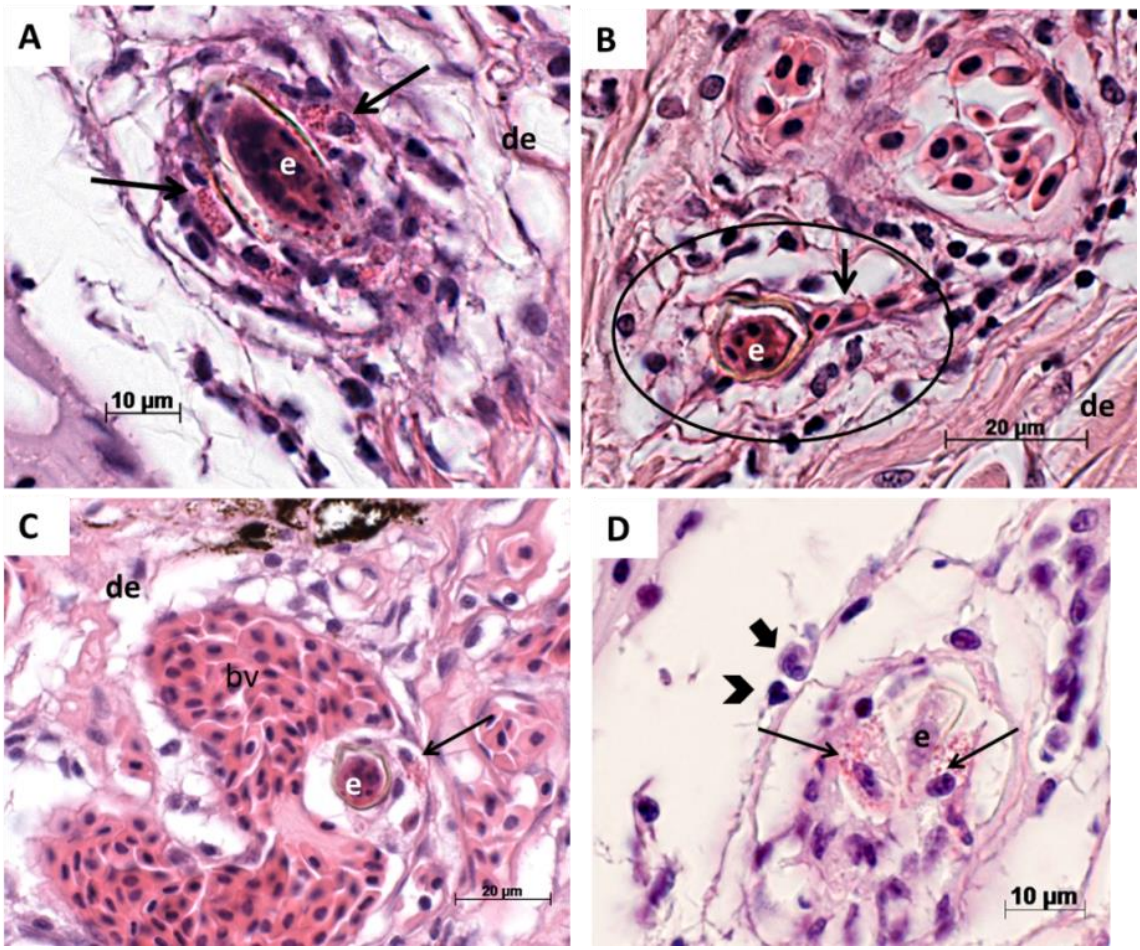

Figure 5.20 The host inflammatory response to migrating, embryonated blood fluke eggs, as seen it in the dermal blood vessels. (**A**) An egg (e) is seen trapped in a blood vessel and surrounded by degranulating eosinophilic granular cells (black arrow). (**B**) An egg is seen occluding the dermal capillary (circle) restricting the blood flow evident by the congestion of red blood cells (RBC) behind the egg (black arrow). The blood vessel is surrounded with a number of lymphocytes. (**C**) A congested blood vessel packed tight with RBCs. An egg (e) can be seen in the blood vessel and an eosinophilic granular cell at the stage of degranulation can be seen in close proximity to the egg (black arrow). (**D**) Within the dermal blood vessels, degranulating eosinophilic granular cells can be clearly seen surrounding an egg (e) which in the image is difficult to discern in its necrotic state. At the periphery of the blood vessel, a lymphocyte (black arrow head) and a neutrophil (thick black arrow) can be seen. *Abbreviations:* bv=blood vessel; de=dermis; e=egg. (H&E)

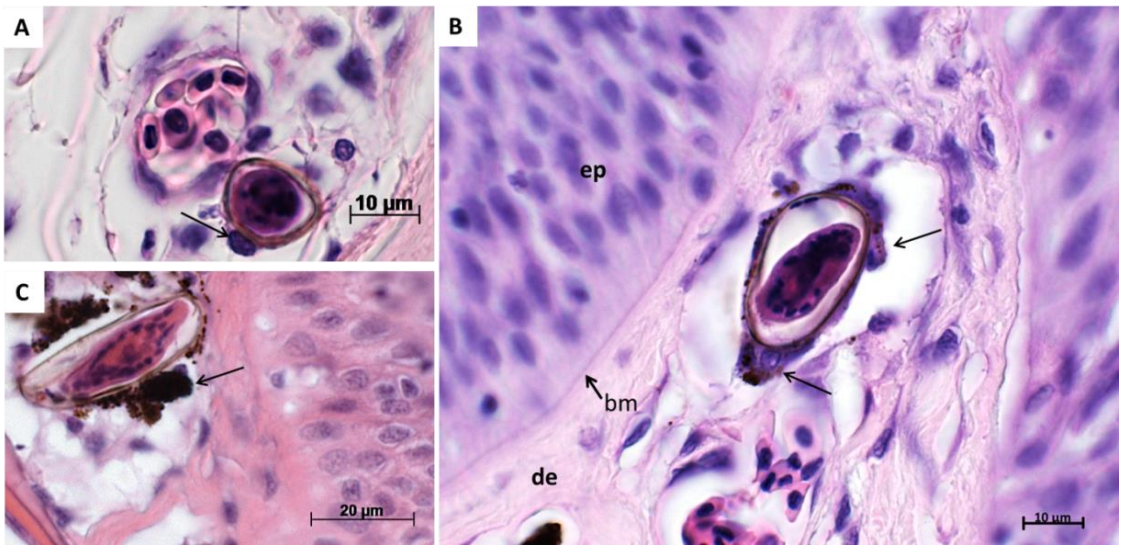

Figure 5.21 (**A**) Cross section through an egg situated in the superficial dermis (de) close to the basement membrane (bm). An inflammatory cell attached to the shell can be seen (black arrow). (**B**) Macrophage (black arrow) attached to shell of a mature egg with a miracidium within. (**C**) An egg containing a miracidium within the superficial dermis positioned above a scale can be seen surrounded by melanin granules (black arrow). *Abbreviations:* bm=basement membrane; de=superficial dermis; ep=epidermis. (H&E)

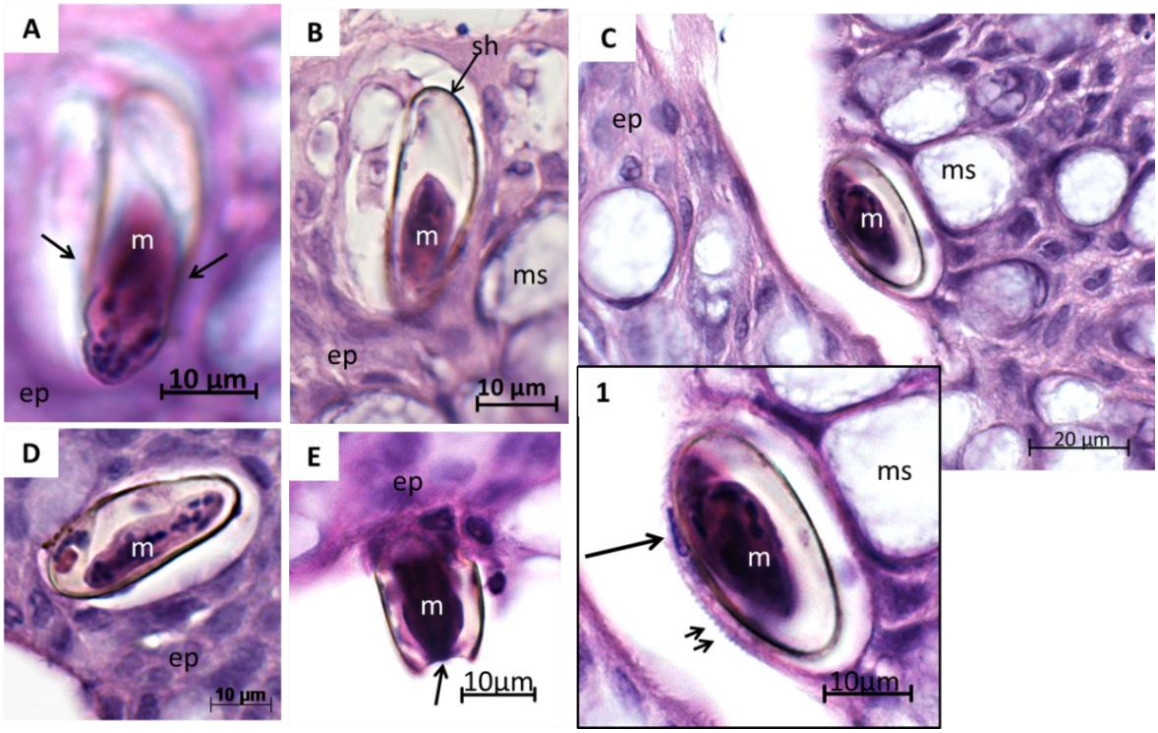

Figure 5.22. The release of blood fluke eggs from its dusky grouper, *Epinephalus marginata*, host. (**A &B**) An egg at the point of exit with a miracidium hatching from the dermis. The image shows a miracidium emerging from the egg located in the lower epidermis; its proximal end is still within the egg shell (sh) (two arrows). (**C**) An egg with a miracidium within is only separated by a single cell from the outer environment. The inset (1) shows how the pressure exerted on the outer epidermal cell is visible by the compressed appearance of the nucleus (long arrow). Microridges can also be seen (short double arrows). (**D**) Egg and miracidium within the outer epidermis. (**E**) Egg with the epidermis (ep) missing terminal part of shell, either true artefactual damage or miracidial hatching in progress. *Abbreviations:* ep=epidermis; m=miracidium; ms=mucous cell; sh=shell. (H&E)

5.4 Discussion

In the present study a blood fluke (Aporocotylidae) has been described through the reconstruction of numerous serial histological sections of the parasite, which had been found lodged within the dermal blood vessels. The largest section of the parasite viewed histologically was a part of the female reproductive system, of 1200 µm in length. The morphological characteristics of this parasite showed no signs of sexual dimorphism, showing both male and female gonadal tissue in the same parasite (*i.e.* ovary and testis). It is therefore safe to assume it to be a hermaphrodite.

Horák *et al.* (2014) stated that the presence of trematodes in the vascular system narrows down which species they belong. In the present study, the blood fluke was described as a digenean trematode according to descriptions by Chitwood & Lichtenfels (1972) and Gardiner & Poynton (1999) of trematodes in tissue sections, while it was described as blood flukes following Smith (2002) key to fish blood flukes. Although no description of blood flukes from the skin, the present parasite is a blood fluke affecting the skin blood vessels.

To date the majority of described adult blood flukes, Aporocotylidae, Odhner 1912, are found infecting the vascular system of fish (Montero *et al.* 1999; Smith 2002), whilst some can also be found in the kidney of sea bream (Padrós *et al.* 2001), and meningeal veins in the brain of common two-banded sea bream (Alama-Bermejo *et al.* 2011). Adult aporocotylids are small and are difficult to study if improperly fixed, stained, and mounted (Bullard *et al.* 2009). It is also worth noting that when blood flukes are suspected, it is usually the heart, gills and visceral mesenteries of the fish that are screened rather than the peripheral vascular system. As suggested by Nolan & Cribb (2004) it is likely therefore, that many blood

flukes remain undetected, not only as a general comment but more so given that they may infect other parts of the circulatory system (Nolan *et al.* 2014).

In the current study an adult blood fluke has been described in dermal blood vessels. It is the first time a blood fluke has been described from that region of the vascular system in general, and from the dusky grouper specifically. The blood flukes that have been described from groupers so far belong to two genera, and all species are described from the heart (Nolan & Cribb 2004). From the present study, it was clear that none of the histological sections of the heart revealed the presence of adult flukes, although evidence of an inflammatory reaction in the heart was indicated by the presence of MMCs and a diffuse myocarditis was observed. This latter condition, however, was not associated with dusky grouper dermatitis and the skin lesions (see **Chapter 4**).

From the 25 fish with skin lesions that were sampled, the parasite was re-constructed and described from tissue sections through skin lesions taken from only five fish. Four of the five fish had both parasite eggs and adults, while one fish had only parasite eggs. Given the typically large skin area of the lesions in relation to the area processed and evaluated histologically, it is possible that the adults, while present in the fish, were not present in the small sub-sample of tissue. Yong *et al.* (2013) observed that often infected hearts with blood fluke eggs, worms are not always found, as often worms might be present and might be overlooked by the observer, while (Nolan & Cribb 2006) observed a low prevalence of blood flukes in Serranids. Given the potential to easily overlook these parasites Bullard (2012) sugested that fish might harbour a number of undesrcribed blood fluke species.

From the recognised morphological features of the present blood fluke it was possible to observe the presence of minute spines and two groves at one side of the parasite in longitudinal sections see **Figs. 5.3 A & 5.5 A**. This is in agreement with Smith's (2002) description of blood flukes, where some species have a smooth tegument with spines found in some sections along the parasite body, from large to minute spines.

From **Table 5.2**, the key morphological features of the blood fluke parasitising the dermal vasculature of a Libyan population of dusky grouper are compared to other aporocotylid genera. From the table, it can be seen that the blood fluke under study here shares some morphological similarities to other genera. The uterus in *Cardicola* for example, is pre- and post-ovarian, whilst in the test specimen, the uterus is pre-ovarian, ovarian and post-ovarian. Specifically, the uterus of the test specimen appears to be coiled and U-shaped which is not dissimilar from those in *Cardicola* and *Sanguinicola* (see Smith 2002).

The seminal vesicle is a typical finding in several blood fluke, it is a blind end sac containing spermatozoids (Smith 2002) although in the present study it was not possible to ascertain whether the seminal sac is indeed blind ended or open further samples are required to make a definitive statement on this.

While these features may suggest that the current specimen belongs to the genus *Cardicola*, and given that *Cardicola orientalis* and *Cardicola opisthorchis* are sinuous (long and slender)(Ogawa et al. 2010; Palacios-Abella et al. 2015). the resemblance to *Cardicola* ends as there are a number of features to indicate the current specimens do not belong to this genus. The serpentine body form of the present blood fluke, for example, is similar to that of species belonging to the genus *Phthinomita* (see Nolan & Cribb 2006) infecting *Plectropomus*

leopardus coral trout (type host) & Epinephelus spp. *i.e.* thread like body and overlapping testis and ovaries, which is similar to the present parasite.

Although the eggs of blood flukes are mostly un-operculated, Yamaguti (1970) considered the absence of the operculum in eggs to be diagnostic for the Aporocotylidae. The absence of an operculum is regarded a diagnostic feature in identifying genera and species belonging to the Aporocotylidae. The eggs of *Pearsonellum* which are operculated, however, are an exception (Smith 2002). Also, while the shape of the eggs can vary, most are ovoid; *e.g.* eggs of *Pearsonellum*, however, other forms are also reported triangular *e.g.* Parasanguinicola Herbert *et* Shaharom, 1995 or fusiform *e.g.* Aporocotyle Odhner, 1900 (Smith 2002). In the present study, none of the eggs were operculated (i.e. those that were intra uterine nor those lodged in the dermal blood vessels, between the dermal connective tissue and epidermal cells. All eggs that were encountered were ovoid and unoperculated. The eggs in host tissue were considered viable at the point of fixation, as they appeared to contain well-developed miracidia.

The parasite eggs collected within the current study were frequently seen to contain vesicles between the embryo and the egg shell, both of which stained strongly acidophilic in H&E and positive with Periodic Acid Schiff (PAS) suggesting a carbohydrate composition such as glycan. Changes in the number and position of the vesicles could be ascertained but these had no consistency in relation to the position of the egg within the uterine tube or the developmental state of the egg. Variation in vesicle number, size and position in relation to the embryo was also observed; the numbers ranged from one to multiple vesicles which varied in size. The function of the vesicle inclusions seen in the eggs at various stages of development remains unclear.

Kusel (1970) also described the presence of vesicles in *Schistosoma japonica* and concluded that they may play a role in the hatching process. Schmidt (1998) also described glycan based vesicles and vesicles in the eggs of *Echinostoma caproni* which formed during the development phase and were present in the egg up until the point of hatching.

In the present study, some of the eggs have, in addition to the vesicles, paired or single, H&E basophilic staining sub-polar structures. These structures were dissimilar to the vesicles, where they are found in eggs within the descending uterus, and deposited eggs in the dermis but not in the eggs found within the epidermis.

It was not possible to collect fresh eggs from the dusky grouper; all observations relating to the eggs are made on formalin-fixed material in histological sections. Using this material, it was possible to describe the internal and external structure of the embryonated eggs. Jurberg *et al.* (2009) found that the cilia of miracidium from histological cross section are not always evident; especially at the early development when cilia are not yet formed in *S. japonica*, it is at the later stage that the cilia become evident. Miracidium cilia were not always described from histological section (Herbert *et al.* 1995), Padrós *et al.* (2001) & Holzer *et al.* (2008) described cilia from unhatched miracidium within eggs lodged in the tips of gill filaments in cultured sea bass (*Lates calcarifer*) and sea bream, respectively . Similar finding were seen in the present study where cilia could be seen on the unhatched miracidium still within the egg shell, in the upper dermis, and epidermis.

The eggs found in the dermal capillaries, dermis and the epidermis, possess morphological characteristics that resemble the intra-uterine eggs.

Typically, once the eggs are released from an adult blood fluke within the heart, they migrate via passive transport to the gill filaments. Until to date, the gill epithelium has been described as the pathway from which blood fluke miracidia have been observed to leave the host (Bullard *et al.* 2001; Padrós *et al.* 2001; Cribb *et al.* 2011; Dennis *et al.* 2011; Yong *et al.* 2013), however, Bullard *et al.* (2001) proposed that in the presence of blood flukes in places other than the heart suggests possible alternative pathways for the miracidia to leave the body of their host. As seen with *Schistosoma'* infecting humans, once the eggs are released some pass through the intestinal wall or the urinary bladder after which they can be passed out into the external environment (Pearce & Macdonald 2002).

A combination of the blood circulatory system and host inflammatory processes can serve to aid the movement of embryonating eggs to the gill filaments, as seen in the life-cycle of *Cardicola forsteri*, which infects the heart of bluefin tuna (Cribb *et al.* 2011). An exception to this typical route of release is seen in *Plethorchis acanthus* (Trematoda: Sanguinicolidae) which infects grey mullet, *Mugil cephalus* L.. Here, the adult parasites exit the blood system and then crawl over the intestinal surface to lay their eggs (Lester *et al.* 2009).

In this study, blood fluke eggs have been observed to be most likely released from nearby adults within the blood vessels, where the eggs were seen in dermal capillaries as well as the epidermis, with some breaching the last epidermal cellular layer. These eggs bear morphological characteristic resembling the eggs in the inter-vascular adult parasite. Thus it was clear that the oviposition takes place in the superficial dermal blood vessels, by gravid adult parasites. Eggs migrate through the dermis *en route* to the outer environment via the epidermis It was not possible to ascertain whether or not the position of the adults within the skin was its ''egg lying'' or ''permanent'' one. The migratory pathway during the life cycle

of this parasite, from point of entry to its final egg laying position was not possible to detect during this study. Nevertheless, in the present study the skin has been identified as the point of exit in the life cycle of this parasite. The juvenile form of this parasite was also not possible to find, which might be due to the life cycle of the juvenile parasite located elsewhere at a different part of the host's body.

Kirk & Lewis (1996) investigated the development of *Sanguinicola inermis* Plehn, 1905 in carp, through serial sectioning of experimentally infected fingerlings at pre-determined time-points post-challenge, to identify the preferred sites of cercarial penetration. *S. inermis* favoured the anterior half of the fish, the skin, fins and the opercular cavity. If the species of blood fluke under investigation here also uses the skin as the principal route of entry, with a subsequent migration and maturation within the dermal blood vessels, then this may explain why most of the of the DGD lesions are found on the trunk and head of dusky grouper. This, however, requires determining through a closed and controlled experiment.

Often adult blood flukes can remain undetected by its host's immune system for long periods of time (Kirk & Lewis 1996). *Schistosoma,* for example, can live for up to 2 years in their final host (Pearce & MacDonald 2002), and the host immune system does not become stimulated until larvae migrate (Kirk & Lewis 1996) or eggs are released (Padrós *et al.* 2001). These stages of the parasite elicit an inflammatory response directed toward the eggs, which can vary in its severity. In any case, the aim of a parasite is to successfully complete its life-cycle and, frequently the host's inflammatory response process would aid the movement of an otherwise immobile stage, such as an egg, to move from its place of deposition to its exit point, where the miracidia can be released. Given that in most blood fluke infections, the host reaction is directed towards the released eggs, these have a limited amount of time to

move passively from their point of release from the adult to their exit point. Kirk and Lewis (1996), from their studies with *S. inermis* infections in carp, indicated that this occurs within a few days. Eggs that are unable to achieve this within a certain timeframe are most likely to be contained and destroyed by the host response. In the present study, some necrotic eggs contained by the host response were seen, while others appeared to be unaffected, in that no attached inflammatory cells were seen. The role of the inflammatory process in the migratory process of the eggs remains unclear in the present study.

Granulomatous formation is observed surrounding sequestered viable *Schistosoma mansoni* eggs, but not immature eggs (Jurberg *et al.* 2009). Jurberg *et al.* (2009) had observed a different patter in the PAS positive reaction during *S. mansoni* eggs embryogenesis, the strong positive reaction of adult eggs is weekend in immature eggs. During PAS reaction of the eggs during the current study, the eggs and embryo, in the dermis and epidermis, stained strongly PAS which might be an indication for their maturity.

From the present study, degranulating eosinophilic granular cells were seen in the blood vessels as well as in the dermis but not in the epidermis.

Eosinophilic inflammatory infiltrates are inflammatory cells often seen in chronic but also acute stages of the immune inflammatory response (Slauson & Cooper 1990), and in reaction to helminthic infection (Dezfuli *et al.* 2015) as well as acute bacterial infection (Reite & Evensen 2006). Padrós *et al.* (2001) described eosinophilic granular cells in the vicinity of *Cardicola* egg infection, while Shin *et al*. (2009) observed de-granulated eosinophil cells in vicinity of damaged parasites. Similarly, in the present study, embryonated blood fluke eggs

where seen undergoing necrosis surrounded by degranulation eosinophils, in dermal blood vessel and between the dermal connective tissue.

Schistosoma eggs seem to employ the inflammatory response to aid in its migratory process within its host towards the exterior via the blood vessel endothelium, the connective tissue, the basements membrane and epithelium of the intestine. In the current study there was evidence of a similar mechanism in play to facilitate egg movement towards the epidermis so that they reach the outer most epithelial cells. It would appear, however, that this mechanism does carry an element of risk, with some eggs being seen to be surrounded by EGCs and undergoing necrosis. The mechanism facilitating the migration of the eggs of the current study is poorly understood. From a study conducted by Ashton *et al*. (2001) on secretions released by the eggs of *S. mansoni*, it is believed that the eggs use the immune response of the host to facilitate its transit through the gut.

While the formation of granulomas has been described for schistosomiasis infections, Lenzi *et al.* (1987) observed that *S. mansoni* eggs reaching the intestine, induced an inflammatory perivascular reaction as part of the mechanism to be expelled into the intestinal lumen. A similar reaction was described to entrapped eggs of *Schistosoma japonica* often found surrounded by granuloma (Holzer *et al.* 2008). These trapped dead eggs were found surrounded by inflammatory cells, including monocytes and eosinophils, causing severe necrosis to the liver to the point where, in late stages of the granuloma, the eggs could often no longer be detected (Pearce & MacDonald 2002).

No evidence for granulomatous responses could be seen in the present study, although ulceration was seen in DGD affected fish (Rizgalla *et al.* 2016). Furthermore the host seem not to exhibit minimal inflammatory reaction towards the adult parasites in the blood

vessels, but in few histological sections it was possible to appreciate adult gravid blood fluke found within blood vessel, surrounded by a perivascular inflammatory infiltrate. Although in that particular section, no eggs were seen within that particular inflammatory infiltrate, it is safe to assume the inflammation to be elicited by migrating eggs, laid by the adult, given that several free eggs were seen in the dermis and epidermis. Herbert *et al.* (1995) explained the lack of host response to adult blood flukes within the blood vessels, is due to by the lack of spines and the probable sedentary life of some blood flukes.

In *Schistosoma* the majority reach the exterior via the intestine (*S. mansoni*) or urinary bladder (*S. haematobium*), the majority of egg seem to pass undetected from the inflammatory cells. In the present study EGC's degratnuating and attached to the eggs, are indication for active recognition its meaning and consequence to the parasite and host response will be addressed in future studies.

In this study a preferable point for lesions manifestation was frequently seen occurring posterior to the eye, and in the mid trunk section, and to a lesser degree caudal peduncle and abdomen (Rizgalla *et al.* 2016). For further details see **Chapters 3 & 4**.

In fish, branchitis is often observed during blood fluke infections which can be severe (Dennis *et al.* 2011) or moderate (Padrós *et al.* 2001). Which might be related to intensity of the parasitosis (Padrós *et al.* 2001). In the present study, grossly, the skin lesions were haemorrhagic and reddened compared to the surrounding ''normal'' skin. This was due to the congested blood vessels in response to the migrating eggs, seen in the dermis and epidermis.

The occurrence of lesions at specific points has been described by Kick *et al.* (2000) during cutaneous human schistosomiasis, an unusual ectopic rare manifestation of blood flukes infection during with tumour like gross symptoms and increased dermal hyperplasia. These extragenital lesions affect mainly the trunk, in which histologically, eggs can be seen embedded in the dermis of groin and trunk, causing ulcers in the genital and perigenital area. Although the site of oviposition is unknown, the eggs might reach the skin via the communication between inferior mesenteric vein and the rectal venouse plexus.

Adults were not observed to be associated with any moderate or severe stage DGD skin lesions and only in one moderate stage lesion were eggs observed. This might be attributed to the life span of the adult parasite, where Kirk & Lewis (1996) observed adult *S. inermis* to die and disappear from tissue sections 70 days post infection (p.i), which might explain the absence of adult parasites in the skin of moderate and sever lesions. Adding to it the sever inflammation and sloughing of the epidermis described during DGD moderate and mild stages (see chapter.4), the mixed inflammatory infiltrate in the dermis, and loss of architecture all might omit the presence of eggs, and the adult parasite.

Although mortalities have been reported from fish e.g. Tuna infected by blood flukes causing severe necrosis to the gill filaments, induced by shed egg shells and trapped eggs with mortalities reaching up to 10-15 % (Dennis *et al.* 2011), there were no indication that might suggest DGD to cause mortalities even in fish showing severe DGD skin lesions (Rizgalla *et al.* 2016).

Some blood flukes exhibit seasonal variation (Bullard *et al.* 2001), in this instance seasonal variation is highly unlikely as the parasite were seen in fish collected in April, May, July and October.

In conclusion, a blood fluke infecting dusky grouper is described from a reconstruction of serial histology sections. The study describes distinctive morphological features, including the reproductive and digestive system, egg morphology, the embryo and the miracidia which are sufficient to place the adult digenean within the *Aporocotylidae*. Furthermore, these unique features suggest that the species might belong to a new genus. Considerable further study is nevertheless required before this can be emphatically stated. Future work, therefore, will attempt to isolate this parasite for more detailed morphological and molecular studies to confirm its identity.

Chapter 6

6.1 General Discussion

The present study was conducted to investigate the health status of wild dusky grouper, an important fish species in the Mediterranean Sea and in Libya. Dusky grouper is popular and regularly consumed in Libya as the main ingredient of a national dish called ''Haraymi'', which is reflected in its year round availability at fish markets despite the existence of a closed season and a minimum size (Reynolds *et al*. 1995).

From the survey work underpinning this study (see **Chapter 1**), there was no indication from the fish market field survey or from the Facebook postings, that Libyan fishermen or spear-fishermen were abiding by either regulations as fish as dusky grouper were readily available all year round and fish small as 20 cm total length (TL) were commonly seen on sale. Although the fish on sale at the markets within Tripoli were caught locally, when fishing was difficult, *e.g.* due to bad weather, stocks of dusky grouper were brought in from further afield, for example from Benghazi, which lies almost 1025 km to the east of Tripoli. Dusky grouper is caught using artisanal methods and by spearfishing, with an estimated 300 spear-fishermen supplying the fish market in Tripoli that served as a focus of this study. Spearfishing activity has also been documented in other Mediterranean countries (Coll *et al.* 2004; Morales-Nin *et al.* 2005; Pawson *et al.* 2008), with dusky grouper cited as one of its main targets (Coll *et al.* 2004). Spearfishing is a significant factor that can, given its highly selective nature, affect the composition of fish communities (Lindfield *et al.* 2014) and has therefore been detrimental to grouper species (Sadovy de Mitcheson *et al.* 2013), but also other fish species *e.g.* the brown meagre, *Sciaena umbra* Linnaeus, 1758 (see Harmelin-Vivien *et al.* 2015).

The dusky grouper spawning season on the western coast of Libya from the current study, appears to extend from May to September, while the nominal Libyan closed season extends from June to July. Variations in the onset of the spawning season are suggested to be temperature-related (Tsikliras *et al.* 2010) and make the setting of dates for a closed season more challenging. The variation observed in size at first female sexual maturation and of sex change events varies across studied stocks. Reñones *et al.* (2010) suggested that the size at which females mature is approximately 38.6 cm TL, while sex change events take place when fish are at least 52.1 cm TL, and the smallest sexually mature male was reported as measuring 58.4 cm TL. In the current study, the smallest sexually mature female was 39 cm TL, and a transient fish measured 58 cm TL although this finding is inconclusive given it represented stage T2 of sexual transition see **Chapter 2 Figure 2.5**, thus no data is available for size at the start of transition. The smallest sexually mature male fish found in the current study measured 58 cm TL. These measured fish give a guide to the sizes at first maturation, however, several factors have to be taken into consideration. First, the sample size of 55 fish sampled over 3 years and across different seasons in the current study is a small number. Second, it is not clear if the fish have all been caught in the same fishing grounds, i.e. in the coastal waters of Tripoli, or comprise members of the same population. Given the site fidelity of dusky grouper (Lembo *et al.* 1999; Spedicato, Carbonara &. Lembo 2005) and the likelihood that some populations are more exploited than others, then the number of fish that were sampled are unlikely to be wholly representative of the entire Libyan dusky grouper population which extends along an 1970 km coastline. Clearly, further research is needed to assess Libyan grouper populations more widely (IUCN).

Some of the parasites identified in the current study could, in the event that culture of dusky grouper becomes feasible, lead to pathologies in the aquaculture environment, presenting

an additional risk factor to be taken into consideration. Similar to the findings of Genc (2007) and Roumbedakis *et al.* (2013), the gills of dusky grouper are colonised by a range of parasitic infections, including isopods and monogeneans, the latter with 100% prevalence. According to Roumbedakis *et al.* (2013) while both wild and cultured dusky grouper seem to be affected by the same parasites, cultured fish often have a higher intensity of infection. Given that gill infecting monogeneans can lead to reduced growth and respiratory distress in cultured grouper species, *e.g. E. coioides, E. malabaricus* (Cruz-Lacierda & Erazo-Pagador 2004) and cultured dusky grouper (Sanches 2008), then parasites do pose a health risk factor that needs to be considered during dusky grouper culture.

A further parasite which could be considered a health risk in both wild and cultured fish is the nematode *Philometra* sp. This was found with a prevalence of 52% in the present study and was commonly found within the ovaries of dusky grouper. The presence of nematodes was associated with a severe necrotic reaction in the ovaries of post-spawning fish that were sampled in October. The extent of ovarian tissue damage that was seen may be sufficient to cause parasitic castration.

An additional finding from the current study, was that of a bacillus bacteria attached both to *Philometra* sp. and in the surrounding ovarian tissue. Given that this condition was found only on one occasion, the significance of the bacteria and their impact on the ovaries in addition to the philometrid infections are unclear. The finding of a bacterium associated with a philometrid infection, however, has been documented elsewhere and have been reported in the gonads of wild red drum *Sciaenops ocellatus* (Linnaeus, 1766) (Perciformes: Sciaenidae) by Bakenhaster *et al.* (2014).

Given that most grouper broodstock are obtained from wild fish populations (Leong 1998; Tupper 2008; Seckendorff 2009) including dusky grouper for culture programmes in Mediterranean and Brazil. In the event of the development of dusky grouper culture programmes in Libya, the findings of the present study suggest that the best time for grouper sampling for the purpose of establishing a broodstock population would begin March to April, when the ovaries, although infected by small male and juvenile female nematodes, can be easily treated using anti parasitic drugs, leading to minimisation of ovarian damage. This thesis, however, has as one of its foci, looked at a digenean infection which results in damage to the dermal tissues as a condition that has been described here as dusky grouper dermatitis. This novel skin condition referred to as DGD is described in detail in **Chapter 4**, while the aporocotylid digenean is described in **Chapter 5.**

The distribution and status of DGD in Libya was investigated in **Chapter 3**, in which the use of social media networks was investigated as an alternative or supplemental approach to disease surveillance, with Facebook profiles employed to assess the distribution of DGD along the Libyan coastline. Of the 23 cities (capture areas) from which spearfishing activity was posted, dusky grouper landed in waters close to 19 coastal cities were found to be positive for skin lesions. As the impact of this condition upon individual fish and on the health of the dusky grouper population is not well established, further investigation is urgently required. The successful and innovative use of social media networks to characterise the distribution of DGD suggest that there may be wider potential for its use in fish disease surveillance, especially for wild populations in areas that are difficult to reach through geographical or geopolitical constraints. The capacity for almost instantaneous reporting that Facebook posts provide could prove invaluable in monitoring and tracing disease as well as in providing an initial alarm raising mechanism in the case of disease

outbreaks. Its potential as a tool to support epidemiological studies has already been acknowledged by several research groups working in human health areas (see for example Brownstein *et al.* 2009; Corley *et al.* 2010; Bernardo *et al.* 2013; Zaid *et al.* 2014; Asghar 2015). To reduce the biases resulting from the use of posted images, it is essential to follow up each original report by directly contacting individuals for information and to confirm details of the posting. This can also provide an opportunity to recruit individuals for future collaborative work in monitoring and reporting on disease as citizen scientists. Currently work is underway to develop a survey tool to provide further deeper understanding of the distribution of DGD along the Libyan coastline. Such a survey might help confirm, as one example of its proposed use, the absence of skin lesions on dusky grouper landed in four neighbouring cities. None of the fish caught in these waters and then posted on Facebook had evident skin lesions.

Facebook offers the potential to use citizen scientists in a wider national study involving governmental entities in a nationwide survey *e.g*. using direct questioners and calling for active collection and posting of data might add yet another dimension to the use of social media in the investigation of a disease affecting a wild fish population. Ethical considerations, in terms of using Facebook data without the consent of its owner and documenting the activity of posting individuals, despite such information having already been placed in the public domain, should nevertheless be taken into account (Zimmer 2010). According to the guidelines published by the Norwegian national research ethics committee in 2014, use of online information already placed within the public domain is considered acceptable. However,researchers should ensure the anonymity of sensitive and private data, with individual researchers deciding upon what should be considered as such. Given the variation in what people might consider public or private, despite its being in the ‘‘public

domain'' (Elgesem 2002), this might pose challenges as to how to treat information. On the other hand, Elgesem (2015) argued that past studies involving people required researchers to ask and obtain their consent, and that therefore collection of data from social media might entail the same actions. Nevertheless, it still remains unclear whether data derived from social media should be treated in the same way as directly collected personal data (Gehner & Oughton 2016), leaving the scientific community in an ongoing debate about the validity of sourcing information from social media (Zimmer 2010).

While for the present study, the consent to use web-sourced / Facebook images had been sought from their owner, the remaining information was mined from open access Facebook accounts and public open access Facebook groups. To mitigate loss of privacy, data deriving from social media should be anonymised (Zimmer 2010). In the current study, to ensure the anonymity of sea fishermen posting on Facebook, the identity of Facebook users was not included in the results, and only following the written consent of their owner, were images used and their source noted only in the acknowledgments.

In **Chapter 4,** the DGD skin lesions that were seen and mapped in **Chapters 2** and **3** are described for the first time and characterised as a new disease: dusky grouper dermatitis (DGD). A combination of field investigations and an independent Facebook survey suggest that DGD affects dusky grouper ranging in size from 40 cm to over 100 cm TL. The skin lesions are described as a multi focal dermatitis, affecting the dusky grouper all year round and there are indications that their aetiology involves infection by a previously undescribed aporocotylid blood fluke. Other types of skin lesion, however, have been described from dusky grouper in the past and these have been associated with a number of bacterial (Marzouk *et al.* 2009; Eissa *et al.* 2011; Soliman *et al.* 2011), viral (Marino & Azzuro 2001;

Vendramin *et al.* 2013; Kara *et al.* 2014) and parasitic pathogens (Sanches 2008). Excluding the present study, none of the lesions described to date, however, have been attributed to a blood fluke infection.

The wide distribution of dusky grouper dermatitis along the Libyan coastline, as determined from the study presented in **Chapter 3**, and, at the time of its discovery, renders it a risk factor to be considered when developing culture programmes as the culture environment may exacerbate the development and course of these skin lesions. Although there has been no evidence to support DGD causing mortalities, the effects on fish health and immunity in both wild and cultured fish have yet to be assessed. The breach of the dermal epidermal protection, during the formation of the skin ulcers makes the fish prone to secondary invaders (Ferguson 2006), which, under culture conditions, could be detrimental. Despite no evidence for these skin lesions reducing the market value of dusky grouper on sale in Libya, this results from the general belief that the lesions are caused by remoras attaching to fish; remoras have been seen attached to dusky grouper during field surveys and this is supported by comments posted on Facebook. If cultured dusky grouper stocks, however, also subsequently present similar lesions, this may reduce the aesthetic appeal and sale price of stock or may even lead to customer rejection. Such product devaluation is often mentioned in conjunction with skin lesions affecting other fish species, for example in the case of well-established skin lesions affecting rainbow trout *Onchorrhychus mykiss* (Walbaum, 1792) *e.g.* RMS or red mark syndrome (Peeler 2014) puffy skin disease (Maddocks *et al*. 2015) and strawberry disease (Ferguson *et al.* 2006).

Although bacteria and fungi were seen in some severe lesions, none could be attributed as the primary cause of the lesions. Adult blood flukes and their eggs, however, were seen in

fish with mild skin lesions and from the host response in these fish it was clear that this blood fluke was capable of eliciting substantial inflammation, a feature of DGD. The effects that these skin lesions have on blood parameters and the wider immune responses of dusky grouper, and the observation that they only affect a specific size of fish that coincides with sexual maturity, requires further assessment as does the question of whether this disease represents an established or a new disease. Given that most wild dusky grouper skin lesions that are described in the literature are suggested to relate to *Nodavirus* disease outbreaks, in which large numbers of fish can be found floating at the water surface (Al-Attar *et al.* 2009; Al-Attar *et al.* 2013; Kara *et al.* 2014) or dead within their caves (Kara *et al.* 2014), it is possible that some of these dusky grouper might have a mixed infection comprising DGD and *Nodavirus*. The potential for a mixed infection might pose a further risk, not only to wild dusky grouper populations, but also to cultured fish, especially broodstock, given the size/age threshold of these lesions. The relationship, if there is any, between *Nodavirus* and DGD therefore also requires further investigation.

From Facebook data, where some of the dusky grouper posted from Algiers and Turkey showed similar skin lesions, it is likely that these populations of dusky grouper populations are also infected with the same or similar species of blood fluke resulting in DGD-type lesions.

In **Chapter 5,** the parasite found in early stage DGD lesions was characterised as an aporocotylid blood fluke through the serial reconstruction of histological sections and by determining the parasite's role in the DGD inflammatory process. While this parasite shares some similarities with some other genera within the Aporocotylidae, the possession of certain unique morphological features suggests that this may represent a new genus.

Although evidence is limited, it is possible that this parasite is responsible for initiating DGD, however, further research is required to support the parasite's role in DGD pathogenesis. Future studies should aim to: 1) provide further appropriately fixed material for histological analysis; 2) isolate infecting blood flukes from the skin of dusky grouper for molecular analysis and for a classical taxonomic-based description of whole specimens in order to determine its position and relation to the 33 described genera of aporocotylid blood flukes; and, 3) attempt to follow the pathways of infection by histological and immunohistochemical-based analyses.

In conclusion the purpose of this body of research has been to provide a first overview of the health status of wild dusky grouper and to identify some of the health issues and to comment on those that have not been previously described. From the specimens examined within this study, two major disease conditions provide clear potential for impact on wild and cultured fish, namely a *Philometra* sp.-induced ovarian necrosis and DGD.

With these identified health issues, does dusky grouper aquaculture have a future? In a culture system, with good control and management, it is possible to mitigate many of these problems. All the fish affected by skin lesions were 40cm Tl and above, and *Philometra* infection is only significant in spawning ovaries, with juvenile fish not being affected by the described pathological changes. As the typical market size for grouper spp. is about 400-1000 g (Tucker, 1999). Depending on the aetiology of DGD, it may be possible to mitigate the impact of skin lesions, by harvesting the fish at sizes less than 40 cm TL (500-1000 g), which is the size/age at which the fish are not affected.

It should be noted, however, that broodstock collected from the wild might pose a serious threat in terms of disease, as the outcome of these lesions in a closed culture environment remains unknown. There is also a need to consider broodstock fertility, which might be affected by philometrid infections. Furthermore, the assessment of the feasibility of a dusky grouper culture programme in Libya, due to its importance as food fish for the local markets, should be revised in an independent study taking the above listed difficulties into consideration. Facebook has offered a new method for assessing the extent of spearfishing in coastal waters, however, the unstable political situation experienced over the duration of the current research in Libya may well have introduced biases in the numbers of people posting from the east and centre of Libya, in relation to the more frequent posts made by people living in the west.

However, from the field survey, spearfishing approximately supply 20% of the market, making important contribution of the fish sold, its effect on the natural population, needs an independent study, to assess the impact it has on dusky grouper stocks. Meanwhile in agreement with Echwikhi *et al.* (2014), the implementation and enforcement of lower size limits, bag limits, and closed seasons, as part of a national strategy for managing dusky grouper fisheries, might be an action to be considered. Another management tool would be the implementation of a maximum size limit, which would protect the larger and thus more fecund individuals, described by Froese (2004) as mega-spawners, to help ensure a successful fish recruitment.

Lack of dusky grouper fisheries statistics is also a typical problem encountered for fisheries relying predominantly upon artisanal fishing methods (Marino *et al.* 2001). The foregoing observations strongly suggest that a national study to assess the status of dusky grouper

populations along the Libyan coastline, and indeed a wider collaborative study examining stocks in the Mediterranean, is urgently needed. Despite the limited information obtained during the current study, fish market and Facebook data provide some evidence that dusky grouper are threatened by overfishing.

The general lack of available data concerning fishery captures agrees with the wider observation that FAO have underestimated the amount of landed fish in Libya (Khalfallah *et al.* 2015). Unfortunately, there is very little literature on pre conflict fisheries governance in Libya (FAO 2006) and in the current conflict situation, data on artisanal fisheries in Libya is lacking. Insufficient knowledge of the fisheries resource base had been identified as one of the problems that Libyan fisheries face. Furthermore, the FAO (2006) report identified the fisheries research and practice as deficient, for which reason a fisheries development project spanning 5 years from 2006 to 2011 had been proposed. In midst of the ongoing political situation, however, the outcome of this project was not possible to follow-up.

Given that despite the endangered condition of this fish and calls for more extended protection across the Mediterranean areas (MEPA 2011), dusky grouper is still captured in the Mediterranean and as evidenced here in Libyan coastal waters. Despite regulations nominally defining minimal capture size and closed seasons, currently the only realistic protection comes from marine protected areas (MPAs), in which moratoria against spearfishing have been implemented and which have witnessed an increase and overspill of juvenile dusky grouper to close by areas (Bodilis *et al.* 2003). Despite their success, it should be noted that MPAs only comprise ~3.8% of the Mediterranean (Abdul Malak *et al.* 2011), of which strictly protected areas represent only 0.01% of the total surface area of the Mediterranean Sea, with the remaining areas open for human activity including fishing

(Harmelin 2000; Badalamenti *et al.* 2000; Francour *et al.* 2001). Added to this is the fact that most of the marine protected areas are coastal and are located in the northern part of the Mediterranean Sea (Abdul Malak *et al.* 2011), which mandates creation of more MPAs in the south and eastern Mediterranean (Abdulla *et al.* 2008).

While several MPAs have been proposed, only three MPAs are currently established in Libya (Haddoud & Rawag 1995; Hamza *et al.* 2011). For MPAs to succeed there is need to provide dedicated manpower and the means to monitor and implement regulations, both of which are currently lacking in Libya. Despite considerable effort from a number of research groups and governmental bodies e.g. the Marine Research Centre, Tajura (MBRC), Libya has a long way to go in order to establish a successful protection strategy. In Libya, several governmental bodies (e.g. The Libyan Authority for Marine Wealth and, the Libyan Environment General Authority) and NGOs (e.g. The Libyan Marine Science Association, Libyan Society for the Protection of Marine Biology, The Libyan Wildlife Trust *etc.*) have an interest in safeguarding the marine environment, and protecting endangered species, *e.g.* sea turtles (*Caretta caretta* (Linnaeus, 1758)), monk seals (*Monachus monachus* (Hermann, 1779), and endangered fish species e.g. dusky grouper. Finally, while the government is acutely aware of the urgent need to assess and regulate marine biodiversity and services, current political instability demands other government priorities, while the associated lack of means and resources to enforce existing regulations impedes any progress in this area. There is an extremely delicate balance to be drawn between the instantaneous food security provided by the income that families gain from fishing/spearfishing for dusky grouper, and the longer term food security that might be provided by tighter restrictions of the fishery.

The most important individuals associated with such a programme are the fishermen and their families. Thus those people interested in implementing conservation measures need to call upon the co-operation and benevolence of individual fishermen by involving them in the process, providing awareness campaigns that highlight the current fragility of this marine fish and stressing that tighter regulations do not threaten the fisheries sector but instead are the only means to ensure long-term continuity. In order to better conserve stocks, there is also an urgent need to support research that provides a better understanding of this fish species and also research that can help support commercial culture and possibly restocking programmes.

The dusky grouper is a seriously understudied fish, furthert and better knowledge, provided by appropriately targeted research, would provide clear benefits for fisheries, species conservation and aquaculture. Improved knowledge and the improvement in protection that such knowledge can provide is the only way to ensure the availability of this fish species for present and future generations, however, this is only achievable with the joint efforts of fishermen, citizen scientists, researchers, NGOs and governmental entities. During the present conflict, however, there are more pressing issues than the sustainability of fisheries, and at present there exist different priorities. This could possibly change following settling of the political situation in Libya.

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