THE POTENTIAL OF COMMERCIAL PRAZIQUANTEL FORMULATIONS AS "OFF LABEL" TREATMENTS FOR *DIPLECTANUM OLIVERI* (MONOGENEA) INFECTING CULTURED *ARGYROSOMUS* SPECIES IN THE SOUTH AFRICAN MARINE FINFISH AQUACULTURE INDUSTRY.

CASPER J. H. JOUBERT

TITLE OF THESIS

THE POTENTIAL OF COMMERCIAL PRAZIQUANTEL FORMULATIONS AS "OFF LABEL" TREATMENTS FOR *DIPLECTANUM OLIVERI* (MONOGENEA) INFECTING CULTURED *ARGYROSOMUS* SPECIES IN THE SOUTH AFRICAN MARINE FINFISH AQUACULTURE INDUSTRY.

Casper Jan Hendrik Joubert

Submitted in partial fulfilment of the academic requirements for the degree

Master's of Science

Department of Biodiversity and Conservation Biology

Faculty of Natural Sciences

University of the Western Cape

Bellville

Supervisors:

Dr Kevin Christison (Department of Biodiversity and Conservation, University of the Western Cape)

Prof Horst Kaiser (Department of Ichthyology and Fisheries Science, Rhodes University)

May 2012

KEYWORDS

1. **Aquaculture**
2. **Monogenea**
3. **Praziquantel**
4. **Bath treatment**
5. **Dusky kob**
6. ***Diplectanum oliveri***
7. ***Argyrosomus japonicus***

ABSTRACT

The potential of commercial Praziquantel formulations as "off label" treatments for *Diplectanum oliveri* (Monogenea) infecting cultured *Argyrosomus* species in the South African marine finfish aquaculture industry.

C. J. H. Joubert

***Magister Scientiae,* Department of Biodiversity and Conservation Biology, University of the Western Cape, 2012**

Aquaculture is a vast industry all over the world and has increased significantly during the past 30 years. In South Africa, finfish aquaculture farms stretch from Gansbaai to as far as Richards bay with the potential of extending into Mozambique. The future success of this fast growing industry in South Africa strongly relies on the development of the supporting sector such as government legislation, sponsorship, participation of the pharmaceutical industry and research and development in aquatic organism health management. *Diplectanum oliveri* Williams, 1989, a monogenean gill parasite of both *Argyrosomus japonicus* (Temminck & Schlegel, 1843) (dusky kob) and *A. inodorus* Griffiths & Heemstra, 1995 (silver kob) is currently regarded in South Africa as the most persistent ectoparasite associated with the culture of both fish species, causing pathological tissue changes in the areas associated with attachment and feeding which can

result in stock losses. The egg production of *D. oliveri* was used to evaluate and develop a method to quantify monogenean infections on fish, by counting the eggs produced by infra-populations of these parasites over a 24-hour period and to determine the reliability of this method as a non-invasive/non-destructive method to quantify the intensity of an individual infra-population of parasites on a single host. Currently, *Diplectanum* spp. on dusky kob are being controlled in local mariculture facilities using methods and drugs that are traditionally used for monogeneans (flukes) and are regarded as effective. Most of these drugs are, however, no longer approved for use in food fish and none of them has proven to be very effective in controlling *D. oliveri* in culture facilities, which can result in subsequent re-infections of epidemic proportion. Currently, there are no anthelmintics registered for aquaculture in South Africa. An registered anthelmintic used in terrestrial animals (sheep, goats, cattle and ostriches) containing praziquantel was tested at various concentrations and exposures against *D. oliveri* on *A. japonicus* to determine the efficacy of two different formulations and the potential for "off label" use. The 20 ppm (high) praziquantel concentration treatments eliminated all adult parasites, but caused significant measureable stress and affected the central nervous system of the fish, which resulted in death of all fish in the solution group after 18 hours. The 2 ppm (low) concentrations failed to remove all adult parasites. Although both the 2 hour (short) exposure/high concentration and 24 hour (long) exposure/low concentration of the suspension formulation were effective, but only the short exposure/high concentration eliminated all adult parasites with little change in behaviour by the treated fish.

DECLARATION

I declare that the dissertation, **The potential of commercial Praziquantel formulations as "off label" treatments for *Diplectanum oliveri* (Monogenea) infecting cultured *Argyrosomus* species in the South African marine finfish aquaculture industry**, is my own work, that has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name..... . Date.....

Signed……..... .....May 2012

Casper Jan Hendrik Joubert

ACKNOWLEDGEMENTS

I would like to express my gratitude to the following people and institutions for their support and contribution to this dissertation.

Marine and Coastal Management (MCM) of the Department of Environmental Affairs and Tourism for granting funds through a Frontier Programme to address Mariculture Research and Development.

Bayer Animal Health, Bayer (Pty) Ltd, South Africa, for financial support towards the project, sponsoring of the pharmaceuticals that were needed for the treatment trials ,continuous support and professionalism.

AASA, The Aquaculture Association of Southern African for assisting in aquaculture production data.

Dr. Angela Graham, Dr. David Huchzermeyer & Dr. Glynn Catton, assisting in the legal aspects for veterinary drug control and management for the practicing veterinarian in South Africa concerning "pet shop medications" as discussed in Chapter 2.

Dr. Arrie du Plessis, New Product Development Manager, Pfizer Animal Health South Africa.

Dr. Ernest Mokantla, Internal Technical Advisor to the Registrar.

Dr. Kevin W. Christison, Specialist Scientist (Marine Aquaculture Diseases) Department of Agriculture, Forestry and Fisheries, for his primary supervision and friendship during this exiting scientific research journey we embarked on.

Dr. Pam Miller, assisting me in the preparation of the research proposal.

Dr. Patrick Page, Faculty of Veterinary Science, University of Pretoria.

Mrs. Gretha Joubert, my wife and my two children for their patience, support and sacrifices during this period and assistance with photography.

Prof. Robert D. Sykes & Dr. Vinny Naidoo, Faculty of Veterinary Science, Onderstepoort, University of Pretoria for their assistances in the legal aspects for veterinary drug control and management for the practicing veterinarian in South Africa as discussed in Chapter 2.

DEDICATION

I dedicate this work to my Creator, *Elohim* that inspired and granted me the opportunity, ability and colleagues to embark on this journey, discovering once again that He is indeed the Beginning and the End of all things.

Colossians 1:16-17

For by God were all things created, that are in heaven, and that are in earth, visible and invisible, whether they be thrones, or dominions, or principalities, or powers: all things were created by Him, and for Him: And He is before all things, and by Him all things consist.

CONTENTS

CHAPTER 1: Introduction

The very simple definition that can be applied to aquaculture is the farming of aquatic organisms, but more specifically, aquaculture is the propagation and rearing of aquatic species in controlled or selected environments (Storey 2005). Although aquatic animals are frequently referred to simply as "fish", accordingly, the term “fish” often includes a large variety of taxa including finfish, molluscs and crustaceans. Mariculture is defined as the farming of marine and/or estuarine organisms in land-based or water-based brackish water and/or marine environments (DWAF 1995).

Aquaculture facilities vary from extensive, semi-intensive to intensive production systems, which include streams, ponds, impoundments, dams, estuaries and net-pens or cage farming. Finfish species are reared under controlled conditions to any specific market size for a sale to restaurants and supermarkets or fish markets.

In 2004, the global aquaculture contribution reached a record high 43% of the total 106 million tonnes of food fish supplied to the world. Since 1970, global aquaculture production had an annual growth of 8.8%, with only 1.2% and 2.8% growth respectively for captured fisheries and terrestrial meat farming. This makes it the fastest growing animal food-production sector (Van Gass 2007).

Commercial mariculture in South Africa is a relative young farming activity, which began in 1948, with the establishment of the Knysna Oyster Company. The growth potential of the industry has, however, only been realised since 1984 (Hecht & Britz 1992). The industry grew rapidly since the 1980s’; producing 345

tonnes in 1980 escalating to 3111 tonnes in 1988, a near tenfold increase in 1984 and only three species were cultured, which quadrupled by 1992 (Hecht *et al.* 1992). The African aquaculture production in 2004 was nearly 1.6 MT, an average annual increase of 6.8% since 1993, with a production figure of 6500 tonnes in 2005 from South African aquaculture.

Aquaculture in South Africa includes both freshwater and marine species. Commonly farmed aquatic species within South Africa include rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792), brown trout *Salmo trutta* Linnaeus, 1758, atlantic salmon *Salmo salar* Linnaeus, 1758, sharp tooth catfish *Clarias gariepinus* (Burchell, 1822), redbreasted tilapia *Tilapia rendalii* (Boulenger, 1897), mozambique tilapia *Oreochromis mossambicus* (Peters, 1852) and nile tilapia *O. niloticus* (Linnaeus, 1758), mullet various spp., common carp *Cyprinus carpio* Linnaeus, 1758, grass carp *Ctenopharyngodon idella* (Valenciennes, 1844), bass *Micropterus* spp. (Lacépède, 1802), galjoen *Dichistius capensis* (Cuvier, 1831), garrick *Lichia amia* (Linnaeus, 1758), dusky kob *Argyrosomus japonicus* (Temminck & Schlegel, 1843), silver kob *Argyrosomus inodorus* Griffiths & Heemstra, 1995, yellowtail *Seriola lalandi* Valenciennes, 1833, oyster *Crassostrea gigas* (Thunberg, 1793), Mediterranean mussel *Mytilus galloprovincialis* Lamarck, 1819, black mussel *Choromytilus meridionalis* (Krauss,1848), and abalone *Haliotis midae* Linnaeus, 1758. Research species include; white stumpnose *Rhabdosargus globiceps* (Valenciennes, 1830), spotted grunter *Pomadasys commersonnii* (Lacepède, 1801) and yellowbelly rockcod *Epinephelus marginatus* (Lowe, 1834). Eel, prawn and shrimp have also been farmed historically (Hecht *et al.* 1992).

The family Sciaenidae is an important family of fish for the South African fishing fraternity and five species are of particular importance; silver kob *Argyrosomus inodorus*, dusky kob *A. japonicus*, squaretail kob *Argyrosomus thorpei* Smith, 1977, geelbek *Atractoscion aequidens* (Cuvier, 1830), snapper kob *Otolithes ruber* (Bloch & Schneider, 1801) and slender baardman *Umbrina robinsoni* Gilchrist & Thompson, 1908. Silver kob is a highly regarded food-fish. According to Heemstra & Heemstra (2004) Silver kob is also the most important linefish in Namibia and is also caught by trawlers, with a distribution that stretches from Namibia to the Kei River and due to over exploitation a marked decline in catch occurred

Dusky kob, also known as 'mulloway' or 'jewel fish' in Australia, has a local distribution from False Bay to Mozambique. Dusky kob is highly palatable and due to its size of up to 75 kg it is particular important to the recreational fraternal and commercial fisheries (Griffiths 1996). According to Heemstra & Heemstra (2004), it is regarded as the most important and popular angling source from Cape Agulhas to Durban with combined catches between recreational and commercial of up to 400 tons per annum. Catch restrictions have also been implemented due to the sharp decline in the local stocks of this species.

There are about 150 different linefish species in South Africa of which 15 species have collapsed and where the other species find themselves either threatened or over-exploited. Due to the popularity of these line fish species and consumer preference, the market has been established for the demand of the above mentioned fish. With the current restrictions on catch quotas due to declining wild

caught fish stock, these linefish species should receive more consideration as important culture species candidates in aquaculture (Van Gass 2007).

Despite the magnitude of aquaculture production globally, pharmaceutical companies are still unlikely to invest significantly toward the research and development of new anthelmintics against monogeneans due to the high associated costs. Yet the problems remain with over 24000 described teleost species with there even larger number of associated monogenean parasites (Buchmann 1998). There are currently no registered drugs available for disease control in aquaculture for South Africa.

The artificial and or semi-artificial conditions generally encountered in finfish aquaculture are often more conducive to the development of diseases caused by viruses, bacteria, fungi, protozoan and metazoan parasites than would have occurred under natural conditions (Paperna 1996; Reno 1998). Pathogen transmission escalates under high density culture conditions where the probability of encountering a susceptible host is greatly enhanced. This is even further exacerbated when considering pathogens with direct life cycles, thereby eradicating the necessity of an intermediate host or vector for a successful transmission (Reno 1998).

Parasitic diseases are of concern in cultured fish of which monogenean parasites cause serious damage to cultured fish with loss of production. In aquaculture facilities, high numbers of these parasites negatively affect production directly through the reduction in production efficiency of the fish due to the resulting pathology, reduced growth rates and the physiological responses to the infestation, or indirectly through elevated production costs required for

containment and treatment thereof. Ultimately, persistent infestations may lead to mass mortalities within the captive fish population (Thoney & Hargis 1991).

Monogenean parasites are found on both the cartilaginous (Class: Chondrichthyes) and bony fishes (Class: Osteichthyes). This problem is global and affects the production in cultured finfish of Australia, South East Asia, the Indian sub-continent (Rajendran *et al.* 2000), Japan (Mansell *et al.* 2005), and Italy (Dezfuli *et al.* 2007).

The majority of these monogenean parasites are ectoparasites and have a direct life cycle inhabiting the skin, fins, oral cavity, nasal tissue and gills of their fish hosts (Buchmann & Bresciani 2006) and are of particular concern for the captive husbandry of numerous fish species (Ernst *et al.* 2002). Under natural "wild" conditions, the parasites are relatively well adapted to their host causing relatively few apparent problems (Wedemeyer 1996). Under intensive, high stocking density aquaculture production conditions there is a significantly increase in prevalence and intensities of the parasites (Ernst *et al.* 2002). There is also an increase in stress factors that render the host more vulnerable to super infections, due to their suppressed immune system (Wedemeyer *et al.* 1990). Parasites under these conditions also display less host specificity. Although viral, bacterial and fungal infections occur in aquaculture, monogenean infections are often responsible for greater losses. Monogenean induced pathology, resulting from the parasites' attachment and feeding in severe infections may also predispose the host to secondary infection which often results in stock losses (Thoney & Hargis 1991).

Production losses attributed to pathology caused by representatives of the monogenean sub class Polyonchoinea have been researched and well documented

to emphasise the impact on the host resulting from these parasite infections (Ernst *et al.* 2002).

Pathology caused by monogeneans includes haemorrhage, mucoid exudates, inflammation, erosions, hyperplasia that lead to disruption and fusion of the secondary lamellae (Dezfuli *et al.* 2007). This causes flashing of sides and opercula against substrate that can lead to trauma, self mutilation an secondary infection. Behaviour changes occur, like head bobbing. Excessive mucus production of gill tissue, gill necrosis, gill hyperplasia, (Rajendran *et al.* 2000), lamellar hyperplasia and fusion (Mansell *et al.* 2005) and severe skin lesions (Ernst *et al.* 2005) contributes to large scale mortalities

It is estimated that an economic loss of up to 20% of the total production cost of yellowtail kingfish *S. lalandi*, is caused by *Benedenia seriolae* Yamaguti, 1934 on *Seriola* spp. in aquaculture of Japan (Ernst *et al.* 2005). In Italy, annual stock losses of juvenile *Dicentrarchus labrax* (Linnaeus, 1758) caused by *Diplectanum aequans* (Wagener, 1857) range between 5 – 10% (Dezfuli *et al.* 2007). Pathology inflicted by *Diplectanum latesi* (Tripathi, 1957) on sea bass *Lates calcarifer* (Bloch, 1790) causes large scale mortalities of brood stock in Thailand, Malaysia and Singapore during the months of November to December (Rajendran *et al.* 2000).

The fins and skin infestation of red sea bream *Pagrus major* (Temminck & Schlegel, 1843) with *Anoplodiscus tai* Ogawa, 1994 causes emaciation, erosions and haemorrhage (Ogawa 1994). *Neobenedenia melleni* (MacCallum, 1927) Yamaguti, 1963 has the ability to infect more than 100 marine teleost species, attaching to skin and eyes, these parasites cause blindness, scale loss, deep skin

ulcers and mortalities (Deveney *et al.* 2001). During heavy infestations of *N. melleni* in common sole, *Solea solea* (Linnaeus, 1758) mass mortalities occur if the fish are untreated (Kearn 2002). *N. girellae* (Hargis, 1955) infecting amberjack *Seriola dumerili* (Risso, 1810), also inflicts the same degree of injury as *N. melleni.* Mugilid fish, infected with *N. girellae* and *Benedenia monticellii* (Parona & Perugia, 1895) causes injury to skin and buccal mucosa of the fish, which can lead to mortalities (Paperna *et al.* 1984).

Benedenia seriolae is considered worldwide as one of the most important and costly disease causing parasite associated with sea-cage farming. Fish hosts for *B. seriolae* include Japanese yellowtail, *Seriola quinqueradiata* Temminck & Schlegel, 1845, *S. dumerili* and the Australian yellowtail kingfish or goldstriped jack, *S. lalandi* and high fin amberjack, *S. riviolana* Valenciennes, 1833 (Ernst *et al.* 2002). It has been reported that in 2001 the Japanese annual "yellowtail" aquaculture industry production was 150 000 tonnes (Whittington *et al.* 2001). Compared to the production cost of Atlantic salmon in Norway, it is more than double in the Japanese "yellowtail" culture industry of which 22% of total production cost is due to the cost towards control *B. seriolae* infections (Ernst *et al.* 2002). While the annual production of Australian kingfish for the term 2004/2005 was more that 2000 tonnes (Chambers & Ernst 2005).

Buchmann & Bresciani (2006) reported that the injury sustained by the skin and fins infected by *B. seriolae* as it feeds on the mucus and epithelial, inflicting large feeding wounds is also consistent with *Entobdella* sp. and *Neobenedenia* sp. This causes the fish to flash against objects which leads to trauma and secondary infection. These injuries further results in a decrease in growth rate of fish as well

as diseased fish have a decreased market value (Chambers & Ernst 2005). In Australian stock losses of up to 200,000 fish in cultured barramundi *L. calcarifer* infected with *N. melleni* have been reported (Deveney *et al.* 2001).

Although extensive damage to the skin is caused by representatives from the larger capsalides, such as *Benedenia, Entobdella* and *Neobenedenia* during feeding, pathology is also associated with these parasites at their attachment sites.

Four genera of the class Monogenea have been confirmed from dusky kob in South Africa (Christison & Mouton 2007). These include species of *Diplectanum* Diesing, 1858, *Calceostoma* van Beneden, 1858, *Benedenia* Diesing, 1858 and *Sciaenocotyle* Mamaev, 1989.

Diplectanum oliveri Williams, 1989 has a simple life cycle. Adults inhabiting the gill tissue produce eggs that are released into the water. Free swimming oncomiracidia hatch from the eggs and actively find the hosts, attach to the body from where they migrate to the gills and mature. Sediment containing parasite eggs incubated at water temperature of 25°C, hatch after four days and the new infection generation produces eggs fourteen days later (Christison 2005).

In light of the global concern about monogenean related production and stock losses, the potential impact and threat on South African finfish aquaculture cannot be underestimated. Early qualitative and quantitative assessment of emerging monogeneans infection is paramount. Quality research to identify and describe the pathogens, their life cycles and epidemiology needs to be developed for better integrated parasite management. The different legitimate options available to farmers and veterinary services for the identification, evaluation and use of drugs

need to be re-evaluated and adapted for the industry. Potential effective chemotherapeutic compounds need to be identified and evaluated to provide the industry with effective treatments that could be integrated with parasite biology for optimum parasite control.

Praziquantel is an anthelmintic normally used with success in mammals against the endoparasitic platyhelminthes, including cestodes and trematodes Due to the drugs relative low toxicity and few side effects, it is increasingly regarded as a option for treatment against monogeneans (Thoney & Hargis 1991). Praziquantel is also widely used in aquaculture due to its relatively high efficacy and low toxicity to the host (Campbell 1986). Schmahl & Mehlhorn (1985) showed that bath treatment with praziquantel caused vacuolization of the tegument in monogeneans and that these alterations were irreversible within 30 minutes after exposure to a concentration as low as 1 ppm. Within seconds of exposure to praziquantel, two major effects become apparent in the adult worms: a rapid, sustained muscular contraction (Fetterer *et al.* 1980) and tegumental disruption (Becker *et al.* 1980) which leads to exposure of parasite antigens on the worm surface (Harnett & Kusel 1986). Both these responses are thought to be linked to praziquantel-dependant disruption of calcium and magnesium homeostasis.

1.1. Research aims

The academic aims of the dissertation are:

1. To provide an overview of the constraints imposed on the aquaculture industry globally and locally, through the restricted availability of registered pharmaceuticals and stringent drug

legislation. Initiatives, worldwide to provide the necessary special accommodation in legislation to accommodate the use of existing pharmaceuticals in aquaculture?

2. To determine if *Diplectanum oliveri* on dusky kob *Argyrosomus japonicus,* causes pathological tissue changes in the areas associated with attachment and feeding, which may lead to loss of production and mortalities in *A. japonicus* in the South African aquaculture industry?

3. To determine if the counting of eggs produced by infra-populations of *D. oliveri* over a 24-hour period, could be used as a reliable non-invasive/non-destructive method to qualify and quantify the intensity of an individual infra-population of parasites on a single host. Does this method have the potential to be utilized as a diagnose tool for *D. oliveri* on aquaculture farm, continual monitoring of parasite intensity and to evaluate the efficacy of drug trials on the egg production and parasite stages?

4. To determine the efficacy of two approved veterinary anthelmintic formulations, used in terrestrial animals (sheep, cattle and ostriches) containing praziquantel as the active ingredient in different combinations of drug concentrations and exposures against *D. oliveri* to reduce parasite egg production and parasites on the host as an "off label" used drug. Do these drug formulations cause altered behaviour in *A. japonicus* and toxicity that may cause mortality?

CHAPTER 2: Challenges with the development and approval of pharmaceuticals for aquaculture in South Africa.

2.1. Abstract

The development of a sustainable aquaculture industry in South Africa strongly relies on the concurrent development of the support sectors such as aquaculture nutrition, aquaculture engineering, innovative marketing approaches and aquatic animal health services. The increasing awareness of food safety, environmental conservation and pollution cause pressure on farmers to produce better quality food.

To some extent the development of the aquaculture industry locally is constrained by new challenges with regard to pathogens, their unknown epidemiology and the limited number of approved chemotherapeutic agents to treat or control them. Local aquaculture consultants and fish farmers are currently forced to use medication, often without authorisation, with very little guidance on efficacy, toxicity and residues in tissue. The long-term solution is to develop and register approved drugs for the specific disease conditions for the global and South African aquaculture industry. In the interim, however, it is necessary to follow global trends whereby drugs are identified for "off label" use.

Keywords: Monogenea; aquaculture production; off label drug use; drug legislation; drug regulations; drug toxicity; drug residue; pet shop medication.

2.2. Emerging challenges in intensive finfish aquaculture

An increase in research and development of the global aquaculture industry has lead to increased attention being paid to parasites or pathogens and their relative economic importance of reducing the productivity of the industry. The scale of commercial production requires high stocking densities which inevitably lead to disease-related problems and direct losses through reduced growth rates and ultimately the mortality of the fish host (Reno 1998). Although progress has been achieved regarding the parasitology of fish in aquaculture, numerous problems remain unresolved (Scholz 1999).

In order to protect their stock against parasitic infections and consequent stock losses and in compliance to the Animal Diseases Act (No 35 of 1984), which requires that any owner in respect of animals shall take the necessary precautions and actions to prevent the infection of their animals with any disease or parasite and prevent the spreading thereof, local fish farmers are currently forced, to use medication "off label" to treat disease. Currently, only a limited number of chemotherapeutic drugs are registered worldwide for aquaculture compared to the number of drugs available for terrestrial production animals, and no drugs are registered for disease control in aquaculture for South Africa.

The legislation and registration of drugs for the aquaculture industry in South Africa needs to be re-evaluated to accommodate the testing of potentially effective, approved drugs for "off label" use to support the successful development of the industry. The success and sustainability of this fast developing sector largely relies on a dedicated and effective support sector to improve aquatic organism health management.

Under Act 101 of 1965, medicines and scheduled substances are subjected to a scheduling classification process. Schedule 1 to 6 may only be sold by members of the pharmacy, medical, dental and veterinary professions. Schedule 0 medicines are also termed "over the counter medicines" (OTC/open shop) and include anthelmintics. Medicines and substances can also be registered as a "stock remedy" under Act No. 36 of 1947, in which case they can be sold without prescription as an OTC product at an agricultural co-op and pet shops. These remedies include certain antimicrobials as an exception to the rule, which under scheduling would be a schedule 4 medicine.

In terrestrial animals treated with anthelmintics, a withdrawal period is stipulated that varies from 7 to 28 days depending on the drug. Meat consumption is prohibited for the period stipulated. The increase in food safety awareness and compliance to international standards resulting in increased requirements on the traceability of the food products produced, further limits the application of medication to the fish, thus leaving the farmer vulnerable against fish disease outbreaks.

There is a demand for improved and integrated methods to control and manage fish parasites. The aim is to have an integration of management methods, immunoprophylaxis, bio-control and anthelmintic treatments (Buchmann 1998). A limited number of tested drugs are available for the treatment of aquatic parasites globally (Schnick *et al.* 2005). There are also limitations to their use because of parasitic resistance, toxicity and persistence of chemical residues. Moreover, many drugs previously widely used in fish farming such as copper

sulphate, malachite green or 2,2,2-trichloro-1-dimethoxyphosphotyl-ethanol are now viewed as environmentally undesirable (Scholz 1999).

2.3. A global initiative to facilitate and promote the approval of drugs and vaccines

Worldwide co-operation to gain approvals of drugs and licensure of biologics for aquaculture was realised. At the Workshop on International Harmonisation for Aquaculture Drugs and Biologics, held in February 1997, a worldwide co-operation to gain legal approval of drugs and registration of biologics for aquaculture was proposed by representatives from Japan, Australia, Europe, Canada and the USA (Schnick *et al.* 2005).

One of the five committees of the International Harmonising Workshop (February 1997) held workshops (Models of Partnership for Registration of Drugs and Vaccines) and round-table discussion (Progress with Registration of Drugs and Vaccines for Aquaculture) at the Eighth International Conference of the European Association of Fish Pathologists (EAFP) on Disease of Fish and Shellfish (Edinburgh, Scotland, September 14-19, 1997) to identify future priorities Drugs and vaccines were categorised into two lists: Category one comprised the current number of drugs and vaccines approved for aquaculture application globally, numbering 70 drugs and 34 vaccines; and category two comprised the priority aquaculture drugs and vaccines currently needed globally numbering 123 drugs and 28 vaccines (Schnick *et al.* 2005).

2.4. Constraints and alleviation in aquaculture drug use and registration globally

Legislation for aquaculture drug development and registration of these drugs in most developed countries is very strict and is regulated in the same capacity as any veterinary medicine. A number of constraints are being looked at in drug use and registration.

All old and new drugs widely used in aquaculture in Europe are reviewed for their Maximum Residue Limit (MRL). Old drugs have to be defended by the sponsor. Study results are submitted to the European Commission (EU) which qualifies the drugs into four categories. A new drug will not be considered for Marketing Authorisations without a MRL assessment as a Veterinary Medicinal Product (VMP). Furthermore, no field trials will be approved without a MRL. Environmental concerns have prompted even more requirements for the Environmental Risk Assessment which makes registration even more costly. Alleviation to the constraints through workshops and round table meetings by all parties involved were organised to discuss the availability and registration of drugs. The Committee on Veterinary Medicinal Products proposed and approved (September 11, 1997) a more pragmatic approach to MRLs in Europe, which alleviate the need for additional metabolic studies as long as the existing studies were done on major food animal species. The only additional study introduced was the required testing to determine if cold storage has an influence on drug residue depletion from the tissue and referred to as, cold residue depletion (Schnick *et al.* 2005).

In Australia, the National Registration Authority can grant a substance exemption from registration which can then be used legally in the industry. A

Minor Use Minor Species (MUMS) permit grants the use of a drug for 12 months when there is insufficient information or resources for full registration (Schnick *et al.* 2005).

In the United States and Canada, all animal drugs are controlled by the U.S. Food and Drug Administration's (FDA) Centre for Veterinary Medicine (CVM). The Animal Medicinal Drug Use Clarification Act (AMDUCA) allows a veterinarian to prescribe certain drugs in an extra-label manner where there is suffering and death but still excludes many products that have the potential for the effective treatment of disease. A recent document provides guidelines of discretion for the extra-label use of medication in feed for minor species. New drugs are developed and registered as a New Animal Drug Application (NADA) which is time consuming and expensive. The Investigational New Animal Drug Application (INAD) permit the compassionate use of a drug where there is suffering, death or harm to endangered species, while at the same time data are recorded on the efficacy and toxicity that will help with the registration of the drug (Schnick *et al.* 2005).

The USA and Canada have through workshops and newly formed coalition's adapted legislation assisting the industries in their quest for drug development and approval. A succession of initiatives followed from 1980 to support and develop drugs for registration. These included the National Aquaculture Act of 1980, the National Aquaculture Improvement Act of 1985, the formation of the Joint Subcommittee on Aquaculture (JSA) (32) (JSA referred to in Storey 2005) and the Quality Assurance in Aquaculture Production Working Group by the JSA in 1990 (Storey 2005).

In 1993 the United States Drug Administration and FDA established the National Research Support Project Number-7 (NRSP-7) Minor Use Minor Drug Program to address the shortage of drugs (AVMA). Liaison between CVM, the NRSP-7 program, investigators and pharmaceutical firms on MUMS was implemented. The constraint of funding on drug development initiated a coalition of pharmaceutical companies, veterinarians, producer commodity groups and others in 1999 for MUMS which led to the Minor Use and Minor Species Animal Health Act in August 2004. The National Aquaculture instituted the NADA that was used as a guide to provide support with the drug approval process and liaise between researchers and pharmaceutical firms (Schnick *et al.* 2005).

Thus drugs in the USA can be divided in the following categories. Firstly, the approved drugs which are confirmed to be effective in their claim. Secondly, unapproved drugs used as; a) an investigational new animal drug (INAD) exemption of either a standard INAD authorized use of an unapproved drug, b) a compassionate INAD authorized use of an unapproved drug, c) an "off label" prescription issued by a licensed veterinarian. Thirdly, the use of drugs that are compounds unapproved for use in fishes, but with a comparatively little risk to aquatic organisms, human consumers, or the environment as low regulatory priority (LRP) (American Fisheries 2011).

2.5. An overview of an initiative to support and develop aquaculture in South Africa

Initiative and recommendation by the South African Commission of Administration in 1990 for the development of an aquaculture policy, lead to the

establishment of the Aquaculture Policy Committee by the Department of Agriculture. A meeting on the 14 April 1991 was attended by the Development Aid, Health, Water Affairs, Environmental Affairs, the Provincial Administrations, Universities, the Foundation for Research Development, the Council for Scientific and Industrial Research, the South African Agricultural Union, producer associations, the Aquaculture Association of South Africa and Agriculture Research Council initiative was terminated at the end of the previous political dispensation in 1994 (Hinrichsen & Brink 2004).

In 1991 the Western Cape Aquaculture Liaison Committee was established which continued as the Southern Aquaculture Workgroup and a similar group was formed in the northern regions. Both groups have not been successful. The "Fishing the Future" aquaculture strategy workshop was held at Onderstepoort Research Institute in 1997 to develop a national strategy and policy. The committee never convened again. The Aquaculture Association of South Africa has attempted from 1994 – 2002, at the national congresses, to create a national aquaculture policy but very little has materialised. The two workshops that were held by the Department of Water Affairs and Forestry (DWAF) in 2001 in Gauteng and the Western Cape are seen as the predecessor for the current cooperation of the DWAF and the National Department of Agriculture to establish a National Aquaculture Policy (Hinrichsen & Brink 2004).

2.6. Regulation of veterinary medicines and stock remedies in South Africa

Veterinary medicinal products in South Africa are currently registered under two acts and are administered by two separate regulatory authorities in two different government departments. The two acts are the Medicines and Related Substances Control Act (Act No 101 of 1965) administered by the Directorate Medicines Control within the National Department of Health whose products are called veterinary medicines; and the Fertilisers, Farm Feeds, Agricultural Remedies and Stock Remedies Act (Act No. 36 of 1947) administered by the Registrar appointed within the National Department of Agriculture whose products are called stock remedies (Swan *et al.* 2004).

2.7. Regulations controlling the use of registered drugs

Regulations exist, controlling the different ways in which veterinary medicines/remedies may be used and obtained, e.g. veterinary medicines, remedies, veterinary restricted remedies, general sale remedies, and extra label use, the use of unregistered medicines or scheduled substances and pet shop remedies.

Medicine scheduled under Act No 101 of 1965 may be used by a medical practitioner, veterinarian, pharmacist, dentist, practitioner and nurse. Veterinary medicine, under this Act 101, may only be used by veterinarians and not by medical practitioners on animals.

The Registrar determines if a drug is registered as an Act No 101 of 1965 medicine or an Act No 36 of 1947 remedy. The Registrar further categorises the

remedies as one; a veterinary restricted drug which can only be purchased from a veterinarian or secondly; a remedy for general sale that may also be purchased from other commercial outlets.

South African companies applying for registration of ectoparasiticide and endoparasiticide products, presenting the trial data obtained for its registration in the northern hemisphere were declined in the past when presented without local efficacy tests. In contrast, a registration file prepared for local submission on the northern hemisphere files for drugs like antimicrobials, will be accepted without local efficacy trials.

2.8. The extra-label/off label use of medicines

Only veterinarians registered with the Veterinary and Para veterinary Professions Act (Act 19 of 1982) may prescribe the use of veterinary medicines for uses other than the intended drug indications or animal species, and may prescribe different dosages to what is suggested by the drug manufacturers. Similarly, a veterinarian may also make use of human medicine for veterinary purposes. The use of drugs under such circumstances must be done after careful consideration of the risks and benefits.

The necessary precautions, particularly in food producing animals must be taking into account. The necessary withdrawal periods must be advised in cases where these drugs are used in any food producing animals, including fishes. “Off label” drug use is only considered if no other registered drug is available and may have implications when it is used in animal stock that is insured. It is the duty of

the veterinarian to explain all the material risks involved with the treatment of the animals and to obtain consent from the owner for the use on his animals.

The Medicines Control Council advised by the registrar of medicines was of the opinion that the "off label" or extra-label use of medicines cannot be advocated. Internationally, medicines that are used in such a manner are acceptable, thus the MCC recognised the right or obligation for the similar use by veterinarians in South Africa who will remain accountable as the prescriber when this practice is applied (SAVC 1998).

2.9. Use of unregistered medicines or scheduled substances

No person may use any unregistered medicine, stock remedy, or scheduled substance without permission. Permission to use unregistered medicines may be given by the Department of Health, Medicines Control Council (MCC), in terms of Section 21 of Act No 101 of 1965. In terms of their mandate the MCC will only consider such an application if the science is sound, the product is not available in South Africa and proper motivation for its use is submitted. Only a veterinarian may prescribe an unregistered drug for use in a patient, who then takes the responsibility for it with extra emphasis on the toxicity and withdrawal period. All treatment outcomes must similarly be reported after the termination of the treatment. The patient is defined as a herd of cattle, flock of sheep or in this case the fish in an enclosed spaced e.g. a pond, tank or aquaculture system.

2.10. Pet shop fish remedies

By definition a stock remedy means a substance intended or offered to be used in connection with domestic animals, livestock, poultry, fish or wild animals (including wild birds) for diagnosis, treatment, prevention or cure of any disease, infection or other unhealthy conditions, or for the maintenance or the improvement of health, growth, production or working capacity, but excluding any substance in so far it is controlled under the Medicines and Related Substances Control Act 101 of 1965 (Swan *et al.* 2004).

According to this definition fish remedies are included. It is not only the ingredients of the drugs that are of concern but also the claim by the producer that the formulation is effective as a treatment for a disease. This claim then renders this drug a veterinary remedy/medication. These remedies contain substances such as malachite green, victoria green, formalin, methyleneoxide, acraflavine, nitrofurazone, furazolidone, potassium dichromate, methylene blue, etc. They are imported and some are locally produced and sold as "over the counter drugs". The importation, registration and regulation are all controlled by the inspectorates of Act 101 and 36. The registration holder has to have a license certificate for every remedy as proof that the remedy is registered under the appropriate act. All these remedies have to display the registration number printed on the container.

Although these drugs are mainly used in the ornamental fish industry, aquaculture farmers might also be tempted to use these drugs in food fish. By definition these drugs may only be used with permission from the MCC, however, the control of pet shop remedies has not been a priority and legislation

did not exist or was found inadequate due to size of industry and shortage of manpower.

It is left to the discretion of the user to comply with the directions of use printed on the container. These medications could thus be used by farmers without prescription by an authority, with very little guidance on efficacy, toxicity and residue retention in tissue of non-target species. There is a considerable variation in efficacy of the same drug between different parasites with even more variation in toxicity between hosts (Buchmann 1998).

The initiative to develop and register drugs for the relatively young and developing aquaculture industry is usually taken by individuals closely associated with this industry, which realises the need for proper, effective and safe products. When a company, usually a small enterprise, takes the initiative and invests substantial amounts of money toward the development and registration of medication, it has to compete with freely available non-regulated medication to the public and carry their own costly registration maintenance fees. This imposes such a burden on the growth and sustainability of the company, where the input cost is out of proportion with the returns and leads to deregistration of the drugs.

Act 36/1947 of 1949 had relatively limited control of the so-called animal remedies. It was suggested in 1994 by the MCC that fish and pigeon remedies should also be controlled. The successful execution of this task would require at least double the current administration, law enforcement officers and one if not two more Technical Advisors. The administration of Act 36/1947 has since been degraded from a full directorate to a sub-directorate and the present staff cannot

even handle the amount of work necessary to control the presently registered products. The possibility for additional staff is remote because of the under appreciation of the importance to control stock remedies by the Department of Agriculture (Sykes 2007). In 1972 the Department of Agriculture sent out a new directive which specified which products could now be registered in terms of Act 36. The position has not changed much from that time, except that products containing furazolidone/furaltadone and the lindane-chlorinated hydrocarbon group have been discontinued.

Medicines that were registered and controlled by Act 36 were excluded from control by Act 101 until 1983. Subsequently, problems that arose between these two directorates resulted in the establishment of the Veterinary Products Policy Committee (VPC) to resolve problems. This committee comprises of representatives of both directorates, the Department of Health, the Directorate of Meat Hygiene, the Directorate of Veterinary Services, the veterinary and pharmacy professions and agriculture industry (AVCASA). The AVCASA represents the interest of more than 90% of animal health product importers, manufacturers and suppliers in South Africa, with a membership of 21 companies in the crop protection and animal health sector.

New products have entered the market. Currently these new products are assessed and an application is made to Act 101/1965 to excuse them from scheduling before they can be registered in terms of Act 36/1949. The situation concerning aquaculture or other minor species markets has traditionally been one that was not controlled by Act 36 because of the assumption of the small size and volume of these products and of the expenses in doing this. More recently the

registrar has decided that the scope exists in the Act to control any product which is sold for the use in animals, whether they are minor species.

The current approach taken by the registrar is that any product that is excluded from the scheduling of Act 101 has to be registered in terms of Act 36 before it can be sold. Special coordinated efforts were initiated by the registrar with companies that supply products to aquaculture industry and have started insisting that the products be registered in terms of Act 36 and are demanding that products that already exist on the market, have to supply certain data by a certain timeline. The question arises, if it is economically viable for companies, who have sold or supplied fairly low volumes of product to small industries like aquaculture, to commit to gathering of data with the cost implication. The advantage of registration is that it will prevent abuse and misuse of these products.

2.11. Long term solution for drug availability in South Africa

In South Africa there are approximately 40 local and international pharmaceutical companies that produce and supply veterinary products to the agricultural sector (Swan *et al.* 2004). Although some of the international pharmaceutical companies have registered products for the aquaculture industry abroad, no such approved products are registered with the Medical Control Council of South Africa for use in South Africa aquaculture.

The long term solution is to develop and register drugs for the specific disease conditions in the South African aquaculture industry, but in the interim it is

necessary to follow the global trend. Advances by developed countries to support the development of drugs and vaccines for aquaculture has established and defined the direction and processes needed. If South Africa aims to participate in the global import/export market, it is necessary to comply with the same standards and regulations that are established by the Codex Alimentarius (Schnick *et al.* 2005).

2.12. Short-term solution for drug availability to the aquaculture sector in South Africa

2.12.1. Initiate co-operation to promote short term solution

The development and registration of therapeutic compounds are very costly and require comprehensive studies to demonstrate human food, animal and environmental safety and efficacy. The development of effective strategies to control fish health problems are also hampered by the limited understanding of their biology, vectors and epidemiology of fish pathogens in the aquaculture industry (Stuttgart 2005). Sustainable growth, disease control, management and profitability will be determined mainly by two concepts (Schnick *et al.* 2005).

The first concept is that there is a need for understanding and co-operation between government departments, fish farmers, pharmaceutical companies and research facilities. The government departments that formulate and regulate legislation for the development, registration and use of aquaculture drugs, need to institute more accommodating legislation to support aquatic animal health. Pharmaceutical companies need to realise that they are indispensable in the development and support of this young industry and that they should not wait

until the industry is fully developed, before they get involved. Research facilities such as universities, private research laboratories and government research facilities need to be identified and possibly upgraded for research accreditation.

The second, equally important concept is quality research to identify and describe the pathogens, their life cycles and epidemiology, in order to develop better disease control methods. This will decrease the unnecessary use of effective drugs and target susceptible developmental stages in the life cycle of the pathogen at selected target intervals, which in turn will provide preservation on the usability of the drugs. The objective of the research is to collect as much basic information on parasite biology and infection dynamics to form a database. This information will then be utilized for the development of essential management strategies that would target susceptible parasite stages to optimally interrupt parasite life cycles. Due to drug resistance or regulatory control, the longevity of drug and chemical treatments are not absolute, but the knowledge of parasite biology, their reproduction and infection dynamics is cumulative and enduring (Ernst *et al.* 2002).

Currently, there are no criteria under Act No 101 of 1965 to support drug use in the small number of users (minor uses minor species) category in South Africa as in the USA by the FDA.

2.12.2. Regulations for the importation of registered drugs into South Africa

The importation of a drug registered in other countries is governed by Act 101 of 1965. If a local company decides to import a drug for use by a farmer, the company (pharmacist or veterinarian) puts forward an application to the Medicines Control Council stating their rationale. The regulations under Act 101/1965 are applied to determine if the product will be controlled by Act 36 /1947.

2.12.3. The import of approved drugs for "off label" use

The option to import an approved drug for treatment is conducted as follows. A drug can be imported by a primary holding company or person. The holding company or person (preferably a veterinarian or pharmacist), applies for an Act No 101 of 1947, Sec. 21 permit to import the drug into the country. Other secondary users (farmer) of this drug may apply for a Sec. 21 permit to obtain stock from the holding company for their own use, but not for secondary sales. Only a pharmacist or a veterinarian is allowed to conduct secondary sales.

A Sec. 21 permit allows a farmer to import a registered product. This product is then used in an extra-label unregistered capacity, the farmer has the responsibility to keep detailed records and submit a report to the Medicines Control Council after six months. The drug may only be imported if there is no similar product registered in South Africa for that particular use. Drugs with a schedule 5 and 6 may only be imported, stored and dispensed by a veterinarian or pharmacist.

Unregistered overseas drugs may not be imported into the country.

Another option to use a drug as an extra-label in South Africa is to compound the drug. A veterinarian or a pharmacist can import the raw material (product) and compound any drug. Act 101/1965 permits the compounding of any drug, as long as it does not exceed 30 days worth of medication. Unregistered drugs from overseas, although they may be effective, may not be imported.

Importing an existing drug from overseas and changing its actual indication of use for registration in South Africa, needs to be supported by clinical generated trial data. Currently, a protocol is compiled and submitted to the Medicines Control Council (MCC) to approve the drug trial, but there is also no guarantee that the data generated from the experimental drug trail can be accepted. The recorded information can be used in future as part of the registration dossier. These data will not be accepted retrospectively. If the data is collected without the approval of the MCC, they will not accept the data for registration purposes. An important component of the registration process is the quality of the product. When a drug is imported, the company manufacturing the drug needs to comply with the principles of Good Manufacturing Practice.

2.13. Conclusion

The aquaculture industry has come a long way over the past decades. The approach and experience generated in this sector was mainly through trial and error. Remarkable advances have been made with the successful spawning, hatching and farming of dusky kob *Argyrosomus japonicus* and silver kob *A.*

inodorus with interest in future species like red roman *Chrysoblephus laticeps* (Valenciennes, 1830) and yellowtail *Seriola lalandi* Valenciennes, 1833, in a relative young finfish mariculture industry. This industry will never be without challenges. Freshwater culture will always be challenged by the fact that South Africa is a water scarce country. Water conservation and the control pollution will always be a priority. Marine sea cage culture will be challenged by factors like, a high energy coast line, seals and other predators. On-shore, closed recirculating farms have made the best advances to date.

The universities, private research laboratories and government research facilities who are prepared to take up their role as the support sector to the South African aquaculture industry are faced with the following challenges. To resolve the inadequate knowledge of parasite biology and reproduction dynamics of existing and new emerging parasites on host species. Continuous vigilant monitoring and health assessment on production facilities would confirm early infections. The development of new parasite-specific anthelmintics is the ideal, but it is not a reality in the near future due to the size of the industry and cost. Thus, the second option needs to be explored where current treatments for disease in aquaculture worldwide and approved veterinary and medical drugs are to be considered for future "off label" use. Possible potential treatments for the control of a specific parasite infection need to be identified and research to develop an arsenal of "off label" treatments for disease control should be designated. Three basic questions arise. Will the treatment be effective against the parasite infection on the specific host? What effect does the treatment have on the host concerning toxicity and residue? And what impact does the drug have on the environment?

It is of no use to prescribe a drug that has not been tested to be effective against the parasite in question due to formulation and/or unknown drug concentration and exposure time. If a drug is effective but its toxicity and tissue residue or withdrawal period is unknown, the farmer, although he might be able to purchase the drug as an OTC treatment are not allowed to use it and would not be able to market a safe "food product". Also a veterinarian would not be able to prescribe the treatment as an "off label" drug and take responsibility according to the regulations on toxicity and residue. It is possible that metabolites of the primary active substance could persist for a longer period in muscle and skin than the parent compound (Buchmann 1998). Once *in vitro* studies are done, field trials are recommended. Currently, legislation disqualifies the data acquired in this manner to be used retrospectively to obtain drug approval.

A current initiative and investigation were undertaken to determine the importance of *Diplectanum oliveri* Williams, 1989 infection on dusky kob in the local aquaculture industry. The pathological changes caused by this parasite on its host will be examined to determine the impact that this parasite has on dusky kob in the aquaculture of South Africa. Once this impact has been established the search for an effective drug would be pursued.

In light of the above discussion of the limitations on drug development, availability and legislation for the use of drugs in the aquaculture industry, two existing veterinary drug formulations for treatment in terrestrial animals were targeted for testing on an emerging monogenean parasite for "off label" drug use.

There was never a better time than now for universities, private research laboratories and government research facilities to facilitate, sponsor and gather the

relevant information on pathogens, parasite biology, infection dynamics, epidemiology and drug testing to support the aquaculture industry.

CHAPTER 3: Gill histopathology of dusky kob *Argyrosomus japonicus* (Temminck & Schlegel, 1843) infected with *Diplectanum oliveri* Williams, 1989 (Monogenea: Diplectanidae) in aquaculture of South Africa.

3.1. Abstract

Diplectanum oliveri, a monogenean gill parasite of both *Argyrosomus japonicus* (dusky kob) and *A. inodorus* (silver kob) is currently regarded in South Africa as the most persistent ectoparasite associated with the culture of both fish species. This parasite causes pathological tissue changes in the areas associated with attachment and feeding. The feeding process and attachment to the secondary gill lamellae result in epithelial hyperplasia, interstitial oedema, vasoconstriction and affects the numerous specialised cells associated with structural integrity, ionoregulation, immunological protection and osmoregulation. These cells include squamous epithelium (pavement cells), chloride cells, mucous cells, granular cells, pillar cells, neuroepithelial cells, taste bud cells and rodlet cells. The pathology caused by *D. oliveri*, particularly at high mean intensities, will impact the production of the fish predisposing them to secondary infection. This parasite has been implicated in causing significant mortalities at culture facilities.

Keywords: aquaculture, *Argyrosomus japonicus*; Diplectanidae; *Diplectanum oliveri*; dusk kob; gill pathology; Monogenea.

3.2. Introduction

In South Africa significant progress has been made toward the successful spawning and commercial production of dusky kob, *Argyrosomus japonicus* (Temminck & Schlegel, 1843) and to a lesser degree silver kob, *Argyrosomus inodorus* Griffiths & Heemstra, 1995, in a developing finfish mariculture industry. *Diplectanum oliveri* Williams, 1989 has been recorded from both fish host species from wild caught and captive held fish. Currently this parasite is regarded as the most persistent parasite in all culture facilities and has also been implicated in fish mortalities (Christison 2005).

The monogeneans infecting fish can be subdivided into two sub-classes, namely the Polyonchoinea Bychowsky, 1937 and the Oligonchoinea Bychowsky, 1937 (Rohde 2005). Polyonchoinean parasites are most commonly found on the host's skin and gills where they feed on epithelial tissue. These epithelial browsers ingest epithelial cells, mucus and limited amounts of blood from traumatized tissue (Smyth & Halton 1983). Attachment of the Polyonchoinea to the secondary gill lamellae is primarily by the utilisation of hooks (Kearn 1968) that can penetrate the epithelium of secondary lamellae. Both attachment and feeding often result in pathology of the gill tissue (Rohde 2005). The Oligonchoineans, by contrast, are predominantly sanguinivorous gill parasites (Kearn 2004) which attach to the secondary gill lamellae of the host by means of suckers and clamps (Kearn 1968) seldom causing a noticeable tissue response (Rohde 2005).

Production losses attributed to pathology caused by polyonchoinean monogeneans of the family Diplectanidae Monticelli, 1903 have been thoroughly

researched and documented. Stock losses of up to 10% in the culture of sea bass infected with *Diplectanum aequans* (Wagener, 1857) have been reported (Dezfuli *et al.* 2007). In addition to this, numerous other diplectanids, including *Lamellodiscus elegans* Bychowsky, 1957 on *Sparus aurata* Linnaeus, 1758 and *Diplodus puntazzo* (Walbaum, 1792) (Ivona 2006) and *Furnestia echeneis* Parona & Perugia, 1989 on *S. aurata* (Paperna *et al.* 1977) have been associated with general pathology and necrosis of the host fish gill tissue. Parasites occupying the gills of the host can have varying influences on the host, depending on the parasite intensity, mechanism of attachment and feeding behaviour. *D. aequans* and *Diplectanum laubieri* Lambert & Maillard, 1974 have been reported to cause gill inflammation, haemorrhage, epithelial hyperplasia, lamellar fusion and increased mucus production on the gills of both *Dicentrarchus labrax* (Linnaeus, 1758) and *S. aurata* (Gonzales-Lanza *et al.* 1991; Dezfuli *et al.* 2007). Rajendran *et al.* (2000) reported similar clinical signs associated with mass mortalities of *Lates calcarifer* (Bloch, 1790) infected with *Diplectanum latesi* (Tripathi, 1957) and also reported on some additional fish behavioural characteristics such as flashing of sides and opercula against substrate and head bobbing.

Attachment to the host tissue by means of the haptor induces inflammation followed by hyperplasia, lamellar fusion, and loss of functional tissue at the attachment sites as observed in the case of *D. aequans* on European sea bass (Dezfuli *et al.* 2007). Further effects include the disruption of the extra- or epicellular biofilm on the surface of the gill epithelium predisposing the tissue to secondary bacterial and fungal infections (Cone 1995).

The primary gill lamellae are comprised of cartilage, muscle, nerves, a vascular system, respiratory lamellae and specialised cells that include squamous epithelium (pavement cells), chloride cells, mucous cells, granular cells, pillar cells, neuroepithelial cells, taste bud cells and rodlet cells, each with their own specialized function (Ostrander 2000). Collectively, these constituents of the primary gill lamellae operate as a functional unit responsible for respiration and the regulation of the homeostasis of the fish host (Ferguson 2006). Consequently the loss of functional gill surface area severely compromises the host's health, production and depending on the severity of the damage may elevate mortality within the host population.

The attachment organ, the haptor, is comprised of large "anchor-like" hamuli, smaller marginal hooks, septae and suckers. The attachment of the haptor to the epithelium can be superficial and may result in a slight indentation of the interlamellar epithelium (Hendrix 2004) eliciting little or no tissue response. Conversely, the attachment may be more aggressive whereby the hamuli penetrate deeply into the host epithelium often eliciting a pronounced inflammatory response and resulting in club-shape filaments, lamellar fusion, hyperplasia and mechanical pressure necrosis (Buchmann & Bresciani 2006).

The hyperplasia, resulting from the inflammatory response may cause embedding and entrapment of the haptor thereby resulting in a more secure attachment to the parasite's benefit (Dezfuli *et al.* 2007). Through the embedding, the parasite acquires a more secure hold, better protection and prevention from detachment (Buchmann 1997). Parasites that benefit from this response include; *Linguadactyla molvae* Brinkmann, 1940 on *Molva dipterygia elongata* (Otto,

1821), *D. aequans* on sea bass, *D. labrax*, *Callorhyncicola multitesticulatus* Manter, 1955 on *Callorhynchus milii* Bory de Saint-Vincent, 1823 (Llewellyn & Simmons 1984) and *Pseudodactylogyrus bini* (Kikuchi, 1929) on the gills of *Anguilla anguilla* (Linnaeus, 1758) (Dezfuli *et al.* 2007).

The global importance of pathology causing monogeneans resulting in production loss and mortalities has been well documented and cannot be underestimated, with special reference to the diplectanids as pointed out by Dezfuli *et al.* (2007) in *D. labrax* and *L. calcarifer* (Gonzales-Lanza *et al.* 1991). In light of the global concern with monogeneans and now *Diplectanum* spp. in the South African finfish culture like *D. oliveri* and possibly *Diplectanum glandulosum* Williams, 1989 (present on wild stock), this study was conducted to determine the histopathological changes caused by *D. oliveri* which are already known to have caused stock losses in captive dusky and silver kob (Christison 2005).

3.3. Materials and methods

3.3.1. Source of fish

Dusky kob, *A. japonicus* (80 mm mean peduncle length ± 1.13 standard deviation), infected with *D. oliveri* were obtained from a private commercial production facility. The fish were temporarily relocated to the Department of Agriculture Forestry and Fisheries (DAFF) Aquaculture Research Facility in Sea Point, Cape Town. At this facility the fish were maintained in a well aerated 10 000 L circular flow through tank with water temperature at 20℃. A commercial

diet was fed daily to the fish at 1% of their body weight. The fish were grown (168 mm mean peduncle length ± 1.16 standard deviation), to facilitate handling and sampling and to allow the parasite intensity to increase.

3.3.2. Macroscopic examination and sampling

Fourteen fish were euthanized by severing the spinal chord (iki jimi). Post mortem dissection and examination was conducted on all the fish, the organs were examined for gross pathological changes. The gills were removed in their entirety and two gill sets (left and right) were preserved separately in 10% buffered neutral formalin for monogenean quantification, identification and histopathology.

3.3.3. Microscopic examination

One set of four gill arches from the left side of each fish was allocated for a quantitative microscopic assessment of parasites on the gill tissue. The gills were examined with an Olympus SZ61 dissection microscope with 9X zoom functionality to estimate the monogenean intensity.

The other set of four gill arches from the right side of each fish was submitted to Amanzi Biosecurity (Private Bag X15, Suite 190, Hermanus 7200, South Africa) where the tissue was processed and slides prepared for histological examination according to standard methods as described by Hayat (1993).

Histology slides were examined with an Olympus CX 41 compound light microscope. All areas of the gill tissue were examined. As a reference, normal gill

tissue was identified according to histology described by Ostrander (2000). Ferguson (2006) and Genten *et al.* (2009) These areas of normal gill tissue were used as reference tissue considering the anatomical structure and histological appearance.

The immediate tissue surrounding the parasites was examined and compared with the reference tissue to determine the extent to which the parasite affected the surrounding tissue with accompanied histopathological changes. This area was identified and discussed as the feeding zone (Dezfuli *et al.* 2007). Secondly, the areas where the parasites had attached themselves to the gill tissue epithelium were also assessed. These attachment sites of the parasites were examined for pathological changes and will be discussed as the attachment site of the parasite (Kearn 1968).

3.4. Results

3.4.1. Macroscopic examination

All the fish were examined for external and internal macroscopic changes. They were all in apparent good health, showing no discoloration or macroscopic lesions.

3.4.2. Microscopic examination of gill tissue for parasites

The quantitative microscopic examination was conducted on the parasites on four gill arches from the left side of all fourteen fish. The infection intensity on

individual fish varied between 41 to 416 parasites per set, with a total of 2977 parasites for all fourteen fish and a mean infection intensity according to Margolis (1982) stating, mean intensity = mean number of *D. oliveri* per infected fish sample. Mean intensity= 213 ± 105.08 (SD), range 41 - 416.

3.4.3. Microscopic examination and description of normal gill filaments and secondary lamellae histology as reference material

Areas of normal gill tissue were examined and used as a reference in comparison to the affected areas. A section through the gill arch, perpendicular to the respiratory lamellae and parallel to the axis of the filament was observed. The long gill filaments (f) (Figure 3.1.A) or primary lamellae are covered with thin disc or plate-like secondary lamellae (l) (Figure 3.1.A), extending perpendicular to the primary lamella. The secondary lamellae are covered with a single layer of squamous epithelium (se) (Figures 3.1.B & 3.2.B) on the outside bordering the water column (wc) (Figures 3.1.B & 3.2.A-B) or tissue-water interface. The basal lamina (bl) (Figures 3.1.B & 3.2.A-B) separates and envelopes the inner planar sheet (ips) (Figure 3.1.B) of pillar cells (pc) (Figures 3.1.B & 3.2.A-B). The vascular spaces (vs) (Figures 3.1.B & 3.2.A-B) or lacunae are formed by cytoplasmic extensions of the adjacent pillar cells (Olson 1991). Circulating erythrocytes (er) (Figures 3.1.B & 3.2.A-B) are visible in most of the vascular spaces. The proximal part of the lamella is imbedded in the axial filament and touches the basal lamina of the filament. The interlamellar area (ia) (Figures 3.1.B & 3.2.A-B) stretches from the interlamellar filament epithelium (ife) (Figures

3.1.B & 3.2.A-B) to the basal lamina of the filament and between two adjacent lamellae. In this area the chloride cells (cc) (Figures 3.1.B & 3.2.A-B) (usually two) that are partially covered by the interlamellar epithelium open via an apical pit. This area appears to be three to four cell layers thick. Other cells in this area are mucous cells, interstitial cells and undifferentiated cells. A portion of the extensive interlamellar vasculature (iv) (Figures 3.1.B & 3.2.A-B) or central venous sinusoid is present with its endothelial wall.

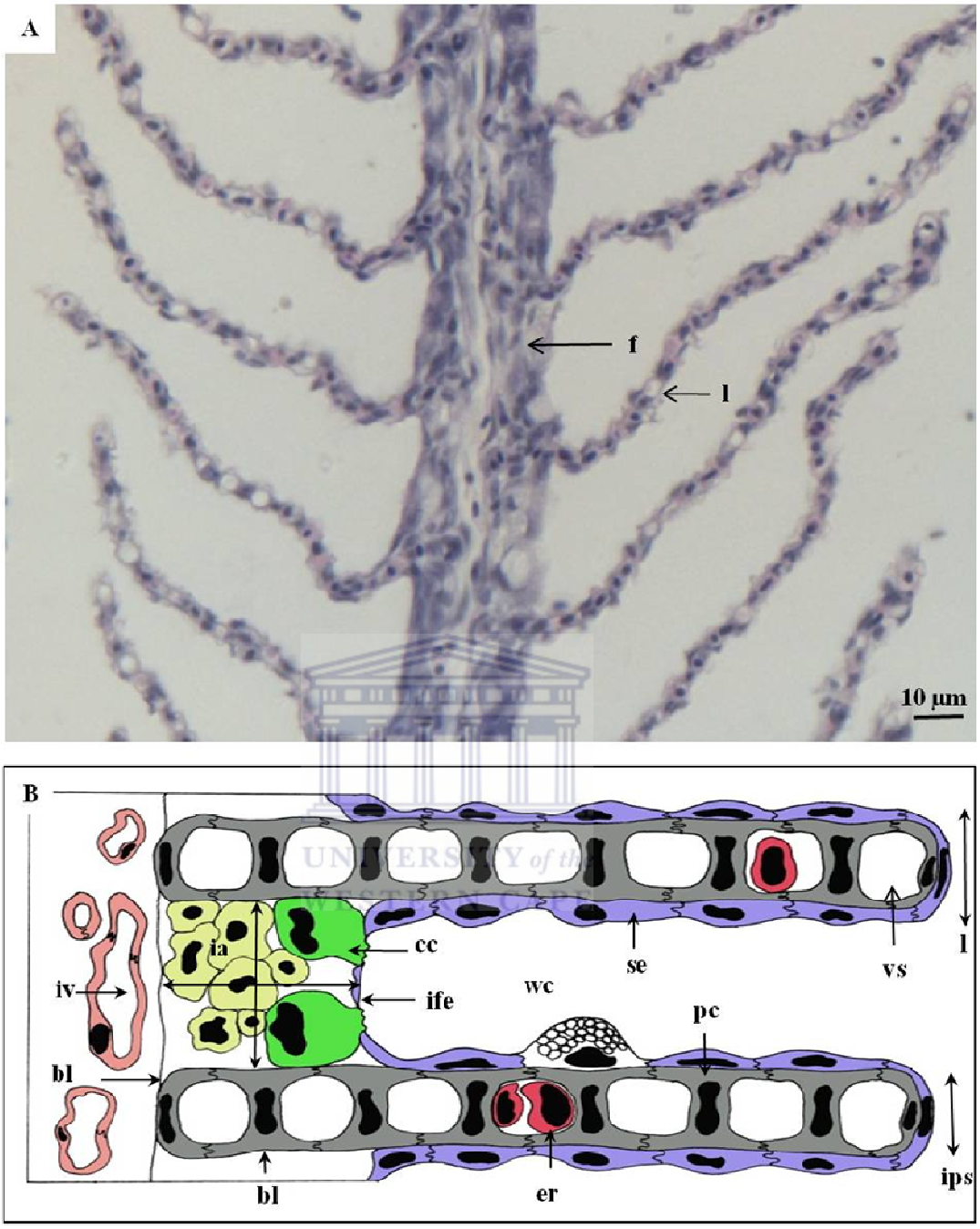

Figure 3.1: The histology of normal gill tissue of *Argyrosomus japonicus*: **A.** Filament or primary lamella with secondary lamellae. **B.** Diagram of one half of filament with lamellae, redrawn from Olson (1991). **Abbreviations:** bl – basal lamina; cc – chloride cell; f – filament; ia – interlamellar area; ife – interlamellar filament epithelium; ips - inner planar sheet; iv – interlamellar vasculature; l – lamella; pc – pillar cell; rbc(er) – red blood cell; se – squamous epithelium; vs – vascular space; wc – water column.

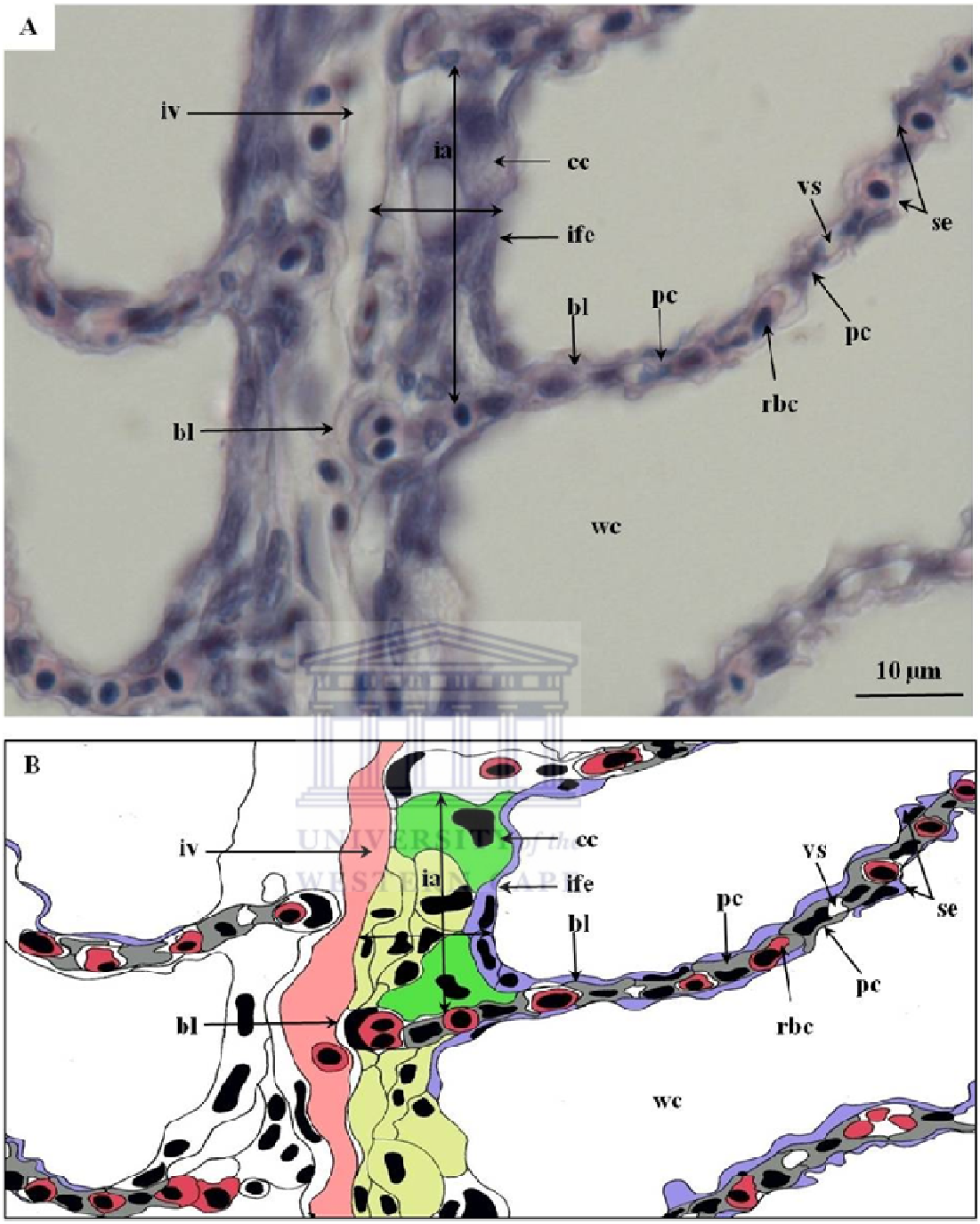

Figure 3.2: Histology of normal gill tissue continues: **A.** Higher magnification of filament with secondary lamellae; **B.** Drawing of specimen **A. Abbreviations:** bl – basal lamina; cc – chloride cell; ia – interlamellar area; ife – interlamellar filament epithelium; iv – interlamellar vasculature; pc – pillar cell; rbc – red blood cell; se – squamous epithelium; vs – vascular space; wc – water column.

3.4.4. Feeding zone

The gill filament adjacent to a diplectanid displays a complete loss of secondary lamellae of approximately the same distance as the length of the parasite with associated further severely affected transition areas (ta) (Figure 3.3.A) on both sides of this feeding zone (fz) (Figure 3.3.B). There is an entire absence of lamellae in the area of intensive feeding (if) (Figures 3.3.B & 3.4) with the loss of proximal and distal parts of the lamellae, interlamellar filament epithelium and chloride cells. Hyperplasia, as a dense layer of immature regenerative hyperchromic cells (irc) (Figures 3.3 & 3.4), ranging between five to ten layers thick are present in the now altered interlamellar area, stretching from the basal lamina.

The areas adjacent to this intensive feeding area still have rudimentary free lamellae (rl) (Figure 3.5.A) and the proximal embedded parts of some lamellae are still present. Both the lamellae and interlamellar filament spaces are covered and filled by a layer of immature regenerative cells (Figure 3.5.A).

The vessel of the interlamellar vascular system (iv) proximal and distal to the intensive feeding area show mild changes resulting in some dilatation with congestion or pooling (po) of blood (Figure 3.3.B). Mild oedema (o) is present between these layers (Figure 3.5.A). The length of parasite (pl) reaches over up to seven lamellae (Figure3.5.B,C).

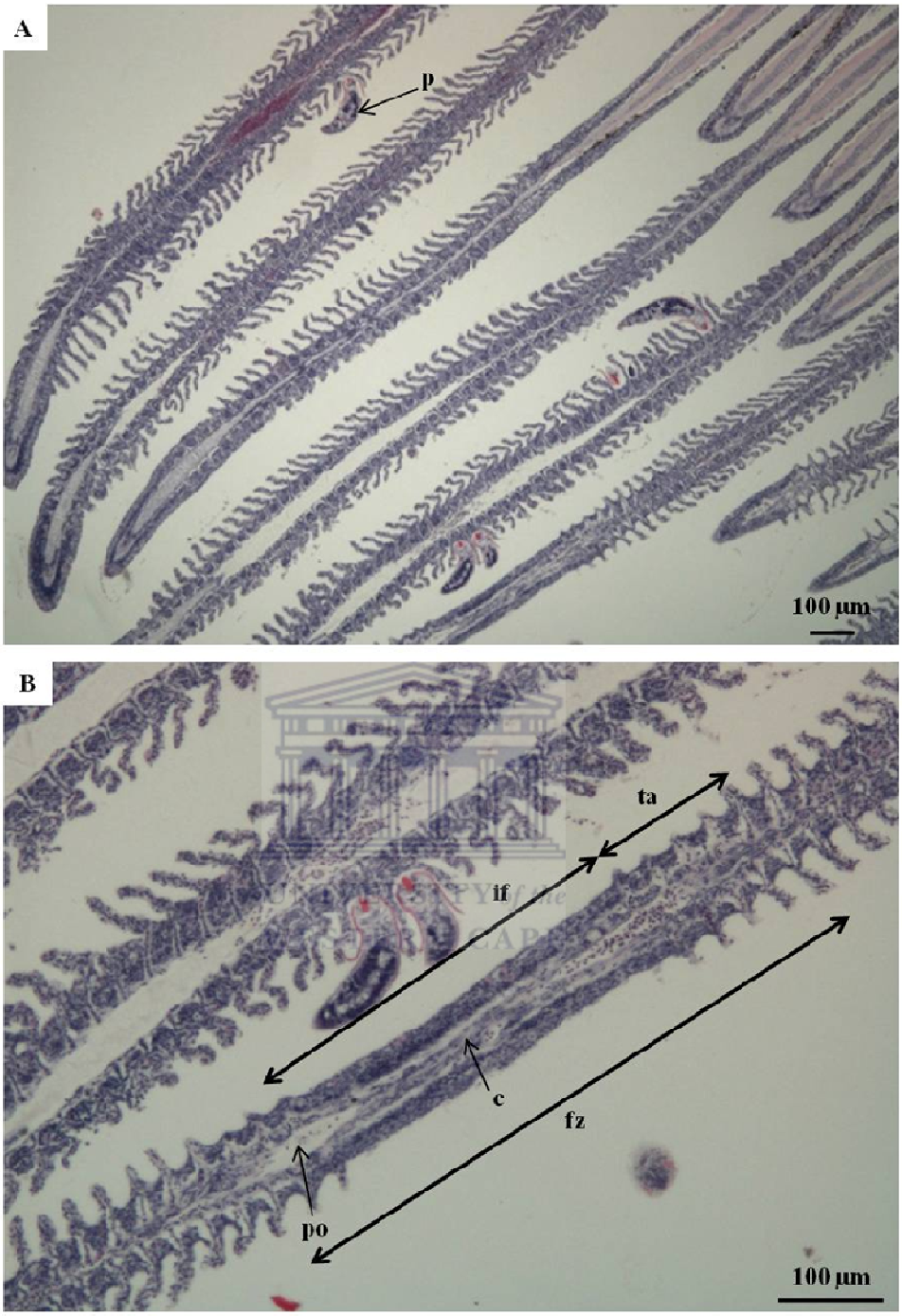

Figure 3.3: Attached *Diplectanum oliveri* on the gill tissue of *Argyrosomus japonicus* and pathological changes: **A.** *D. oliveri* attached to gill filaments; **B.** The total feeding zone consisting of an intense feeding zone and a transitional area illustrating cellular changes in the feeding zone. **Abbreviations:** c – constriction; fz – feeding zone; if – intense feeding zone; p – parasite; po– pooling; ta – transition area.

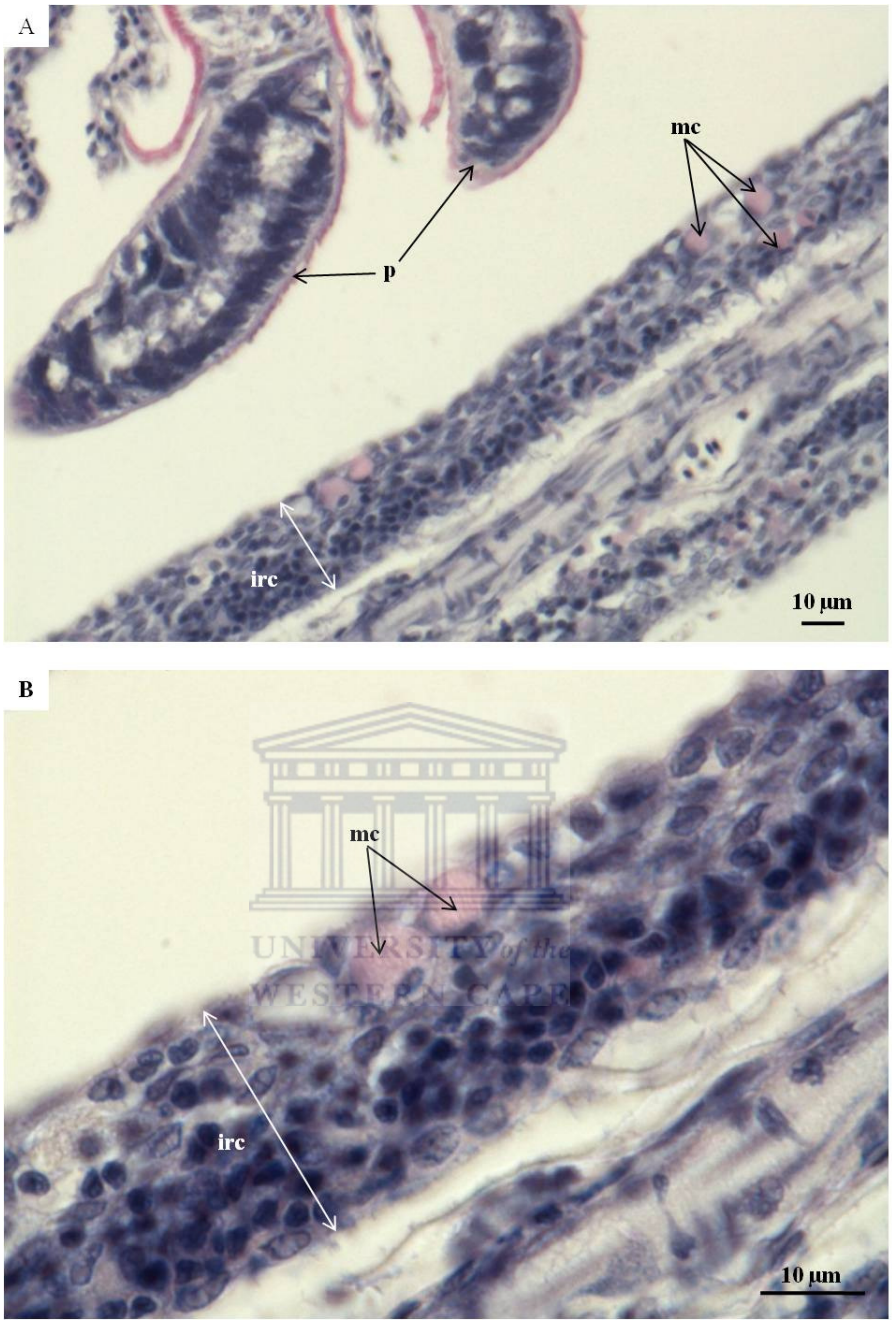

Figure 3.4: Pathological changes in the feeding zone at higher magnification on the gill tissue of *Argyrosomus japonicus* infected with *Diplectanum oliveri*: **A, B.** Cellular changes in the intense feeding zone. The area presents with the complete loss of secondary lamellae. Hyperplasia is seen with the presence of immature regenerative cell. **Abbreviations:** irc – immature regenerative cell; mc – mucous cells; p – parasite.

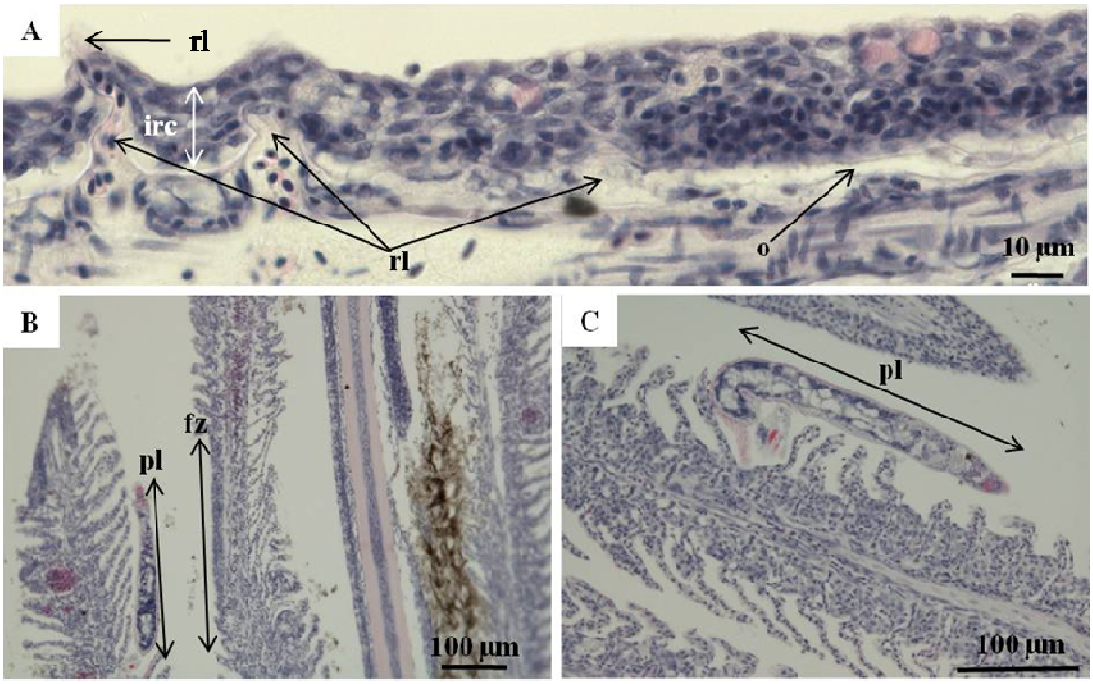

Figure 3.5: The pathological changes in the transitional area of the feeding zone in relationship to the length of *Diplectanum oliveri*: **A.** Magnification of the transition area of the feeding zone; **B.** The length of the parasite compared to its feeding zone on the adjacent filament and the number of lamellae it spans from attachment site on the attached filament; **C.** The parasite length compared to the interlamellar spaces. **Abbreviations:** fz – feeding zone; irc – immature regenerative cells; o – oedema; pl – parasite length; rl – rudimentary lamellae.

3.4.5. Attachment site of parasite

Diplectanum oliveri attaches and secures itself with the haptor or posterior attachment organ containing the posterior dorsal (dd) and ventral (vd) (Figure 3.6.A) squamous discs, hamuli and hooklets (redundant in the adult). The attachment (a) takes place in the interlamellar space on the interlamellar filament epithelium. The parasite is in contact with the two adjacent lamellae laterally using the dorsal and ventral squamous disc respectively and on the interlamellar filament epithelium proximally (Figure 3.6.B). The squamous discs and body surface are covered with scales (s) facing anterior (Figure 3.6.A). Attachment

appears non-invasive (Figure 3.6.A). A mild cellular response is provoked by the attachment of the parasite on the host. The intermediate lamellae (il) show some hyperplasia (Figure 3.6.A) without any large scale inflammatory response, yet the adjacent lamellae (al) are shorter in length. Mild oedema (o) is present in the region of the sinus space between the squamous cell epithelium and basal lamina of lamellae (Figure 3.6.C). Consecutive lamellae show more severe hyperplasia and loss of functional tissue.

Interstitial oedema is also present between the interlamellar filament epithelium and basal lamina of filament axis. The hamuli of *D. oliveri* do not penetrate to the level of the axial basal lamina. While Dezfuli *et al.* (2007) observed that the hamuli of *D. aequans* penetrated deep into the interlamellar filament epithelial tissue, causing more severe pathology.

In the immediate area of attachment the extensive interlamellar vasculature shows a mild degree of constriction (c). Blood congestion or pooling (p) is also presented proximal and distal to the constriction area (Figure 3.7.A). Blood pooling may lead to clotting (cl) intra vascular in the area of attachment (Figure 3.7.B).

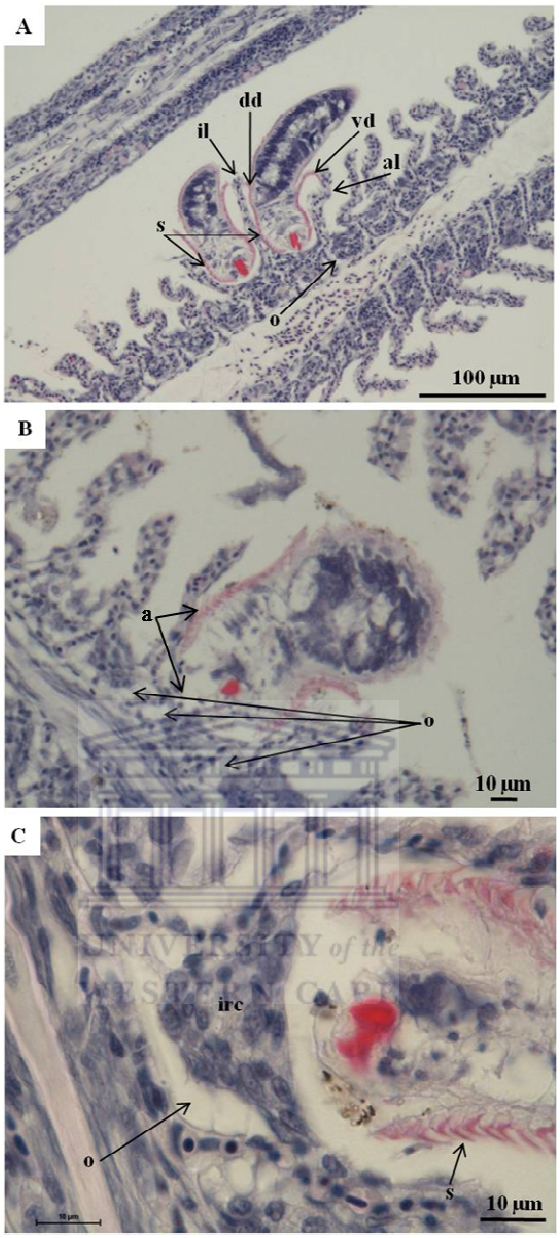

Figure 3.6: The attachment of *Diplectanum oliveri* in the interlamellar gill tissue of *Argyrosomus japonicus* with associated pathology: **A.** Attachment of two parasites in adjacent interlamellar spaces, making close contact with both adjacent lamellae and intermediate lamella with dorsal and ventral squamous disc; **B.** Magnification of contact and attachment of parasite with lamellae and interlamellar epithelium; **C.** Mild hyperplasia between epithelium and basal membrane of filament. Interstitial oedema at attachment site, **A, B. Abbreviations:** a – attachment; al – adjacent lamella; dd – dorsal squamous disc; il – intermediate lamella; irc – immature regenerative cells; o – oedema; s – scales on the surface of parasite; vd – ventral squamous disc.

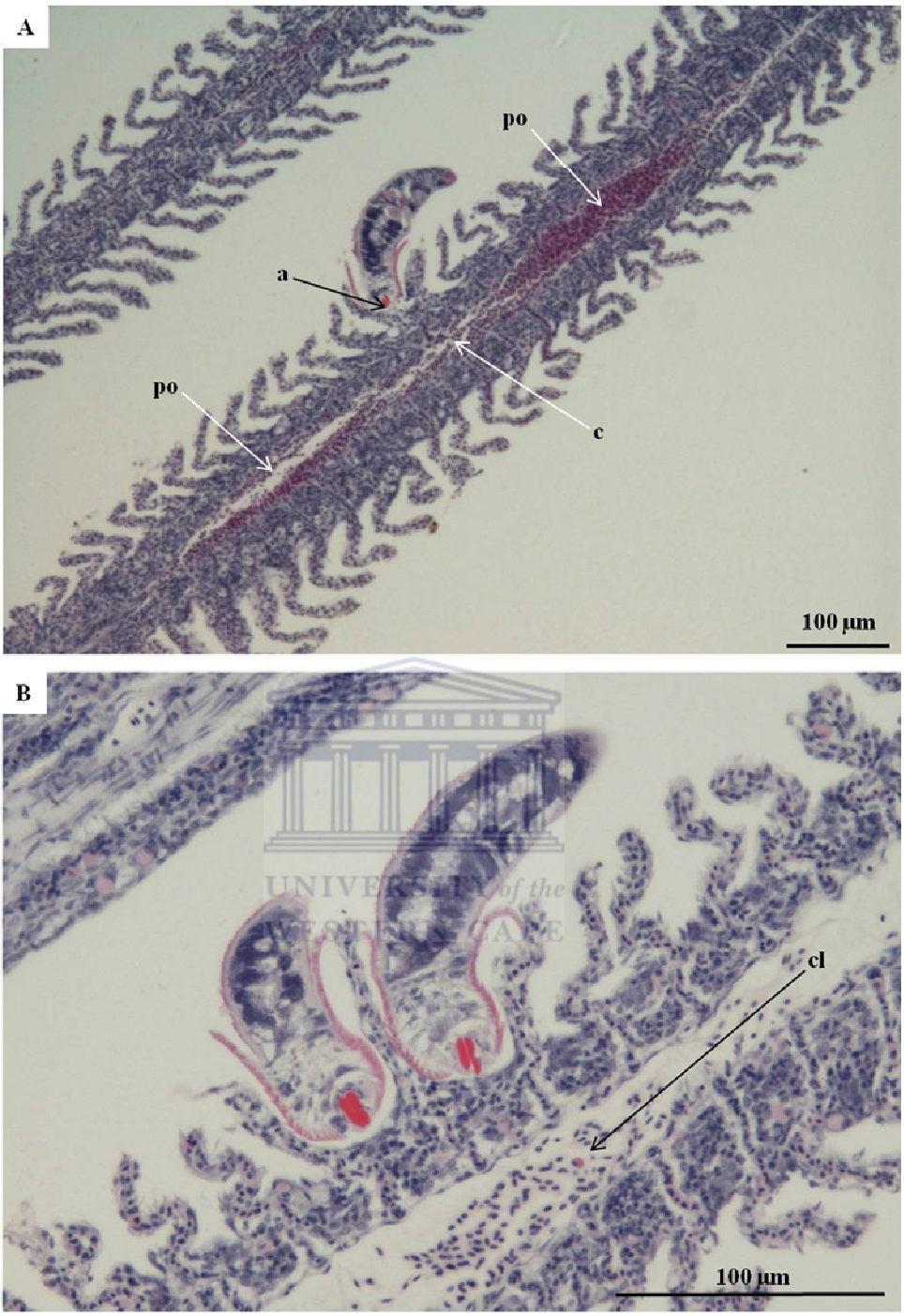

Figure 3.7: Circulatory changes at attachment site: **A.** Constriction of the interlamellar vasculature system, with blood pooling proximal and distal to constriction; **B.** Mild blood clotting in the interlamellar vasculature system. **Abbreviations:** a – attachment; c – constriction; cl – blood clot; po – pooling of blood.

3.5. Discussion

Diplectanum oliveri is a parasite that is becoming increasingly prevalent in the developing marine finfish aquaculture industry of South Africa. It has been confirmed on dusky kob (*A. japonicus*) and silver kob (*A. inodorus*).

There are possibly two ways in which the oncomiracidium can reach the gill tissue. Either, once attached to the body surface, the developing immature parasite migrates to the gill tissue where it completes its development or by directly attaching to the gill arches via the water column passing through the mouth and gills during respiration (Rohde 2005). In the immature stage, the hamuli are underdeveloped and the anterior and posterior attachment organs, as well as the marginal hooks are used to secure the parasite to the host (Kearn 1968). When the parasite reaches its position in the interlamellar spaces, the marginal hooks become redundant and the attachment function is taken over by the fully developed, scale covered dorsal and ventral posterior squamous discs and the hamuli (Paling 1966). The attachment organ is therefore in contact with the interlamellar epithelium and two adjacent lamellae laterally (Dezfuli *et al.* 2007). Effects of the parasite on the host are observed mainly in the area where feeding takes place and referred to as the feeding zone.

The feeding zone is observed primarily directly opposite the parasite on the adjacent filament and to a lesser extent on the secondary lamellae surrounding the attachment area. The feeding zone is divided into two zones. The intense feeding zone and on either side a transition area (Figure 3.3) which extends from where the intense feeding zone terminates to where normal secondary lamellae are found

again. Destruction of tissue takes place on the adjacent filament directly opposite the parasite (Figure 3.3.B) and in the adjacent areas to the parasite on the same filament. It is possible for the parasite to reach and feed on the invaded lamellae as well as adjacent hemibranchs.

In the intense feeding zone there is a complete loss of lamellae approximately the length of the parasite on the adjacent filament. This includes the proximal imbedded and free distal part of lamellae (Figure 3.5.A). In figure 3.3.B, there is a complete loss of approximately fourteen lamellae on the adjacent filament. This differs from what Dezfuli *et al.* (2007) observed for *D. aequans*, where the secondary lamellae are still present in the vicinity of the parasite but extensive lamellar fusion occurs.

It seems possible that a single parasite can reach and feed on approximately 7 secondary lamellae on either side on the adjacent filament, which can lead to the destruction of 14 lamellae. Thus, from the parasite load totalling 5954 for the 14 fish, it can be calculated that an average estimate of up to 5.95×10^3 lamellae been destroyed per fish.

The feeding zone that consisted of functional respiratory lamellae and an interlamellar area of up to five cell layers thick, with its specialized cell like interlamellar filament epithelium, chloride cells, undifferentiated cells, immature cells and mucous cells have been altered and replaced by a dense layer of immature regenerative hyperchromic cells with mucous cells. This hyperplastic layer is up to ten cell layers thick stretching from the basal lamina in some places (Figure 3.4). The loss of the specialized cells is likely to have an effect on the homeostasis of the fish. The parasite's feeding activity and the destruction of the

lamellae can lead to micro haemorrhaging and provides a significant area that can become secondarily infected.

The areas next to this intensive feeding area still have rudimentary free lamellae and the proximal embedded parts of some lamellae are still present. In the transitional area the hyperplastic layer (irc) continues with a decrease in layer thickness. Rudimentary lamellae of different lengths are covered by the hyperplastic layer which also fills the interlamellar spaces (Figure 3.5.A).

It also appears that there is a degree of constriction in the diameter of the filament, with a narrowing of the interlamellar vascular system which can result in altered circulation. Dilatation of the interlamellar vasculature system is visible proximal and distal to the constriction (Figure 3.3.B).

When *D. oliveri* attaches and secures itself by means of its posterior attachment organ (haptor) containing the posterior dorsal and ventral squamous discs and hamuli (Figure 3.6) in the interlamellar area, it can induce inflammation and an immune response (Wedemeyer 1996). Inflammation by definition is characterized by swelling (oedema), redness (hyperaemia), pain, heat and loss of function (West 1988). This response can be activated by the trauma caused by the haptor and/or surface antigens of the parasite.

A mild increase of immature regenerative cells is present in the interlamellar area, thus from the interlamellar filament epithelium to the basal lamina (Figure 3.6.C). Mild interstitial oedema (o) is also present between the interlamellar filament epithelium and basal lamina of filament axis, as well as in the sinusoidal area of the lamellae (Figure 3.6.B) which is an indication of a mild inflammatory

response. There is no decrease in the cellular distance from the interlamellar epithelium to the basal lamina of the filament where the parasite attaches. This depth of attachment and pathological changes differed from that of *D. aequans* (Dezfuli *et al.* 2007).

The pathology caused to the gill tissue at the attachment site may be an indication of the intensity and nature of the haptor attachment. As Paling (1966) demonstrated, the dorsal and ventral hamuli, with their associated muscles, are used in a scissor-like action to penetrate and secure the parasite to the gill epithelium. *Diplectanum aequans,* which penetrates deep into the interlamellar filament epithelial tissue, reaching the basal lamina, where this action causes cell detachment, erosions, pressure necrosis and hyperplasia. The intensity of this attachment action might be the reason why *D. aequans* reaches the level of the basal lamina and resulting in tissue damage. The difference between the pathology caused by *D. aequans* and *D. oliveri* at the attachment site, suggests that the attachment action and intensity of *D. oliveri* differ from *D. aequans* to cause less pathology and that the parasite might not burrow itself into the tissue towards the basal lamina using the hamuli as is in the case of *D. aequans* causing a less severe inflammatory response.

Observing the intermediate lamella between the two attached parasites, there are surprisingly few changes, with only a decrease in length of this lamella. The intermediate lamellae (il) show little hyperplasia (Figures 3.6.A & B) or any large scale inflammatory response, yet the adjacent lamellae (al) are shorter in length and thicker, possibly due to the parasite's feeding activity. Consecutive lamellae show more severe hyperplasia and loss of functional tissue. It would be expected

that if contact between the scales of the parasite and the lamella invoked any severe inflammatory reaction it would be evident in the adjacent and intermediate lamellae which are in direct contact with the body surface of the parasite. Due to only a mild inflammatory response, it appears to be either a very loose attachment to the surrounding epithelial tissue or with no surface antigen response (Figure 3.6.A).

In the immediate area of attachment the extensive interlamellar vasculature shows a mild degree of constriction (c). Blood pooling (po) is also present proximal and distal to the constriction area (Figure 3.7). Furthermore, there appears to be some clotting (cl) taking place intravascular in the area of attachment (Fig. 3.7). These circulatory changes can result in a decrease in blood flow, which could affect the gaseous exchange and ion regulatory function of the gills.

Thus, although there are mild to moderate histopathological changes at the attachment site, the impact of *D. oliveri* is observed in pathology due to the parasite's feeding and grazing behaviour on the secondary lamellae that can result in altered homeostasis, loss in production and stock losses.

In terms of parasite intensity, the parasite counts were relatively low compared to what has been observed in some routine post mortem gill examinations on fish from some aquaculture farms. In recirculation aquaculture systems reinfection of the host by the parasite occurs. This mode of action was utilised during the initial preparation phase of the experiment to establish an adequate parasite load on the experimental fish population. Reinfection can therefore lead to a rapid increase of parasites on the host with devastating consequences.

Furthermore, on macroscopic examination no gross tissue changes were observed, yet there was severe microscopic pathology present. Despite the pathological changes that would impact respiration and osmoregulation, there is also the predisposition of the host to secondary bacterial and other pathogens. If water quality deteriorates fish will be further compromised.

3.6. Conclusion

The data presented here suggests that *D. oliveri* causes significant tissue destruction of the secondary lamellae due to feeding behaviour, with much less impact at the attachment site. This leads to the loss of specialized functional gill tissue and inflammation. The loss of the functional tissue affects the acid-base balance, calcium metabolism, osmoregulation, respiratory surface area and epidermal integrity which can lead to secondary infection (Ostrander 2000). Inflammation is followed by epithelial hyperplasia and fusion of parasitized lamellae which results in "ventilation-perfusion mismatch" (Wedemeyer 1996). The replacement of specialized cells with immature regenerative cells leads to loss of specialized functional cells and vascular abnormalities (Ferguson 2006). Inflammatory oedema causes alteration in the distance between the lamellar interface and blood circulation within pillar channels affecting the critical "diffusion-distance". Although *D. oliveri* is not a sanguinivorous parasite, the destruction it causes to the secondary lamellae due to feeding can result in micro haemorrhage and chronic blood loss. The pathology caused by *D. oliveri* provided evidence for concern.

Therefore, in the developing young aquaculture industry of South Africa, with the ever increasing importance of the monogeneans as parasites causing pathology that can ultimately lead to stock losses, it is of importance to investigate the possibility to develop a non-invasive/non-destructive method to determine not only the qualitative nature of the parasite infection, but also to attempt a quantitative assessment of parasite intensity on the fish. This tool can then be used in various ways as an indicator for early diagnosis of parasite infection, repeated ongoing monitoring of the infection intensity of the parasite on the host and for assessing treatment success of drugs on parasites.

CHAPTER 4: An evaluation of the egg production of *Diplectanum oliveri* (Monogenea: Diplectanidae), a gill parasite of dusky kob (*Argyrosomus japonicus*), as a non-invasive/non-destructive measure to determine parasite intensity.

4.1. Abstract

An innovative approach to quantify monogenean infections on fish, by counting the eggs produced by infra-populations of these parasites over a 24-hour period, may be used as a reliable non-invasive/non-destructive method to quantify the intensity of an individual infra-population of parasites on a single host. The egg production of *Diplectanum oliveri,* a gill parasite of dusky kob *Argyrosomus japonicus* was investigated *in situ* with five infected fish under a controlled lighting schedule of twelve hour darkness and twelve hour light cycle for a period of 48 hours. The comparison of interval data suggested that egg counts from sediment can be used as a repeatable representative measure for both quantitative and qualitative estimations of parasite infra-population density or infection intensity.

Keywords: Monogenea; Diplectanidae; *Diplectanum oliveri*; *Argyrosomus japonicus*; dusky kob; egg production; egg-laying rhythm; parasite intensity; infra-population; non-invasive technique.

4.2. Introduction

Monogenean parasites have been recognised globally as important pathogens in the finfish aquaculture industry (Ogawa 2002). Important pathology caused by these parasites on their respective hosts, includes ulcerative skin lesions (Ernst *et al.* 2005), anaemia (Tubbs *et al.* 2005), severe hyperplasia, fusion and necrosis of the gills (Dezfuli *et al.* 2007 & see Chapter 3). In light of this, the development of reliable criteria for the diagnosis of monogenean species as well as reliable quantitative methods that estimate the intensity of these parasites on their hosts remains important.

In terrestrial production animals, veterinary laboratories are commonly requested to diagnose endoparasitic helminthiasis and monitor their prevalence and intensity in the herd. This monitoring is based on the collection of faecal samples from the host containing eggs of the intestinal parasites, which are then processed in various ways to separate and extract the maximum possible number of eggs from the sample. These data are used to estimate parasite intensity (Ward *et al.* 1997). Coprological examination to determine verminosis is also a standard and routine procedure for veterinarians treating domestic animals like birds, cats and dogs.

Faecal flotation techniques are most frequently employed for this purpose and predominantly utilise the specific gravity of the parasite egg, to separate the eggs from the faecal matter by mixing solutions of varying densities. If the specific gravity of a flotation solution is greater than the specific gravity of the egg, the egg will float. Most helminth eggs and protozoan oocysts will float in a solution with a specific gravity (SG) of between 1.05 and 1.23 (Dryden *et al.* 2005).

Beugnet *et al.* (2008) suggested a magnesium sulphate solution (SG 1.28). For heavier eggs like those of *Fasciola* spp. (Linnaeus, 1758) and *Paramphistomum leydeni* Näsmark, 1937, the authors suggested a hypertonic solution of potassium iodomercurate (SG 1.44), but this solution can alter the morphology of the egg, and thus their specific gravity.

These diagnostic methods are frequently being refined to increase specificity and accuracy of an estimate of the actual parasite infra-population size in the individual host. Such research includes optimising suspension and flotation solutions for a wide spectrum of parasite eggs (Cringoli *et al.* 2004), improved techniques for parasite egg recovery (Dryden *et al.* 2005), methods for counting eggs (Ward *et al.* 1997) and determining required sample sizes (Mes 2003).

The objective of this refinement is to produce a technique that is simple, cheap, accurate, easy to use and versatile so that it can be used in laboratories and at production facilities. Practitioners often prefer commercial kits due to their simplicity and cost effectiveness, although they may not be as accurate as techniques that incorporate more sophisticated laboratory equipment (Dryden *et al.* 2005). Accurate egg detection techniques not only have qualitative importance but aim to be useful as quantitative measures to determine, for example, parasite loads that would qualify the use of anthelmintic preparations (Ward *et al.* 1997).

According to Thienpont *et al.* (1979), the following points must be considered when estimating infra-population size from faecal samples. The number of eggs only relates to the mature egg-laying parasites and do not reflect the numbers of non-egg producing males and immature juveniles in the sample. Large variation exists between the numbers of eggs produced by different parasite species.

Ascarids produce large numbers of eggs compared to *Fasciola* spp., which only produce few eggs in the same time interval. The eggs are only detected in the samples once the prepatent period has elapsed from the primary infection, where newly infective larvae have developed into mature egg producers and proceeded into the patent production period. The immune response of the host can cause inhibition of the egg production of the parasite, which can lead to an underestimation of parasites. The egg production of worms is often not continuous throughout the year but cyclic with variation between seasons and may also have variation within a 24-hour day/night period in the case of a diurnal egg-laying rhythm.

In addition to these, a faecal sample may also contain a variety of eggs from representatives of a diversity of platyhelminth, nematode and acanthocephalan classes. Other elements like larval stages, part of/or entire worms, protozoa, fungi and pseudoparasites or artefacts may also be present in the sample, which will complicate identification and interpretation of the results (Thienpont *et al.* 1979). Provided all of the above factors are considered in the interpretation of the data collected, the evaluation of faecal samples for parasite eggs still remains a valuable and important tool. Considering the terrestrial model, where the development of the techniques are influenced by the characteristics of the faeces of the host and the parasite eggs. The animal faeces are used as the basis for these techniques, whether collected directly as rectal samples or indirectly as excreted faeces. This is a fairly simple process of collection due to the relative ease of access to samples.

Endoparasite egg morphology generally facilitates the relatively uncompromised passage through the gastro-intestinal tract into the environment where the larvae hatch. The egg surface is generally smooth and does not possess any protrusions or structures that may compromise its delivery to the external environment. This characteristic is an advantage which is utilized when preparing the sample for the isolation of the eggs from the faeces by means of flotation.

The terrestrial model was also developed as an alternative to depart from an invasive/destructive method of diagnosing helminthiasis on postmortem examination towards non-invasive/non-destructive technique. Although the technique is not perfect it has become an important tool to estimate parasite intensity and to improve animal health and survival rate.

The diagnosis of helminthiasis in aquatic animals often relies on invasive or destructive methods, where fish are euthanased and the diagnosis of parasites is based on postmortal examination of skin and gill scrapings and both macroscopic and microscopic examination of the internal organs of the host. This allows for qualification and quantification of the infra-population of parasites that may be present but the disadvantages of this method are that the host animal cannot be sampled repeatedly. Hence this method cannot be applied to brood stock animals as these animals are often very valuable and are not kept at high enough densities in tanks to allow for representative sampling.

Another invasive technique currently used, is the harvesting of gill tissue or "gill clippings" from the live host to diagnose parasites as a repeatable representative method. This may not be a reliable method and has the following disadvantages. This sample size or biopsy of the gill tissue is often very small to

minimise trauma to host. With a small biopsy, low parasite intensity and an uneven distribution of parasites, the diagnosis is based on a “hit and miss” result. The humane application for the technique would entail anaesthesia, which implies more handling, chemical exposure and possible fish mortalities. Also, the parasites may be affected. Biopsy size, parasite preference to different parts of the gill and variation in collecting sites of the gills, places the reliability of this technique in question.

Thus, the development of an accurate non-invasive / non-destructive technique to diagnose and quantify helminthiasis in aquatic animals is important. The advantages include the limited handling of the animals where representative samples are taken from the environment which in turn lowers the chance of injury and stress in the fish. This is an important aspect when considering the need for sampling from valuable brood stock animals. In its application these techniques utilize the same aquatic production animals in a repeated manner to obtain representative samples. These sample assessments can be useful in monitoring the efficacy of an anthelmintic by comparing the pre- and post-treatment of individuals or groups repeatedly. Routine sampling is a valuable tool for the continuous monitoring of the parasite status, seasonal variation and epidemiology (Bouloux *et al.* 1998).

The sensitivity of the techniques can be influenced by many factors. The importance lies in that the examination is conducted with the most applicable methods for the parasites to be studied and performed under repeatable conditions to ensure acceptable results (Thienpont *et al.* 1979). Detailed knowledge of the

parasites and their egg production will dictate the specification and development of techniques to quantify intensity (Beugnet *et al.* 2008).

A potential of this innovative approach to quantifying monogenean infections on fish, by counting the eggs produced by infra-populations of these parasites over a 24-hour period, may be a reliable method to quantify the intensity of an individual infra-population of parasites on a single host.

The direct life cycles of these parasites can lead to a rapid increase of the population within high-density aquaculture systems (Ogawa 2002). Monogenean eggs are released into the environment and through gravitation they can contaminate the bottom substrate or become entangled in structures and cage nets. This contamination of the environment increases the chance for re-infection of the host. Contamination of the environment with the eggs varies due to factors like, rate of production (Tubbs *et al.* 2005) and species specific egg morphology (Roubal 1994). The contaminated sediment will be evaluated as the source of eggs to determine whether it can be used as a reliable method to establish a correlation to the parasite intensity on the host, similar to the faecal evaluation methods applied in the terrestrial model.

A marked variability has been observed in the egg production rates among monogeneans, as was noted with terrestrial helminths. Tinsley (2004) stated a minimum egg production rate of 2 eggs per worm per day (e/w/d) at a temperature of 13°C for *Acanthocotyle greeni* Macdonald & Llewellyn, 1980 (Macdonald & Llewellyn 1980) whereas a maximum of 2880 e/w/d at 20°C was recorded for the polystomatid, *Polystoma nearcticum* Paul, 1938 which is only sustainable for a few hours coinciding with the reproductive cycle of its host. *Heterobothrium*

okamotoi Wang *et al,* 1997 has the ability to accumulate up to 1503 eggs *in utero* over two days (Ogawa 1997), whereas, *Zeuxapta seriolae* (Meserve, 1938) (Monogenea: Heteraxinidae) can accumulate up to 1015 eggs *in utero*, before releasing the eggs into the environment. The release of eggs at a specific time may coincide with the host's behaviour (Mooney *et al.* 2006).

Zeuxapta seriolae released 92.8% of the eggs during dark phase of which 71.6% were released in the 3 hours after darkness ("dusk") starting at 18h00 as if there is a trigger stimulus (Mooney *et al.* 2006). Gannicott & Tinsley (1997) demonstrated the absence of an egg-laying rhythm with the monogeneans *Entobdella soleae* (van Beneden & Hesse, 1864) and *Discocotyle sagittata* (Leuckart, 1842) (Kearn 1985). It is believed that these egg-laying and hatching rhythms exist to enhance the probability of the parasite larvae to find a host by coincide with the territorial behaviour of the host (Kearn 1973, 1986).

Although the focus of this study is on egg production, it is of interest to mention variation in embryonation and egg hatching to complete the life cycle for the next generation of infection. Ernst *et al.* (2005) showed a strong influence of temperature and salinity on the embryonation period of *Benedenia seriolae* Yamaguti, 1934. Cecchini (1994) also found a strong correlation between temperature and egg hatching period with *Diplectanum aequans* (Wagener, 1857) a gill parasite of sea bass, *Dicentrarchus labrax* (Linnaeus, 1758).

Mooney *et al.* (2006) demonstrated that *Zeuxapta seriolae,* an oligonchoinean gill parasite of the yellowtail kingfish, *Seriola lalandi* Valenciennes, 1833, has a predictable egg-laying rhythm. This was only the second study (Mooney *et al.* 2006) that demonstrated an egg-laying rhythm in the Monogenea. Macdonald &

Jones (1978) demonstrated an egg-laying and egg hatching rhythm in *Diplozoon homoion gracile.* Both parasites are representatives of the Oligonchoinea (Mooney *et al.* 2006). This present study was the first time that the egg production of *Diplectanum oliveri* Williams, 1989, a representative of the Polyonchoinea was investigated to determine whether an egg-laying rhythm exists.

Understanding the factors affecting the amount of eggs produced (eggs/worm/hour) and egg production as a function of time (egg-laying rhythm) of different parasites encountered in different aquaculture production systems is important. This knowledge can lead to the development of more accurate and specific sampling techniques that in turn can be implemented in a practical diagnostic non-invasive tool to estimate the prevalence and intensity of parasites in aquaculture production facilities.

This study aims to assess the potential of monogenean egg counts as a non-invasive, non-destructive approach to diagnosing monogenean infections on fish and estimating parasite intensity. Determining the egg production by counting the eggs produced by infra-populations of parasites over a 24-hour period, may be a reliable method to estimate the intensity of an individual infra-population of parasites on a single host. Also important, is to determine whether the parasite produces eggs at a constant rate throughout the day/night cycle over the (two) consecutive days and thus whether an egg-laying rhythm exists? If a rhythm exists, it will also influence the quantity of eggs in the collection at different time intervals and will give an indication of the best time to collect a representative sample with a high certainty to find eggs.

4.3. Materials and Methods

4.3.1. Collection and acclimatisation of fish and parasites

Dusky kob, *Argyrosomus japonicus* (Temminck & Schlegel, 1843) infected with *D. oliveri* were obtained from a private commercial production facility. The fish were temporarily relocated to the Department of Agriculture Forestry and Fisheries (DAFF), Aquaculture Research Facility in Sea Point, Cape Town. At this facility the fish were maintained in a well aerated 10 000 L circular flow-through tank with water temperature at 20°C. A commercial diet was fed daily to the fish at 1% of their body weight. The fish were conditioned over a period of two months to a 12-hour dark and 12-hour light illumination cycle; with the dark phase from 18h00 to 06h00.

4.3.2. Laboratory

Following acclimation to the experimental conditions and confirmation of infection with *D. oliveri*, the fish were transported to Blouberg Veterinary Clinic (BVC), due to practical and logistical constraints, where the experiment was conducted. The conditions at BVC were simulated as close as possible to those at DAFF. Fresh sea water was collected and transported from DAFF to BVC. The water was filtered through a series of 2 x 10 µm fibre wound filters (Figure 4.1.A) and 1 x 1 µm ceramic filter into a 1000 L reservoir tank (Figure 4.1.B). The fish were kept in 25 litre plastic containers. The aim was to keep the ambient and water temperature at an average of 20 ± 1.0°C for the duration of the experiment by means of thermostatic submerged aquarium heaters in the reservoir tank and

wall mounted heaters (Figures 4.1.B & E). Each container was fitted with an air stone connected to a common air supply and covered with netting to prevent the escape of fish (Figure 4.1.D). The laboratory was heated by two wall mounted ceramic heater elements (Econo-Heat 400 W) and ambient air temperature was kept at a constant 20°C (Figure 4.1.E). Even illumination throughout the room was provided by means of Biolux (Osram) fluorescent tubes on a timer switch for the light cycle (Figure 4.1.F).

Figure 4.1: General experimental laboratory components. **A.** Filter canisters containing fibre wound and ceramic filter inserts. **B.** 1000 L reservoir tank. **C.** & **D.** 25-L plastic containers. **E.** Econo-Heat ceramic heater element. **F.** Biolux (Osram) fluorescent lighting.

4.3.3. Experiment

Prior to the start of the experiment, the fish were purged for 48 hours to reduce faecal production during the experiment which could obscure parasite eggs during microscopic examination. The eggs of *D. oliveri* were collected from 5 fish at consecutive 3-hour intervals for 48-hours. The 48-hour period consisted of two 12-hour dark/light cycles, starting with the dark phase at 18h00 and light phase at 06h00 to coincide with the pre-conditioning phase.

Individual fish (n=5) (168 mm mean peduncle length ± 1.16 standard deviation), were kept in 25-L, well aerated containers at 20°C (Figures 4.1.C&D). There was no replacement or filtration of the water in the containers during any of the 3-hour periods to prevent any loss of eggs. Water quality (ammonia concentration, dissolved oxygen, oxygen saturation, pH, salinity, conductivity and temperature) was checked daily using a Palintest Photometer and a YSI 85 handheld digital meter with an attached YSI combination conductivity and dissolved oxygen probe.

Eggs produced by the parasites on individual fish were collected every 3-hours, by decanting the total volume of water through a 25 µm monofilament nylon mesh sieve (Figures 4.2.A&B). The individual filtered sediments from each tank were rinsed into separate 250-mL sample bottles (Figures 4.2.C&D). The samples were preserved in 10% buffered neutral formalin (Figure 4.2.E) prior to being marked and logged to link each sample to its day of sampling and the number of the tank and fish, respectively (Figure 4.2.F). Samples were stored for counting of the parasite eggs.

Figure 4.2: Procedure for the collection and preservation of *Diplectanum oliveri* eggs. A & B Collecting and rinsing of tank sediment. C & D Decanting filtered sediment into 250-mL jars. E & F Preserving and labelling collected sediment samples for further parasite egg counting.

A duplicate set of tanks (n=5) (Figure 4.1.D) was used to facilitate the quick transfer of fish at the end of every 3-hour interval. The clean, empty containers were placed back into the laboratory room after every decanting with their paired containers and filled with water for the next rotation in three hours. Fish were handled wearing latex gloves to prevent trauma to skin and to prevent the accidental removal of eggs.

4.3.4. Parasite egg counts

All the sample bottles containing the parasite eggs were randomly selected for counting and their entire contents was decanted through a custom-made filter (sieve) which consisted of a 30 mm section of a 4 mm walled, 75 mm diameter PVC tubing with a synthetic 25 µm mesh glued onto the rim of one end. To assist with orientation of the sample and parasite egg counting, a disc was cut from a transparency film printed with a grid pattern forming five millimetre squares and placed in the bottom of a petri dish. The sieved sample containing the sediment was placed on top of the grid in the dish and the parasite eggs were counted using an Olympus SZ61 dissection microscope with 9X zoom functionality.

4.3.5. Statistical analysis

Differences in the number of eggs produced from five independent infra-populations of *D. oliveri* between respective 3-hour intervals for two consecutive 24-hour periods were tested by repeated measures ANOVA. Furthermore, repeated measures ANOVA was also used to test differences in egg production

rates between night and day. All pair wise multiple comparison procedures were executed with the Tukey's HSD test. The Levene's test for equality variance was applied to show that variances are equal. This test was used within-subject variance which eliminates the differences between fish.

4.4. Results

Throughout the experiment, the water quality parameters were compared to the preferred water quality limits for the experimental culture of marine fish and were found to be within acceptable limits. The water was maintained at 7.1 $mg.L^{-1}$ ± 0.17 (6.97 – 7.79) dissolved oxygen; 93.2% ± 1.19 (92.0 – 97.1), oxygen saturation; 8.1 ± .035 (8.02 – 8.16) pH; 0.027 $mg.L^{-1}$ ± 0.02 (0.00 – 0.06) total ammonium concentration; 33.6 ‰ ± 0.198 (33.0 – 34.0) salinity and temperature of 20°C ± 0.20 (19.8 – 20.70). Water quality data are given as mean ± standard deviation (minimum - maximum).

The egg production of *D. oliveri* varied considerably between fish. The average parasite egg production per fish per day was 12706 ranging from 1894 – 40446 *D. oliveri* eggs per fish per day. This equates to an average of 1588 *D. oliveri* eggs produced per 3-hour time interval ranging between 237 and 5056 *D. oliveri* eggs produced per fish per 3-hour interval (Figure 4.3). The variation in magnitude of the egg counts reflected the difference in the mean intensity of the parasite infra-population on the respective fish.

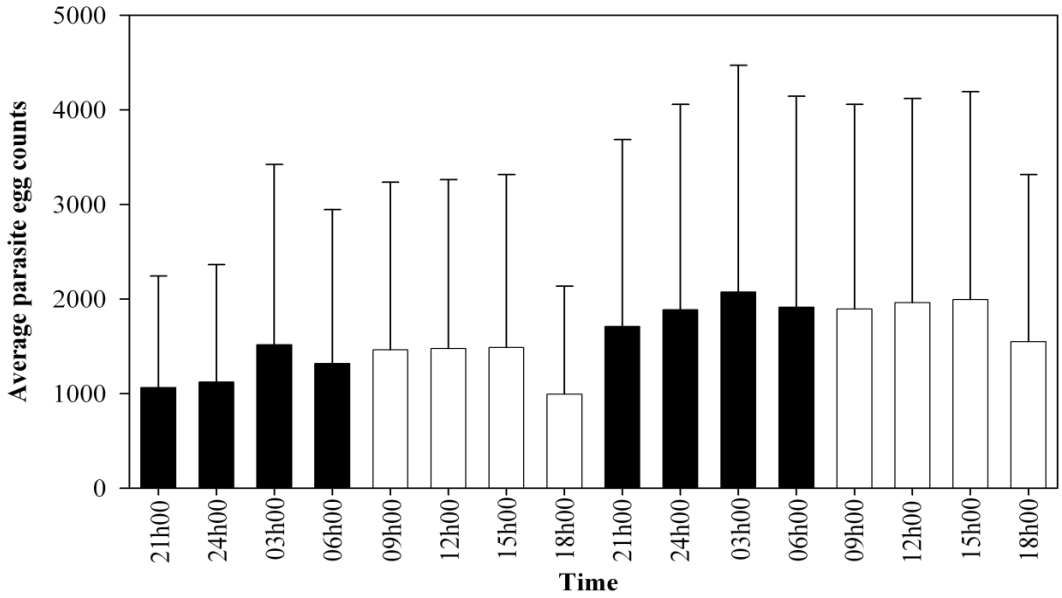

Figure 4.3: Average egg counts of *Diplectanum oliveri* from five fish over 48-hours at 3-hour intervals with a night:day cycle of 12:12 hours starting with the night phase at 18h00. Vertical bars with no fill represent day phase and bars with black fill the night phase. Error bars represent standard deviation.

Table 4.1: Repeated measures analysis of variance for mean egg production of *Diplectanum oliveri* from five fish for the same time interval for day 1 and 2 at a 3-hour interval with a night:day cycle of 12:12 hours starting with the dark phase at 18h00. SS=sum of squares, DF=degrees of freedom, MS=statistical mean, F=F statistic for ANOVA. Bold red = no significant difference between time intervals/phase

	SS	DF	MS	F	P
Phase (Dark:Light)	13494	1	13494	0.00097	0.975576
Days (Day 1:Day 2)	6453048	1	6453048	0.46249	0.506183
Phase*Days	108119	1	108119	0.00775	0.930947
Error	223243637	16	13952727		
Time intervals	**1134419**	**3**	**378140**	**4.39603**	**0.008214**
Time intervals / Phase	**1251553**	**3**	**417184**	**4.84994**	**0.005003**
Time intervals / Days	26850	3	8950	0.10405	0.957309
Time intervals / Phase / Days	52975	3	17658	0.20529	0.892241
Error	4128882	48	86018		

The number parasite eggs produced for each 3-hour interval for day 1 and 2 were compared to determine whether the parasite egg production for the same time interval was constant for the two consecutive days. The mean number of

eggs produced within the same time interval for the two days was consistent and therefore the mean egg production during day two did not differ from that of day one (Figure 4.3, Table 4.1). Consequently, the data for the same 3-hour interval for day 1 and 2 were averaged (Figure 4.4) for the subsequent repeated measures ANOVA analysis to determine whether egg production was constant during the dark and light phases of a 24-hour cycle. Furthermore there was a difference in the mean egg production between the 3-hourly egg collection intervals and there was a significant interaction between the collection intervals and the respective dark:light phases.

The average egg production of *D. oliveri* (Figure 4.4) was used to compare the mean egg production by the parasites on the five fish during the dark and light phases. The comparison indicated a significant difference in egg production between the day and night phase, with 49.1% production during the night phase and 50.9% for the day phase (P=0.000886) (Table 4.2, Figure 4.4).

Table 4.2: Repeated measures analysis of variance for 3-hourly egg production of *Diplectanum oliveri* infra-populations on five fish averaged between the two days.

	SS	DF	MS	F	P
Intercept	**201806222**	**1**	**20180622**	**15.80695**	**0.000886**
Phase Dark:Light	13494	1	13494	0.00106	0.974423
Error	229804803	18	12766934		
Time intervals	**1134419**	**3**	**378140**	**4.85174**	**0.004629**
Time intervals / Phase	**1251553**	**3**	**417184**	**5.35270**	**0.002664**
Error	4208707	54	77939		

Table 4.3: Pairwise multiple comparison (Tukey HSD test) for 3-hourly egg production of *D. oliveri* infra-populations on five fish averaged between the two days following one way repeated measures ANOVA. Values in bold red represent comparisons with significant differences.

	09h00 L1	12h00 L2	15h00 L3	18h00 L4	21h00 D1	24h00 D2	03h00 D3
12h00 L2	0.999						
15h00 L3	0.999	1.000					
18h00 L4	**0.037**	**0.015**	**0.009**				
21h00 D1	0.999	0.999	0.999	1.000			
24h00 D2	0.999	0.999	0.99	0.999	0.979		
03h00 D3	1.000	1.000	1.000	0.997	**0.037**	0.301	
06h00 D4	1.000	1.000	1.000	0.999	0.598	0.986	0.838

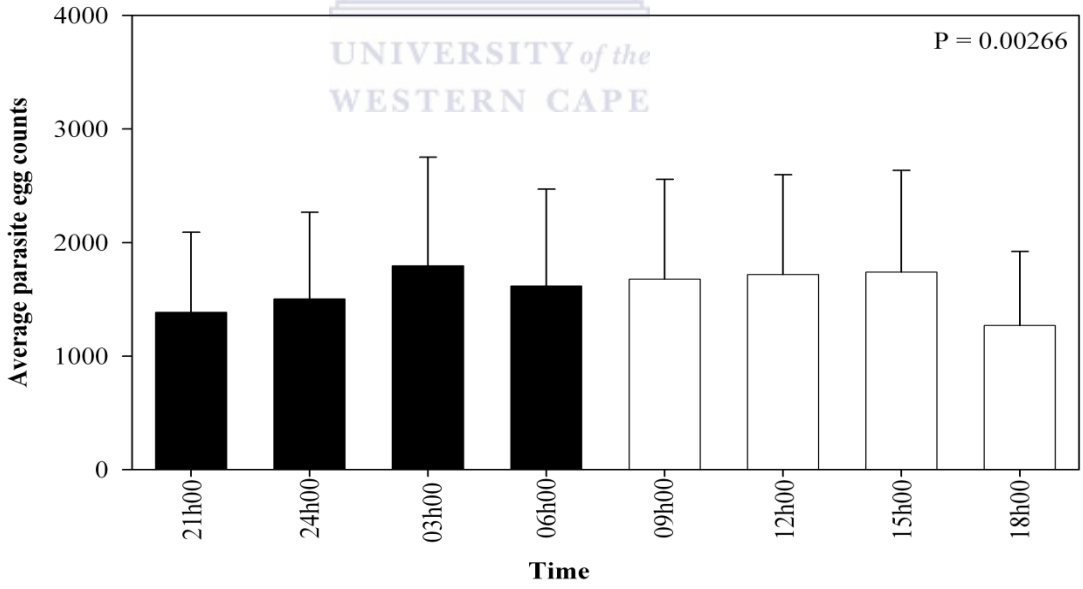

Figure 4.4: Average egg production of the parasites between the five fish for the same time interval for day 1 and 2 of *Diplectanum oliveri* at a 3-hour interval with a day:night cycle of 12:12 hours starting with the dark phase at 18h00. The vertical bars with no fill represent the light phase and bars with black fill the dark phase. Error bars represent standard deviation.

A gradual increase in mean egg production was detected from the start of the dark phase to reach a peak at 03h00 (Figures 4.3 & 4.4). This is followed by a decrease in the 06h00 samples, which in turn is followed by a gradual increase to reach another peak at 15h00. The lowest mean was recorded from the 18h00 samples. Multiple pair wise comparisons were performed between different time intervals over 24 hours to determine if there were any differences in average egg counts between intervals (Table 4.3). Differences were detected according to the analyses, when comparing the maximums at 03h00 with 21h00 of the dark phase and comparing the minimum 18h00 samples with the 09h00, 12h00 and 15h00 samples respectively during the light phase. The p-values of analyses are tabulated for the pairwise comparisons (Figure 4.5 & Table 4.1).

4.5. Discussion

Considering the faecal examination used in terrestrial animals as a basic and routine procedure to determine helminthiasis in production animals, a model for aquaculture application was suggested to provide information about the qualitative and quantitative nature of infection for monogenean parasite infections in aquaculture and specifically for *Diplectanum oliveri* infections on captive *Argyrosomus japonicus* in South Africa.

The observation that parasite eggs gravitated and accumulated in the sediment was utilised by using dusky kob (*A. japonicus*) infected with *D. oliveri* to determine whether a quantitative assessment of eggs in the sediment could be used as an indicator of parasite intensity.

It was determined that the eggs released directly into the water gravitated to the tank bottom. As in the case with faecal samples in terrestrial animals, parasite egg samples could be obtained from the sediment in the holding tanks. This method for obtaining parasite eggs from the tank sediments can therefore be viewed as a direct method to collect both qualitative and quantitative data regarding the parasite burden on the host. *Diplectanum oliveri,* an oviparous monogenean, produces eggs which are not joined together by a filament to form an 'egg-string' that could become entangled around the air stone and tubing. This characteristic simplified sedimentation and collection of eggs which was an advantage of the suggested collection method. *Diplectanum oliveri* eggs are coma-shaped with the tail ending in a structure containing a sticky substance. The function of this structure is not understood, but it is suggested that it could be for adhesion to surrounding structures and also to decrease the sedimentation rate of the eggs to the substrate. This will prolong the period of the egg in suspension which can lead to wider distribution through currents and tides.

The morphology of the monogenean egg is an important factor when considering the methods for sampling and processing techniques of sediment to make this method more versatile with a wider application. Adaptations in egg morphology play an important role to decrease scattering and increase the chance of infection and re-infection (Roubal 1994). These will affect sedimentation characteristics and attachment to structures to decrease egg concentration in the sediment which may influence the interpretation of the sediment sample.

It was also established that eggs could be isolated repeatedly from every water sample decanted through an appropriate mesh filter. The measures that were taken

to limit the waste products in the samples, provided a filtrate that contained very little waste material and eggs could be identified and counted. This technique indicated that counting of eggs recovered from filtrate is a potentially useable method.

Juvenile *D. oliveri* develop into hermaphroditic, egg producing adults. Thus, all adult parasites contributed to the eggs in the sediment. The results illustrated that each infra-population of *D. oliveri* produced eggs into the environment continually throughout the day. The egg release rate measured for consecutive three hour intervals ranged between 10% and 14% of the daily egg collection. However, a statistically supported increase in mean egg production (Figure 4.4 and Table 4.3) was measured staring at 21h00 to 03h00 during the dark phase with a decrease at 06h00. This decrease was also noticed between the 15h00 and 18h00 samples of the light phase. This data suggested that a very weak egg-laying rhythm could exist. Conducting this trial series over more days may give more transparency to the existence of a more pronounced egg-laying rhythm. Mooney *et al.* (2006) and Macdonald & Jones (1978) demonstrated that egg-laying rhythms exist in the two oligonchoinean monogeneans *Z. seriolae* and *D. homoion gracile*. This could be the first record of a member of the monogenean subclass Polyonchoinea possessing an egg-laying rhythm. The results do not depict a pronounced egg-laying rhythm, therefore the specific time for diagnostic sampling is not critical and samples taken at different time intervals can be used to estimate the infra-population intensity of *D. oliveri* on the gills of *A. japonicus*.

Although a difference in average percentage parasite egg production between the night and day phase was detected, the difference is only 1.8% and is not considered to have any biological importance.

The evaluation of parasite eggs collected from the environment as a proxy for parasite infra-population intensity on individual fish, or component population intensity on all fish that are in contact by sharing the same water, has advantages with respect to the handling of fish and the associated negative impacts thereof. As with the terrestrial method, the estimation of parasite egg counts, has numerous applications with regard to aquaculture and captive husbandry of finfish including the ongoing routine sampling as a continuous monitoring process of the parasite status to determine seasonal variation and epidemiology, to decide on the timing of anthelmintic treatment and to monitor the efficacy of an anthelmintic by comparing the pre- and post-treatment groups. Furthermore, understanding the parasite, life cycle and especially the egg release into the environment and hatching dynamics will provide a better insight into developing better and more efficient integrated parasite management systems (Cecchini 1994). This information provides an opportunity to treat the second generation of parasites that were not killed by the previous treatment and consequently preventing this generation from reaching maturity. This, in turn, it will decrease the unnecessary use of chemotherapeutic drugs (Cecchini *et al.* 1998).

The application of this method will be affected by numerous factors including the size and value of the fish. Small fish can be randomly selected from pools, tanks, ponds or raceways and transferred to a container similar to the conditions described in the methods and materials for sampling.

Due to logistic constraints, potential stress and injury, this would not be feasible for the broodstock as these fish can reach up to 1.8 m and weigh 75 kg (Heemstra & Heemstra 2004). A sampling method of siphoning dedicated areas on the bottom of broodstock tanks needs to be considered. The size, number and position of these collection areas, together with the design of the respective land-based recirculation systems will have to be considered. A possibility of siphoning the bottom of the tanks the previous day and then sample the next day will reduce food and faecal contamination of bottom sediments and will give an indication of eggs released by the parasites on the fish during the preceding 24 hours. Although more work needs to be done as a general tool, this approach has merit and application for the industry.

4.6. Conclusion

It is important to understand the parasite life cycle of every parasite species encountered in the different types of aquaculture production systems that can cause pathology, decrease of production capacity and loss of stock. Processes like transmission, infection dynamics, egg production and egg-laying rhythms are part of the parasite's life history. It is imperative to have proper techniques to accurately diagnose parasite intensity qualitative and quantitatively in cultured animals. This study showed that sediment examination can be used as a measure for a qualitative and quantitative assessment of parasite intensity on the host.

This knowledge of parasite egg production can now be used to develop more accurate and specific sampling techniques that can be implemented as a practical

diagnostic non-invasive tool to determine the mean abundance of parasites in the aquaculture production facilities for parasite control. This method can also be used as the basis to expand the technique to evaluate additional parasite species. This non-invasive method of testing can now be utilised to test existing drug formulations potentially effective against parasites that can lead to the effective implementation of “off label” drug use, while the development of new chemotherapeutic drugs are perused.

CHAPTER 5: The efficacy of praziquantel "off label" use on *Diplectanum oliveri* Williams, 1989 on dusky kob, *Argyrosomus japonicus* (Temminck & Schlegel, 1843).

5.1. Abstract

In the light of limited chemotherapeutics available for the treatment of monogenean infections in finfish, the immediate short term option to evaluate the efficacy of an "off label" use drug as a treatment solution for *Diplectanum oliveri* infection on dusky kob was pursued. The efficacy of the solution and suspension formulation was compared in their ability to reduce parasite egg production rates and parasite intensity on the gills of the fish. The effect of the different drug formulations on the host fish were also evaluated concerning changes in habitus of fish. Blood glucose concentrations were monitored as a stress indicator, with increasing in drug treatment concentrations. The high concentration treatments eliminated all adult parasites, but caused significant measureable stress and affected the central nervous system of the fish, which resulted in death of all fish in the solution group after 18 hours. The low concentrations failed to remove all adult parasites. Although both the short exposure/high concentration and long exposure/low concentration of the suspension formulation were effective, only the short exposure/high concentration eliminated all adult parasites with little change in behaviour by the treated fish.

Keywords: Monogenea; *Diplectanum oliveri*; *Argyrosomus japonicus*; dusky kob; praziquantel; "off label" drug use; parasite reduction.

5.2. Introduction

Diplectanum spp. and other monogenean infections on farmed dusky kob in South Africa are currently being controlled using methods and therapeutants that are traditionally used to control monogenean infections in other parts of the world (Buchmann 1998 a & b).

All therapeutants including drugs and disinfectants need to be registered or approved for their specific application (Swan *et al.* 2004). These registrations generally make provision for environment, operator and ultimately consumer safety by approving the use of active ingredients, observing or establishing drug retention times and determining the efficacy of the therapeutants on both target and non-target organisms, thereby providing the competent authorities with the regulatory mandate to regulate the use of these products in accordance with their consumer and operator safety standards and guidelines for their country (see Chapter 2). These standards and consequently the list of approved drugs and therapeutants for use in aquaculture are not uniform globally although increased global trade in aquaculture products or commodities may lead to more uniform registration and approval processes in the near future. Currently veterinarians and fish farmers have to comply with the regulations within their own country but also to that of the country or region to which they intend to sell their product.

Due to the diversity of cultured species, the diversity of pathogens and the difference in response of both the pathogens and hosts to various drug formulations, amongst aquatic animals, as well as drug developing costs, specific registered drug formulations do not exist for specific treatments (Thoney & Hargis

1991). A wide range of responses are observed by monogeneans on their various hosts when exposed to different drugs. Consequently, effective concentrations and exposures should be regarded as specific to the pathogen / host unit and will differ between hosts and pathogen species (Buchmann 1998 a & b). Many drugs employed as treatments in aquaculture facilities can be ineffective in treating parasite infections due to inadequate efficacy of the drug formulations and sub-therapeutic dosages (concentration and exposure) (Thoney & Hargis 1991). Sub-therapeutic treatment concentrations and/or exposure periods invariably lead to imminent re-infections and ultimately parasitic resistance to the drugs.

5.2.1. The relationship between treatment exposure and therapeutic concentration

Table 5.1: A presentation of the wide variation in different treatment exposures of praziquantel that are used with single, uninterrupted treatment exposures that varies from a minimum exposure of 90 minutes to as long as 48 hours.

Exposure and concentration	**Results**
90 min bath at 20 ppm	Removed several capsaloideans, including *Benedenia. posterocopla* from cow nose rays, *D. migrelli* from lemon shark (Thoney 1989) and *Dermophthirioides* from sawfish (Thoney 1990).
3h bath at 10 ppm	*Dactylogyrus eztensus, D. vastus, Diplozoon paradoxum*. (Schmahl & Mehlhorn 1985).
3h bath at 10 ppm	Eliminated *Gyrodactylus* sp. from *Rana catesbiana* (Thoney & Hargis 1991).
5h bath at 10 ppm	Significantly reduced numbers of *Ancylodiscoides vistulensis* on European cat fish (Szekely & Molnar 1990).
8h bath at 10 – 30 ppm	*Pseudodactylogyrus bini* of which 80 – 90% lost motility. Little difference between two concentrations. More than 60% of worms were eliminated from host during 8h in 1 ppm (Buchmann 1987).
24h bath at 2.5 ppm	Removed *Benedenia seriolae* and *Zeuxapta seriolae* from yellow tail Kingfish (Sharp *et al.* 2004).
24h bath at 5 – 10ppm	Treatment of *Neascus*, a digenean (Lewbart 1998).
24h bath at 10 ppm	Screening 22 anthelmintic (Buchmann 1993).
30h bath at 2 ppm	Removed *Haliotrema abaddon* from *Glaucosoma hebrracum* water quality toxicity after 36h (Stephens *et al.* 2001).
48h baths at 2.5 ppm	Removed *Benedenia seriolae* and *Zeuxapta seriolae* from yellow tail Kingfish (Sharp *et al.* 2004).

Table 5.2: A presentation where the response to the treatment had the best results when two consecutive praziquantel treatments were separated by and determined time interval:

Exposure and concentration	Results
2h bath at 20 ppm	Two baths of 2 hour exposure each at 20 ppm, 48h apart removed all skin and gill parasites from the yellow stripy *Lutjanus carpono tatus*. (Whittington & Ernst 2002)
40h bath at 5 ppm	Two baths of 40 hour exposure each at 5 ppm, 48h apart removed four monogeneans from the shovelnose ray (Chisholm & Whittington 2002).

Although praziquantel is a drug widely use with success against a wide range of monogeneans, the drug can be toxic at high concentrations as indicated in the table below. Symptoms include stress related behaviour, paralysis, disorientation and mortality (Table 5.3).

5.2.2. Toxicity of praziquantel at various concentrations

Table 5.3: Behavioural changes and symptoms presented by the host when exposed high concentrations of the drug to demonstrate praziquantel toxicity.

Concentration and exposure	Results
600 – 1200 ppm	Paralyzed eels within 60 seconds (Buchmann 1987).
120 ppm	Paralyzed eels within 18 minutes (Buchmann 1987).
100 ppm	Stressed European catfish (Szekely & Molnar 1990).
20 ppm at 90 minutes	Over 100 species of tropical fish in aquarium tolerated treatment reasonably well, but for sensitive fish such as the clupeoids and other juvenile fish, the concentration was too high. Many of these species showed no signs of stress at 10 ppm for 3h (Thoney & Hargis 1991).

The results in the above tables (Tables 5.1 & 5.2) demonstrate the use of a wide range of effective praziquantel concentrations, treatment exposures and

intervals amongst different parasite-host relationships as summarized below. Single exposure treatments, that ranges from a minimum of 90 minutes at a high concentration of 20 ppm (Thoney 1989 & 1990) on the one extreme, to the other extreme of a maximum of 48 hour at a low concentration of 2.5 ppm (Table 5.1) (Sharp 2004). A further variation exists, where two treatments are separated by interval exposure (Whittington & Ernst 2002; Chisholm & Whittington 2002) (Table 5.2). The above results indicate that there are wide ranges of concentrations and exposures of treatment of praziquantel, the extremes being: 1) A single bath of 90 minutes with 20 ppm, 2) A single bath of 48 hours with 2.5 ppm, 3) Two 2 hour baths with 20 ppm, 48 hours apart and 4) Two 40 hour baths with 5 ppm, 48 hours apart.

Chisholm & Whittington (2002) indicated that the size of the parasite or exposed body surface of the monogeneans display a trend when compared with treatment response. It appears that the smaller spp. to be less affected by the treatment than the larger spp. The smaller spp. and smaller post oncomiracidium stages (with their micro-habitat in their hosts' gills, especially in between the secondary lamellae) seems to be more resistant to treatment. When treated with praziquantel, these smaller stages contract deeper into the interlamellar space, which can provide better protection against treatment. The larger parasite spp. display more sensitivity to treatment possibly due to a larger surface area being exposed to the treatments. It would be of interest to see if there is a homogenous distribution in occupation of the post-oncomiracidium, juvenile and adult stages on the branchial tissue. Chisholm & Whittington (1996) also suggested that the

water current may be less effective in flushing out the smaller stages due to size and location.

Diplectanum oliveri is a gill parasite of approximately 0.5 mm in length and is regarded as a small parasite in aquaculture. Effective treatment for this species would suggest a longer exposure or a more repetitive treatments regime. If results support, that when lower concentrations can be as effective as higher concentrations when using different concentrations and exposures, it will be of economical importance and less chance for toxicity to the fish. A drug concentration of 1ppm already has an effect on the parasite (Schmahl & Mehlhorn 1985).

In this study, the efficacy of the active ingredient praziquantel was tested with two formulations, at two concentrations and two exposure periods against *Diplectanum oliveri* Williams, 1989 on the dusky kob, *Argyrosomus japonicus* (Temminck & Schlegel, 1843).

5.3. Materials and Methods

5.3.1. Source of fish and parasites

Argyrosomus japonicus fingerlings (80 mm mean peduncle length ± 1.13 standard deviation), infected with *D. oliveri* were obtained from a commercial production facility and maintained at the Department of Agriculture, Forestry and Fisheries (DAFF), Aquaculture Research Facility in Sea Point, in a well aerated 10 000 L circular flow through tank with water temperature at 20°C. A commercial diet was fed at 1% of the collective biomass per day as a maintenance

diet. The fish were grown (165 mm mean peduncle length ± 1.16 standard deviation) to facilitate handling and sampling and to allow the parasite intensity to increase.

The fish were acclimated to a 12-hour dark and 12-hour light illumination cycle, with the light phase starting at 18h00 for a period of 10 weeks. Prior to start of the experiment, parasite infection was confirmed by post mortem examination of gill tissue of fish in the population kept at the Sea Point research facility from which fish were selected at random for the experiments to confirm the presence of mature egg producing diplectanids on the gill filaments.

The 35 fish were randomly divided into five groups of seven fish. Each fish in a group was identified by a combination of fin clippings and placed in their respective holding tanks to acclimatise. The fish were not fed for the rest of the nine day trial to ensure optimum water quality throughout the trial. Water quality (dissolved oxygen, oxygen saturation, ammonia concentration, pH, salinity and temperature) in the aquaria was checked daily using a Palintest Photometer and a YSI 85 handheld digital meter with an attached YSI combination conductivity and dissolved oxygen probe. All sediment (faeces and debris) were siphoned with a daily 10% water replacement. The trial was conducted over seven days. Evidence from the Chapter 4 suggested a very slight egg-laying rhythm for *D. oliveri* with an increase in the period 00h00 to 03h00. Therefore, the 03h00 sample time was used for egg collection in this experiment. At the end of the experiment, the fish were euthanized by severing the spinal chord for postmortem examination and parasite collection.

5.3.2. Laboratory

The experiments were conducted in a laboratory maintained at a constant temperature of 20°C and photoperiod of 12 hours dark: 12 hours light. Temperature was maintained by two wall mounted ceramic heater elements (Econo-Heat 400 W) (Figure 4.1.A) and photoperiod was maintained by fluorescent Biolux (Osram) tubes on a timer switch for the dark / light cycle and produced light evenly throughout the laboratory (Figure 4.1.B). Fresh sea water was filtered through a series of 2 x 10 µm fibre wound filters and 1 x 1 µm ceramic filter (Figure 4.1.C) into a 1000 L reservoir tank where it was well aerated and maintained at 20°C (Figure 4.1.D). Five glass aquarium tanks (~80 L), each with a submerged air-lift, foam bio-filter, were used as holding tanks for housing the experimental fish during the periods that the fish were not isolated for parasite egg collection and were randomly assigned to the four different treatment combinations and the control.

For the duration of the egg collection phase, individual fish were kept in plastic containers, each with its own air supply (Figure 4.1.E). All plastic containers were filled from the reservoir 24 hours before the start of the trial for the water to stabilise with regard to oxygen saturation and room temperature of 20°C.

5.3.3. The drug formulations

In this study, we tested the efficacy of a commercial anthelmintic commonly used for the treatment of endoparasitic helminths in terrestrial live stock (sheep, cattle and ostriches) at two concentrations and two exposure periods against *D.*

oliveri on the dusky kob, *A. japonicus*. The two respective formulations supplied by the pharmaceutical company, comprised of praziquantel in solution and suspension respectively. Four trial combinations were chosen to incorporate both short and long exposures to low and high drug concentrations (Table 5.4).

Table 5.4: The four different combinations of each formulation that was used (solution and suspension):

Treatment exposure	Drug concentration	Treatment group
2 hours	2 ppm	SL: Short exposure and low concentration.
24 hours	2 ppm	LL: Long exposure and low concentration.
2 hours	20 ppm	SH: Short exposure and high concentration.
24 hours	20 ppm	LH: Long exposure and high concentration

5.3.4. Experimental design

The individual fish were transferred randomly from their aquarium into each of the plastic containers containing approximately 20 L of water on closed system. The water in these tanks was not filtered to prevent any loss of eggs. After a period of three hours the fish were transferred back into their original holding tanks. Water samples were taken and water quality checked (dissolved oxygen, oxygen saturation, ammonia concentration, pH, salinity and temperature) using a Palintest Photometer and a YSI 85 handheld digital meter with an attached YSI combination conductivity and dissolved oxygen probe to ensure that the water quality remains constant and have no influence on the results. All the sediment containing containers were removed from the room for further processing. All

handling of the fish was done as quickly as possible to minimise stress and all fish were handled wearing latex gloves to prevent trauma to skin and to prevent the removal of eggs that might adhere to ones hands. Each container was poured and rinsed carefully through a synthetic woven mesh of 25 µm to collect all of the parasite egg containing sediment (Figures 4.1. A & B). The individual filtered sediments from each tank were rinsed into separate 250 mL sample bottles (Figures 4.1. C & D) that were marked and logged to link each sample to its day of sampling and the number of the trial, tank and fish respectively (Figures 4.1. E & F). The samples were preserved in 10% buffered neutral formalin and stored for counting parasite eggs contained in the sediments.

This egg collection protocol as described above was repeated for 3 days prior to the administration of the treatment combinations and for three days post treatment to determine the affect of the respective treatment combinations on parasite egg production by comparisons within and between the pre-treatment and post-treatment parasite egg production rates. Treatments were hence administered on the fourth day of the trial. The two treatment concentrations (2 $mg.L^{-1}$and 20 $mg.L^{-1}$) were prepared in duplicate (n=2) for ~70 L containers. The respective drug concentrations were calculated and slowly mixed into 2 L sea water samples to prevent excessive precipitation of the drug in sea water. Subsequently, these samples were then made up to 70 L with filtered sea water. Each individual fish was exposed to its allotted drug concentration and exposed to the drug for the appropriate amount of time in the 70 L treatment tanks before being returned to their respective holding tanks. Water samples (50 mL) were taken from all four treatment tanks at the start (T_0) and end (T_2 - 2 Hr exposure and T_{24} - 24 Hr

exposure) of each respective treatment combination to confirm that the concentration of praziquantel remained constant throughout the treatment experiment.

Samples were also taken from the control container (C_0) and (C_{24}) to provide a base line value. These samples were sent to Biochemical and Scientific Consultants cc. in Hilton, KwaZulu Natal, South Africa for analysis by high-performance liquid chromatography (HPLC).

The whole trial described above was repeated a few days later with a new batch of 35 fish from the same stock tank at DAFF for the second drug formulation.

5.3.5. Evaluation of changes in parasite egg production

The sample bottles with the fixed tank sediments containing the parasite eggs were randomly selected for counting. The entire content of each bottle was decanted through a custom made filter (sieve) which consisted of a 30 mm section of a 4 mm walled, 75 mm diameter PVC tubing with a synthetic 25 µm mesh glued onto the rim of one end. To assist with orientation of the sample and parasite egg counting, a disc was cut from a transparency film printed with a grid pattern forming five millimetre squares and placed in the bottom of a petri dish. The sieved sample containing the sediment was placed on top of the grid in the dish and the parasite eggs were counted using an Olympus SZ61 dissection microscope with 9X zoom functionality.

With a homologous daily parasite egg production, it could be argued that the parasites are acclimatised and prevailing conditions are favourable for continual egg production. Therefore, the average daily parasite egg production for the first

three days (pre-treatment days) was compared between days, within each treatment group and control to determine if daily parasite egg production was homologous. Then the different pre-treatment groups were compared between the different treatments for each drug formulation and control to determine if egg production remained homologous. The above comparisons were done to determine if parasite egg production for all the pre-treatment groups are homologous and therefore comparison of data between days, between treatment groups and between drug formulations could be averaged.

Comparing the pre-treatment groups with the post-treatment groups within and between drug formulations would give evidence of the efficacy of the different treatments and formulations to reduce egg production of the parasites. The changes in parasite egg production between treatment groups and drug formulations was compared and expressed as a percentage change.

5.3.6. Evaluation of the changes in parasite numbers on the gills of fish

All the parasites on one set of gill arches (gill arch 1-4) were counted in the following manner.

a. Parasites on the two hemibranchs of one set of gill arches were counted to provide both the anterior and posterior values.
b. The parasites were divided into 3 groups according to their stage/size of development to determine if age classes respond different to different treatments:

i) Group 1: newly attached immature post oncomiracidium, not protruding above the secondary lamellae.

ii) Group 2: juveniles or sub-adults that are long enough to protrude from the interlamellar space extending past the border of the secondary lamellae, up to adult size without visible reproductive organs.

iii) Group 3: mature adults with visible reproductive organs.

The totals of all three stages of the parasite were compared with the control group to determine the effect of the different treatments on the parasite numbers. The effect on the parasites was compare between treatment groups within formulations. The control groups were compared to determine if parasite populations were homologous for comparison between formulations. Percentage difference in eggs production of the parasite was compared with percentage difference in parasites reduction between treatment groups within drug formulations.

5.3.7. Evaluation of the changes in the parasite population dynamics

The extent of the variation within the population composition concerning the three different stages of each parasite infra-population was evaluated. The changes between the three different parasite stages within each treatment were evaluated. The changes of similar stages of the parasite were compared to evaluated differences between treatments and within formulations and to verify the effect of the different treatments on the different age classes.

5.3.8. Evaluation of the habitus of the fish

The habitus of the fish were evaluated during and after (recovery) the treatment exposure, using the following criteria:

a. Activity / movement: Ranging from hyper active (distressed) to very little movement. Numerical values were allocated to this habitus characteristic. The value allocated for hyper activity was 5, with decreasing values to reach a value of 1 representing very little movement.
b. Recumbency: The body position in relationship to the normal position along the horizontal axis and the failure to correct to normal position (dorsal, lateral and ventral recumbent). Numerical values range from 3 for normal dorsal recumbency, 2 for lateral and 1 for ventral recumbency.
c. Disorientation: Four grades from none too severe. Allocated values range from 0 for no disorientation to 3 in case of severe disorientation.
d. Head bobbing: Fish gasping for air at the surface, present or not.

The above description of the habitus was tabulated as follows (Table 5.5).

Table 5.5: The habitus of fish were evaluated during and after the treatment, using the following criteria:

Habitus	Description	Numerical value
Activity / movement	hyper active/distressed	5
	normal	4
	suppressed	3
	sluggish	2
	very little	1
Recumbency	dorsal	3
	lateral	2
	ventral	1
Disorientation	none	0
	slight	1
	moderate	2
	severe	3
Head bobbing	if present	

5.3.9. Evaluate the blood glucose concentration in the fish in response to the different drug concentration

The source of fish was from the same batch of fish discussed previously, that was relocated and maintained at the Department of Agriculture Forestry and Fisheries (DAFF) Aquaculture Research Facility in Sea Point.

Eleven 363 litre circular flow through tanks were set up with adequate aeration and water temperature of 20°C at the same aquaculture research facility in Sea Point. Water quality and day/night illumination conditions were kept the same. Twelve fish were allocated to each of the ten treatment tanks and one control tank.

The tanks (n=11) were paired to facilitate the concurrent testing of both the solution and suspension formulations. Three additional concentrations were added in this trial to determine the stress response of the fish to the increasing treatment concentration Five drug concentrations: 2 ppm, 5 ppm, 10 ppm, 15 ppm, 20 ppm and a control group were calculated and prepared for both formulations prior to start of the trail. Once the trail started water circulation was stopped to prevent to loss of drug but aeration was continual throughout.

The duration of the experiment comprised of a 24-hour treatment phase followed by a recovery phase in non-medicated water. The fish were sample at a rate of six fish per hour, starting two hours into the exposure. The fish were randomly selected for blood sampling from the tanks and identified with a fin clipping before returning them to their corresponding tanks. Blood samples of 0.5 ml were obtained by means of veno-puncture of the caudal vain, using a 23Gx5/8¨ needle and 2 mL syringe. Plasma concentration of glucose was determined by an One Touch Select digital glucose meter and results were expressed in $mmol.L^{-1}$. After 24 hours all fish were sampled and circulation to tanks restored.

5.4. Results

Throughout the experiment, the water quality parameters were compared to the preferred water quality limits for the experimental culture of marine fish and were found to be within acceptable limits. During the solution trial the water was maintained at 7.20 $mg.L^{-1}$ ± 0.153 (7.01– 7.44 $mg.L^{-1}$) dissolved oxygen; 95.11% ± 1.136 (93.1– 96.7%) oxygen saturation; 8.16 ± 0.041 (8.1– 8.22) pH; 0.14 $mg.L^{-}$

^{1} ± 0.038 (0.01– 0.19 mg.L^{-1}) total ammonium concentration; 33.91 ‰ ± 0.084 (33.7 – 34 ‰) salinity and temperature of 19.98°C ± 0.094 (19.8 – 20.1°C).

Likewise for the suspension trial the water was maintained at 7.19 mg.L^{-1} ± 0.148 (7.02 – 7.44 mg.L^{-1}) dissolved oxygen; 95.01% ± 1.003 (93.6 – 96.7%) oxygen saturation; 8.16 ± 0.047 (8.1 – 8.22) pH; 0.15 mg.L^{-1} ± 0.027 (0.19 – 0.1 mg.L^{-1}) total ammonium concentration; 33.90 ‰ ± 0.072 (33.8 – 34 ‰) salinity and temperature of 19.96°C ± 0.076 (19.8 – 20.1°C). Water quality data given as mean ± standard deviation (minimum - maximum).

The Praziquantel concentrations for the respective treatment combinations tested were confirmed and remained constant throughout the experiment (Table 5.6.):

Table 5.6: Praziquantel concentrations (mg.L^{-1}) for each treatment combination for both before and after each respective trial.

Water Sample	Praziquantel Concentration (mg.L^{-1})	
	Solution	Suspension
Tank 1 - T_0	1.9	2.1
Tank 1 - T_2	1.8	2.0
Tank 2 - T_0	1.8	1.8
Tank 2 - T_{24}	2.0	1.8
Tank 3 - T_0	18.0	22.8
Tank 3 - T_2	18.2	21.9
Tank 4 - T_0	18.5	19.6
Tank 4 - T_{24}	18.1	19.6
Control	0	0

5.4.1. Effects of respective drug formulations and combinations on parasite egg production

The egg production of the respective infra-populations of *D. oliveri* from individual fish within each treatment group was determined for the three days prior to the administration of the respective treatment concentration and exposure combinations as described (Table 5.4). Subsequent to the administration of the treatments on day four, infra-population intensities of *D. oliveri* and consequently treatment success of the various treatment combinations were estimated by a comparison of the initial parasite infra-population egg production to that for the three days following treatment using repeated measures analysis of variance (Tables 5.7, 5.8 & Figure 5.1).

Table 5.7: Repeated measures analysis of variance for mean egg production of *Diplectanum oliveri* following exposure to four different Praziquantel solution combinations.

	SS	DF	MS	F	P
Drug Combination	304117	3	101372	1.54785	0.227904
Error	1571812	24	65492		
Time	**1634575**	**5**	**326915**	**42.37000**	**0.000000**
Time / Drug Combination	**1628121**	**15**	**108541**	**14.06757**	**0.000000**
Error	925886	120	7716		

Table 5.8: Repeated measures analysis of variance for mean egg production of *Diplectanum oliveri* following exposure to four different Praziquantel suspension combinations.

	SS	DF	MS	F	P
Drug Combination	**1768298**	**4**	**442074**	**7.1071**	**0.000377**
Error	1866063	30	62202		
Time	**5051514**	**5**	**1010303**	**115.7343**	**0.000000**
Time / Drug Combination	**2550247**	**20**	**127512**	**14.6071**	**0.000000**
Error	1309426	150	8730		

Both the solution and suspension formulations showed clear differences of the infra-population parasite egg counts between the measurement intervals or time. There was also a significant interaction between the time or measurement interval and the respective drug combinations for both formulations (Tables 5.7 & 5.8). No difference could be observed between the various combination of the Praziquantel solution (Table 5.7), however, significant differences were found between the efficacy of the respective praziquantel suspension combinations (Table 5.8).

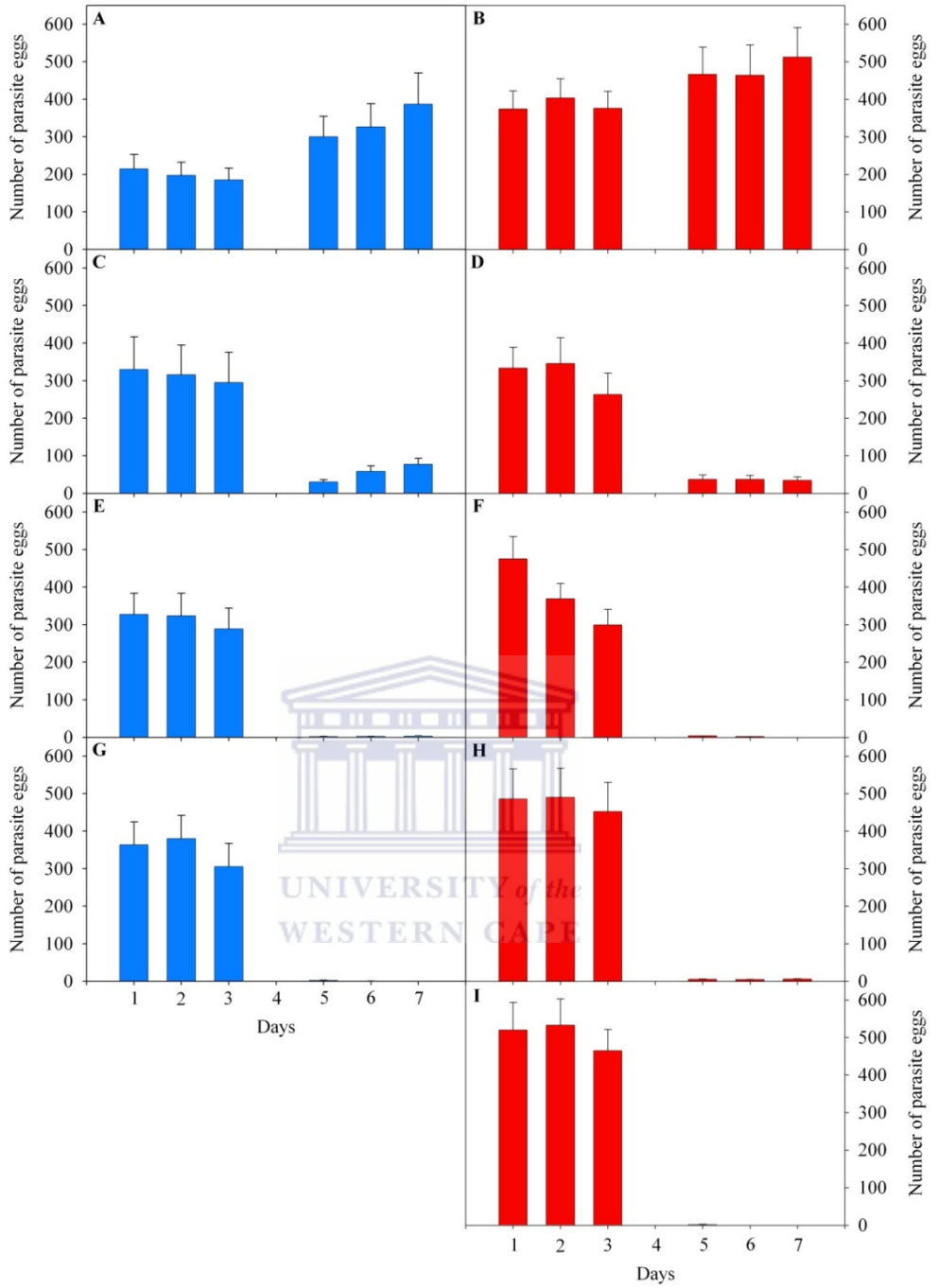

Figure 5.1: Average egg production of *Diplectanum oliveri* on the gills of *Argyrosomus japonicus* before (day 1-3) and after (day 5-7) exposure (day 4) to varying combinations of two commercial praziquantel formulations (Solution – blue bars & Suspension – red bars). A & B – Untreated control; C & D – SL (Short exposure, Low concentration); E & F – SH (Short exposure, High concentration); G & H - LL (Long exposure, Low concentration); I – LH (Long exposure, High concentration). Error bars represent standard error.

Table 5.9: Pairwise multiple comparison (Tukey HSD test) for 3-hourly egg production of *Diplectanum oliveri* following exposure to four different Praziquantel solution combinations.

	SL Day 1	SL Day 2	SL Day 3	SL Day 5	SL Day 6	SL Day 7	LL Day 1	LL Day 2	LL Day 3	LL Day 5	LL Day 6	LL Day 7
SL Day 2	1.000											
SL Day 3	1.000	1.000										
SL Day 5	**0.000**	**0.000**	**0.000**									
SL Day 6	**0.000**	**0.000**	**0.001**	1.000								
SL Day 7	**0.000**	**0.000**	**0.002**	1.000	1.000							
LL Day 1	1.000	1.000	1.000	**0.016**	**0.050**	0.100						
LL Day 2	1.000	1.000	1.000	**0.019**	**0.058**	0.115	1.000					
LL Day 3	1.000	1.000	1.000	0.073	0.189	0.323	1.000	1.000				
LL Day 5	**0.004**	**0.008**	**0.019**	1.000	1.000	1.000	**0.000**	**0.000**	**0.000**			
LL Day 6	**0.004**	**0.008**	**0.019**	1.000	1.000	1.000	**0.000**	**0.000**	**0.000**	1.000		
LL Day 7	**0.004**	**0.008**	**0.020**	1.000	1.000	1.000	**0.000**	**0.000**	**0.000**	1.000	1.000	
SH Day 1	1.000	1.000	1.000	**0.003**	**0.011**	**0.025**	1.000	1.000	1.000	**0.001**	**0.001**	**0.001**
SH Day 2	1.000	1.000	1.000	**0.002**	**0.005**	**0.012**	1.000	1.000	1.000	**0.001**	**0.001**	**0.001**
SH Day 3	1.000	1.000	1.000	**0.039**	0.110	0.203	1.000	1.000	1.000	**0.012**	**0.012**	**0.013**
SH Day 5	**0.004**	**0.008**	**0.019**	1.000	1.000	1.000	**0.005**	**0.005**	**0.024**	1.000	1.000	1.000
SH Day 6	**0.004**	**0.007**	**0.018**	1.000	1.000	1.000	**0.004**	**0.005**	**0.023**	1.000	1.000	1.000
SH Day 7	**0.004**	**0.007**	**0.017**	1.000	1.000	1.000	**0.004**	**0.005**	**0.022**	1.000	1.000	1.000
C Day 1	1.000	0.999	1.000	0.588	0.843	0.946	1.000	0.996	1.000	0.320	0.321	0.329
C Day 2	0.964	1.000	0.999	0.756	0.940	0.987	0.969	1.000	1.000	0.479	0.481	0.489
C Day 3	0.916	0.967	1.000	0.856	0.976	0.997	0.926	0.942	1.000	0.605	0.607	0.615
C Day 5	1.000	1.000	1.000	0.925	0.134	0.240	1.000	1.000	1.000	0.847	**0.016**	**0.016**
C Day 6	1.000	1.000	1.000	**0.017**	0.930	0.105	1.000	1.000	1.000	**0.005**	0.748	**0.005**
C Day 7	1.000	1.000	1.000	**0.001**	**0.004**	0.804	1.000	1.000	0.999	**0.000**	**0.000**	0.477

Table 5.9: Continued

	SH Day 1	SH Day 2	SH Day 3	SH Day 5	SH Day 6	SH Day 7	C Day 1	C Day 2	C Day 3	C Day 5	C Day 6
SL Day 2											
SL Day 3											
SL Day 5											
SL Day 6											
SL Day 7											
LL Day 1											
LL Day 2											
LL Day 3											
LL Day 5											
LL Day 6											
LL Day 7											
SH Day 1											
SH Day 2	1.000										
SH Day 3	1.000	0.996									
SH Day 5	**0.000**	**0.000**	**0.000**								
SH Day 6	**0.000**	**0.000**	**0.000**	1.000							
SH Day 7	**0.000**	**0.000**	**0.000**	1.000	1.000						
C Day 1	1.000	0.770	1.000	0.317	0.304	0.300					
C Day 2	0.766	0.999	0.996	0.475	0.460	0.455	1.000				
C Day 3	0.648	0.478	1.000	0.601	0.585	0.580	1.000	1.000			
C Day 5	1.000	1.000	1.000	0.845	**0.014**	**0.014**	0.978	0.868	0.708		
C Day 6	1.000	1.000	1.000	**0.005**	0.739	**0.004**	0.762	0.491	0.308	1.000	
C Day 7	1.000	1.000	1.000	**0.000**	**0.000**	0.462	0.059	**0.018**	**0.007**	0.973	1.000

Table 5.10: Pairwise multiple comparison (Tukey HSD test) for 3-hourly egg production of *Diplectanum oliveri* following exposure to four different Praziquantel suspension combinations.

	SL Day 1	SL Day 2	SL Day 3	SL Day 5	SL Day 6	SL Day 7	LL Day 1	LL Day 2	LL Day 3	LL Day 5
SL Day 2	1.000									
SL Day 3	1.000	0.999								
SL Day 5	**0.000**	**0.000**	**0.002**							
SL Day 6	**0.000**	**0.000**	**0.002**	1.000						
SL Day 7	**0.000**	**0.000**	**0.002**	1.000	1.000					
LL Day 1	1.000	0.991	0.412	**0.000**	**0.000**	**0.000**				
LL Day 2	1.000	1.000	1.000	**0.004**	**0.004**	**0.004**	0.952			
LL Day 3	1.000	1.000	1.000	0.087	0.087	0.077	0.103	1.000		
LL Day 5	**0.005**	**0.002**	0.097	1.000	1.000	1.000	**0.000**	**0.000**	**0.000**	
LL Day 6	**0.004**	**0.002**	0.091	1.000	1.000	1.000	**0.000**	**0.000**	**0.000**	1.000
LL Day 7	**0.004**	**0.002**	0.084	1.000	1.000	1.000	**0.000**	**0.000**	**0.000**	1.000
SH Day 1	1.000	0.975	0.315	**0.000**	**0.000**	**0.000**	1.000	0.998	0.681	**0.000**
SH Day 2	0.916	1.000	0.282	**0.000**	**0.000**	**0.000**	1.000	1.000	0.640	**0.000**
SH Day 3	0.997	1.000	1.000	**0.000**	**0.000**	**0.000**	1.000	1.000	1.000	**0.000**
SH Day 5	**0.005**	**0.003**	0.102	1.000	1.000	1.000	**0.000**	**0.001**	**0.023**	1.000
SH Day 6	**0.005**	**0.003**	0.099	1.000	1.000	1.000	**0.000**	**0.001**	**0.022**	1.000
SH Day 7	**0.005**	**0.003**	0.106	1.000	1.000	1.000	**0.000**	**0.001**	**0.024**	1.000
LH Day 1	1.000	0.802	0.108	**0.000**	**0.000**	**0.000**	1.000	0.944	0.341	**0.000**
LH Day 2	0.546	1.000	0.065	**0.000**	**0.000**	**0.000**	1.000	1.000	0.235	**0.000**
LH Day 3	0.989	0.997	0.999	**0.000**	**0.000**	**0.000**	1.000	1.000	1.000	**0.000**
LH Day 5	**0.004**	**0.002**	**0.090**	1.000	1.000	1.000	**0.000**	**0.001**	**0.020**	1.000
LH Day 6	**0.004**	**0.002**	**0.084**	1.000	1.000	1.000	**0.000**	**0.001**	**0.018**	1.000
LH Day 7	**0.004**	**0.002**	**0.084**	1.000	1.000	1.000	**0.000**	**0.001**	**0.018**	1.000
C Day 1	1.000	1.000	0.999	**0.003**	**0.003**	**0.003**	1.000	1.000	1.000	**0.001**
C Day 2	1.000	1.000	0.975	**0.001**	**0.001**	**0.001**	1.000	1.000	1.000	**0.000**
C Day 3	1.000	1.000	1.000	**0.003**	**0.003**	**0.002**	1.000	1.000	1.000	**0.001**
C Day 5	0.987	0.997	0.504	0.309	**0.000**	**0.000**	1.000	1.000	0.854	0.199
C Day 6	0.990	0.998	0.531	**0.000**	0.319	**0.000**	1.000	1.000	0.872	**0.000**
C Day 7	0.756	0.859	0.140	**0.000**	**0.000**	0.160	1.000	0.968	0.411	**0.000**

Table 5.10: Continued

	LL Day 6	LL Day 7	SH Day 1	SH Day 2	SH Day 3	SH Day 5	SH Day 6	SH Day 7	LH Day 1	LH Day 2
SL Day 2										
SL Day 3										
SL Day 5										
SL Day 6										
SL Day 7										
LL Day 1										
LL Day 2										
LL Day 3										
LL Day 5										
LL Day 6										
LL Day 7	1.000									
SH Day 1	**0.000**	**0.000**								
SH Day 2	**0.000**	**0.000**	1.000							
SH Day 3	**0.000**	**0.000**	1.000	1.000						
SH Day 5	1.000	1.000	**0.000**	**0.000**	**0.000**					
SH Day 6	1.000	1.000	**0.000**	**0.000**	**0.000**	1.000				
SH Day 7	1.000	1.000	**0.000**	**0.000**	**0.000**	1.000	1.000			
LH Day 1	**0.000**	**0.000**	1.000	1.000	1.000	**0.000**	**0.000**	**0.000**		
LH Day 2	**0.000**	**0.000**	1.000	1.000	1.000	**0.000**	**0.000**	**0.000**	1.000	
LH Day 3	**0.000**	**0.000**	1.000	1.000	1.000	**0.000**	**0.000**	**0.000**	1.000	1.000
LH Day 5	1.000	1.000	**0.000**	**0.000**	**0.000**	1.000	1.000	1.000	**0.000**	**0.000**
LH Day 6	1.000	1.000	**0.000**	**0.000**	**0.000**	1.000	1.000	1.000	**0.000**	**0.000**
LH Day 7	1.000	1.000	**0.000**	**0.000**	**0.000**	1.000	1.000	1.000	**0.000**	**0.000**
C Day 1	**0.001**	**0.001**	1.000	0.998	1.000	**0.001**	**0.001**	**0.001**	1.000	0.907
C Day 2	**0.000**	**0.000**	1.000	1.000	1.000	**0.000**	**0.000**	**0.000**	0.998	1.000
C Day 3	**0.001**	**0.001**	0.999	0.999	1.000	**0.001**	**0.001**	**0.001**	0.967	0.918
C Day 5	**0.000**	**0.000**	1.000	1.000	1.000	0.203	**0.000**	**0.000**	1.000	1.000
C Day 6	0.201	**0.000**	1.000	1.000	1.000	**0.000**	0.208	**0.000**	1.000	1.000
C Day 7	**0.000**	0.094	1.000	1.000	1.000	**0.000**	**0.000**	0.104	1.000	1.000

Table 5.10: Continued

	LH Day 5	LH Day 6	LH Day 7	C Day 1	C Day 2	C Day 3	C Day 5	C Day 6	C Day 7
SL Day 2									
SL Day 3									
SL Day 5									
SL Day 6									
SL Day 7									
LL Day 1									
LL Day 2									
LL Day 3									
LL Day 5									
LL Day 6									
LL Day 7									
SH Day 1									
SH Day 2									
SH Day 3									
SH Day 5									
SH Day 6									
SH Day 7									
LH Day 1									
LH Day 2									
LH Day 3									
LH Day 5	**0.000**								
LH Day 6	**0.000**	1.000							
LH Day 7	**0.000**	1.000	1.000						
C Day 1	1.000	**0.001**	**0.001**	**0.001**					
C Day 2	1.000	**0.000**	**0.000**	**0.000**	1.000				
C Day 3	1.000	**0.001**	**0.001**	**0.001**	1.000	1.000			
C Day 5	1.000	0.193	**0.000**	**0.000**	0.993	1.000	0.995		
C Day 6	1.000	**0.000**	0.195	**0.000**	0.995	1.000	0.997	1.000	
C Day 7	1.000	**0.000**	**0.000**	0.094	0.576	0.940	0.609	1.000	1.000

When pairwise multiple comparison (Tukey HSD test) for the 3-hourly egg production of *D. oliveri* between all pre-treatment days for treatment groups of both formulations including control groups were compared, no significant differences were detected. No difference between pre- and post-treatment days of the control groups for both the formulations occurred, which made comparisons between formulations possible (Tables 5.9 & 5.10). The egg production of all the pre-treatments groups compared to their post-treatment production within treatments were different, which indicated that all the treatments combinations of both formulations had an effect on parasite egg production (Tables 5.9 & 5.10).

The data were transformed and expressed as an average percentage reduction in parasite egg production to determine to what extent the drug formulation effected parasite egg production within treatment groups. This was calculated by using the average post-treatment egg production divided by the average pre-treatment value as a percentage. Because the average percentage parasite egg production of the pre-treatment groups was similar within respective treatment groups, comparisons between formulations were done. There were no differences between average percentage reductions, when comparing between the same pre-treatments of different formulations.

Post-treatment comparisons of average percentage reduction in egg production were estimated. Data was non-parametric analysis was run on a Kruskal-Wallis One Way Analysis of Variance on Ranks. A significant difference was found between treatments ($df = 6$, $p < 0.001$).

Table 5.11: P values for solution and suspension formulation, All Pairwise Multiple Comparison Procedures, Kruskal-Wallis One Way Analysis of Variance on Ranks for average reduction. Statistical level = 0.05

	SL Sol	SL Susp	LL Sol	LL Susp	SH Sol	SH Susp	LH Susp
SL Sol							
SL Susp	1.00000						
LL Sol	0.17549	1.00000					
LL Susp	**0.00856**	0.09383	1.00000				
SH Sol	**0.00005**	**0.00117**	0.77743	1.00000			
SH Susp	1.00000	1.00000	1.00000	1.00000	0.09662		
LH Susp	**0.00001**	**0.00037**	0.39700	1.00000	1.00000	0.04125	

Comparisons within treatment groups and between formulations indicated no difference in average percentage reduction of parasite eggs. Comparing within solution formulation, SL differ from SH. Comparing within suspension formulation, treatment groups SL differ from SH and LH respectively (Table 5.11).

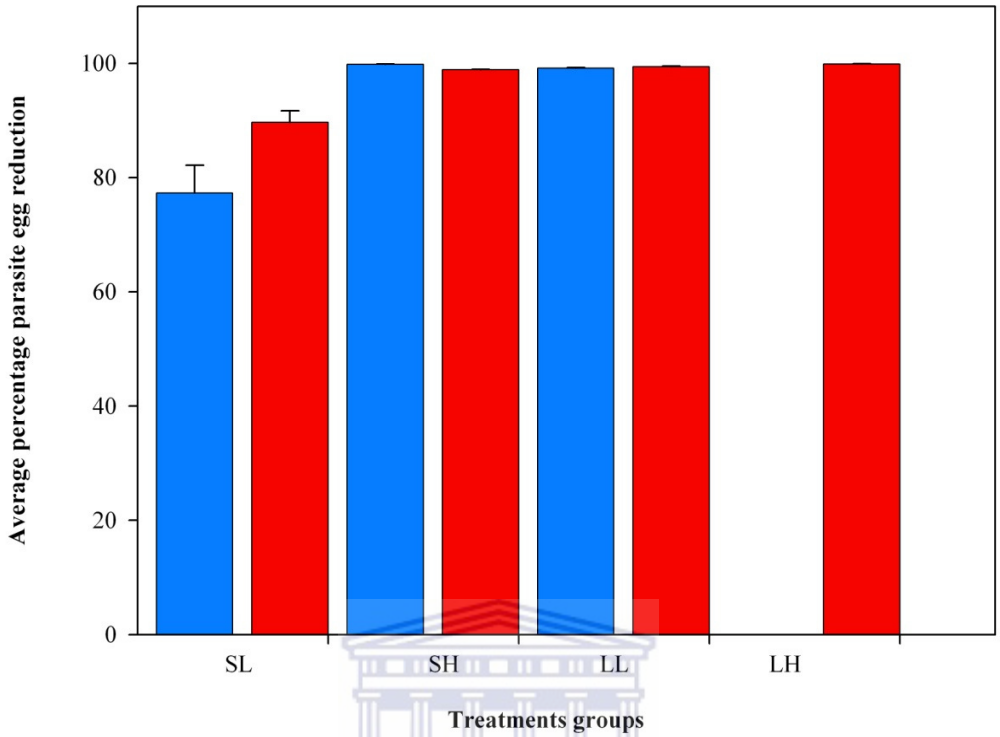

Figure 5.2: Average reduction (%) in egg production of *Diplectanum oliveri* following exposure to varying combinations of two commercial praziquantel formulations (Solution – blue bars & Suspension – red bars). SL (Short exposure, Low concentration); SH (Short exposure, High concentration); LL (Long exposure, Low concentration); LH (Long exposure, High concentration). Error bars represent standard error.

5.4.2. Evaluation of parasite counts on gills of fish

As discussed in the materials and methods section, the total parasite population is divided into and describe as three different sub-populations. The post oncomiracidia (S1), juvenile parasites (S2) and mature adults (S3).

The total of all three stages for each individual fish were calculated. Theses totals were averaged for the seven fish within the treatment group and compared with the

control population for both formulations to determine if the formulations had any effect on the total average parasite counts.

Table 5.12: Source of Variance, One Way Analysis of Variance for solution. The difference in mean values among treatment groups are greater than expected by chance (P = < 0.001).

	SS	DF	MS	F	P
Drug Combination	2790706	4	697677	44.6844	**0.000000**
Error	468403	30	15613		

Table 5.13: P values for solution formulation, All Pairwise Multiple Comparison Procedures (Holm-Sidak method). Statistical level = 0.05

	SL	LL	SH	LH
LL	0.980			
SH	0.993	0.860		
LH	**0.002**	**0.001**	**0.006**	
Control	**0.000**	**0.000**	**0.000**	**0.000**

There was a significant reduction in the total average parasite counts of all treatment groups of the solution formulation compared to the control group There were also differences between SL, LL and SH compared to LH respectively (Tables 5.12 & 5.13).

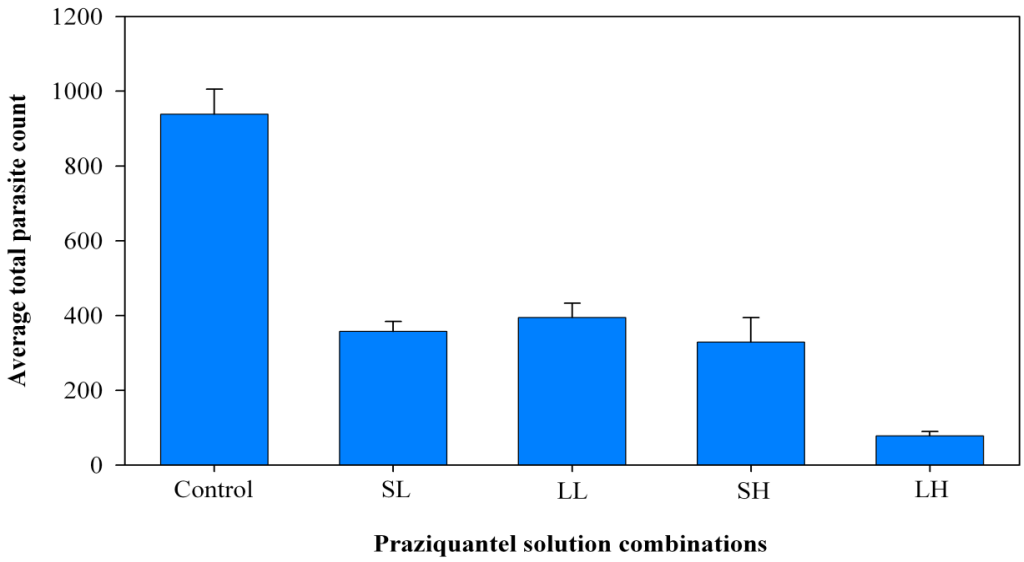

Figure 5.3: The average reduction in total parasite on the gill tissue of dusky kob, in the different treatment groups after treatment with solution formulation. SL (Short exposure, Low concentration); SH (Short exposure, High concentration); LL (Long exposure, Low concentration); LH (Long exposure, High concentration). Error bars represent standard error.

The data for the average reduction in total parasites between different suspension treatments was non-parametric and analysis was run on a Kruskal-Wallis One Way Analysis of Variance on Ranks (Table 5.14)

Table 5.14: P values for suspension, All Pairwise Multiple Comparison Procedures, Kruskal-Wallis One Way Analysis of Variance on Ranks, (df = 4, N = 35, H = 26,30476, p = 0.0000) Statistical level = 0.05

	SL	LL	SH	LH
SL				
LL	0.900125			
SH	1.00000	1.00000		
LH	**0.005226**	0.761330	0.066772	
Control	1.00000	**0.022763**	0.346312	**0.000014**

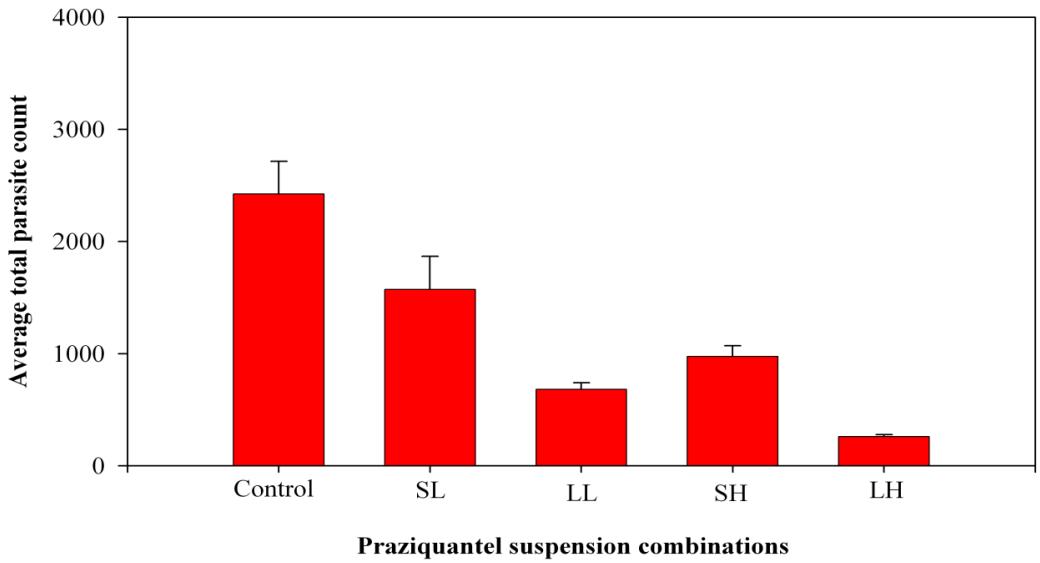

Figure 5.4: The average reduction in total parasite on the gill tissue of dusky kob, in the different treatment groups after treatment with suspension formulation. SL (Short exposure, Low concentration); SH (Short exposure, High concentration); LL (Long exposure, Low concentration); LH (Long exposure, High concentration). Error bars represent standard error.

Significant reduction only occurred in the LL and LH treatment groups when compared with the control population. The SL treatment group also differ from the LH treatment group (Figure 5.4).

The data for each formulation was transformed and expressed as an average percentage reduction in total number of all parasite stages to determine to what extent the drug formulations affected the total number of parasites within the treatment groups. The sum of the parasite stages for each fish were divided by the averaged control population and converted to a percentage for each fish. Because the control groups of the solution and suspension were not homologous ($P<0.05$), a comparison between formulations was not done.

Table 5.15: Source of Variance, One Way Analysis of Variance for solution. The difference in mean values among treatment groups are greater than expected by chance (P = < 0.001).

	SS	DF	MS	F	P
Drug Combination	4932	3	1644	12.42	**0.000000**
Error	3177	24	132.3		

Table 5.16: P values for solution formulation, All Pairwise Multiple Comparison Procedures (Holm-Sidak method). Statistical level = 0.05

	SL	LL	SH
LH	**0.0000613**	**0.0000121**	**0.000217**

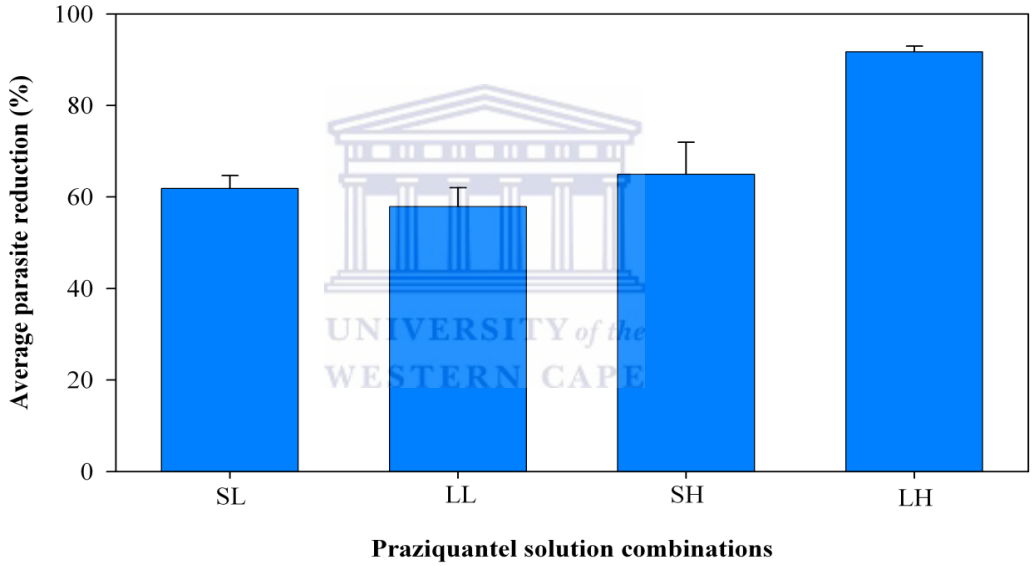

Figure 5.5: The average parasite reduction (%) in parasite in the different treatment groups after treatment with solution formulation. SL (Short exposure, Low concentration); SH (Short exposure, High concentration); LL (Long exposure, Low concentration); LH (Long exposure, High concentration). Error bars represent standard error.

There was a statistical difference when comparing SL, LL and SH with LH solution treatment (Figure 5.5).

The data of the average percentage reduction in parasites treated with the different suspension treatments was not parametric. A non-parametric analysis was run on a Kruskal-Wallis One Way Analysis of Variance on Ranks (Table 5.17)

Table 5.17: P values for suspension, All Pairwise Multiple Comparison Procedures, Kruskal-Wallis One Way Analysis of Variance on Ranks, (df = 3, N = 36, H = 15.40841, p = 0.0015). Statistical level = 0.05

	SL	LL	SH	LH
SL				
LL	0.440965			
SH	1.000000	1.000000		
LH	**0.001338**	0.343334	**0.018877**	

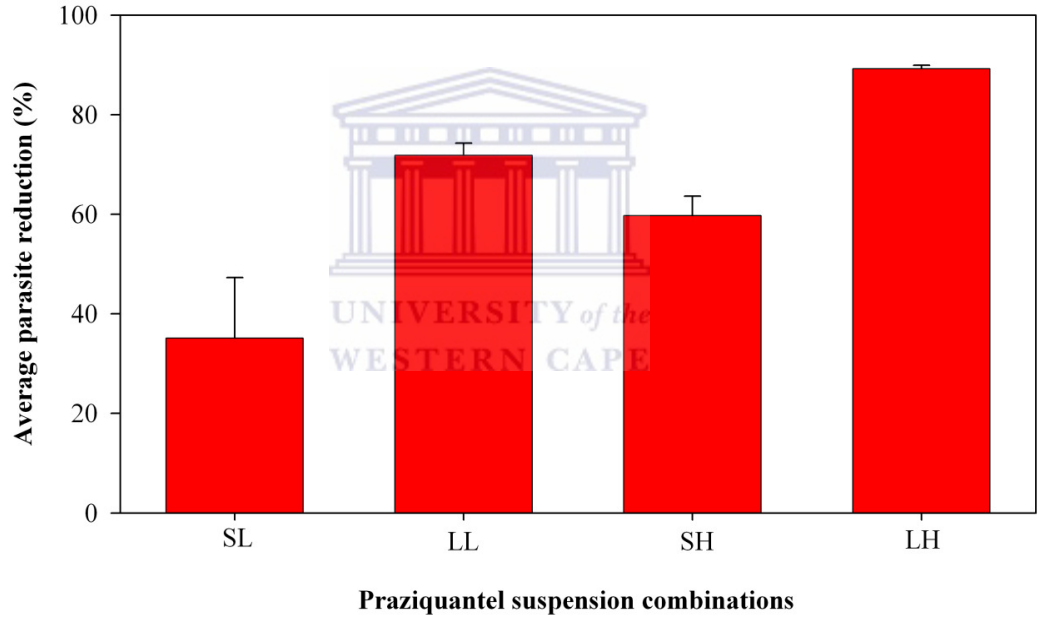

Figure 5.6: The average parasite reduction (%) in parasite in the different treatment groups after treatment with suspension formulation. SL (Short exposure, Low concentration); SH (Short exposure, High concentration); LL (Long exposure, Low concentration); LH (Long exposure, High concentration). Error bars represent standard error.

Comparison within the suspension formulation, between treatment groups revealed that SL, and SH differed from LH respectively. There is no statistical difference when SL, LL and SH are compared between treatment groups (P<0.05) (Figure 5.6).

The average percentage reduction in egg production of parasites within treatment groups were compared with the average percentage reduction of the total parasite population within treatments for the two formulations. The average percentage egg reduction was more pronounced than the average percentage parasite reduction. This demonstrates that the egg producing adults are more susceptible the treatment than the oncomiracidium and juvenile stages (Figures 5.7. A & B).

To determine how the different parasite stages were affected by the different treatments, the population dynamics between the post-oncomiracidium, juvenile and adult stages were evaluated. This was done by calculating the reduction of the three different parasite stages and expressed this as an average proportionate percentage parasite stage change in relation to the control population. For comparisons between formulations, the average proportionate percentage parasite stage of the two control populations were compared to determine if populations were homologues.

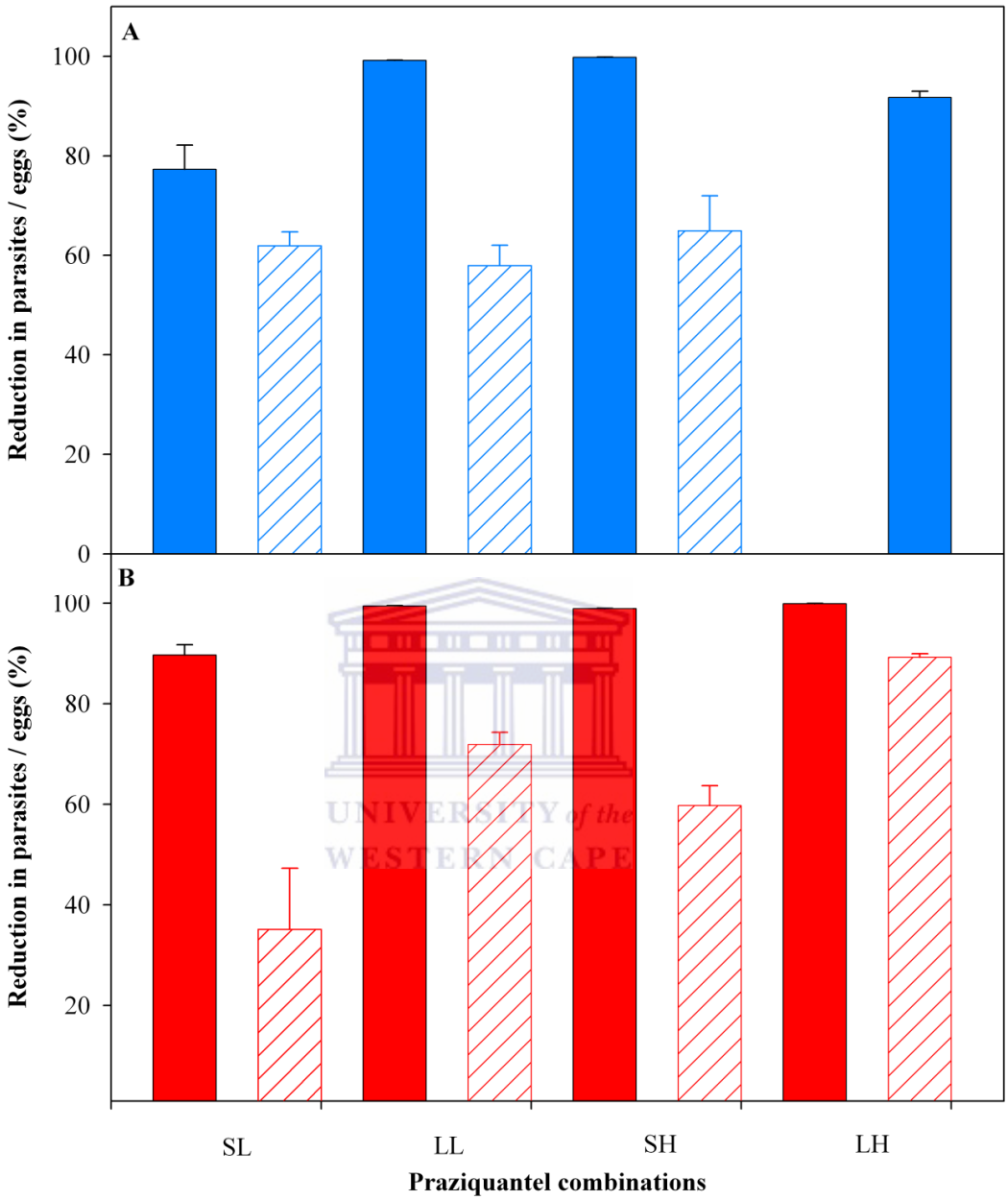

Figure 5.7: A- solution, The average percentage reduction in parasite egg production is compared with the average percentage reduction of total parasites for the solution formulations. There is a statistical difference ($P < 0.05$) within compared groups SL, LL and SH. No egg data available for the LH treatment group comparison dew to group mortality as a result to toxicity. **B-suspension,** The average percentage reduction in parasite egg production is compared with the average percentage reduction of total parasites for the suspension formulations. There is a statistical difference (p-values) within compared groups SL, LL, SH and LH. Solid bars – Egg reduction, Patterned bars – Parasite reduction. Error bars represent standard error.

5.4.3. Evaluation of parasite population dynamics on gills of fish

The proportionate percentages of the three stages of parasites on individual fish were calculated within treatment groups for both the formulations to determine how the parasite age groups change within the sub-population on the fish. The comparison between formulations of the proportionate percentages of post-oncomiracidia (P=0.107), juveniles (P=0.802) and adults (P=0.0645) of the control groups were homologous (Figure 5.8).

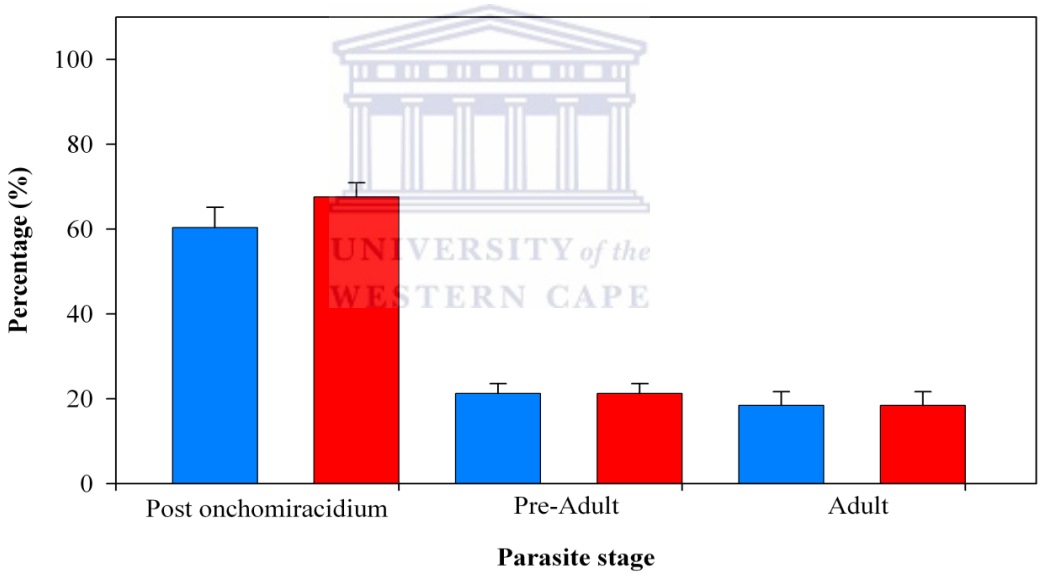

Figure 5.8: The average proportionate percentage parasite stage changes in the control populations: The three different parasite stages are presented by **1;** post-oncomiracidia.**2;** juveniles and **3;** egg producing adult parasites. The comparisons within stages between the two drug formulations of the control populations demonstrated no significant statistical difference dynamics. The population stages dynamics were homologous. Blue – Solution, Red – Suspension.

The control groups were averaged. The parasite populations were compared between formulations. In both the formulations the same trend was detected. The

mature egg producing adults were the most effected stage followed by the juveniles and then the least effected post oncomiracidia.

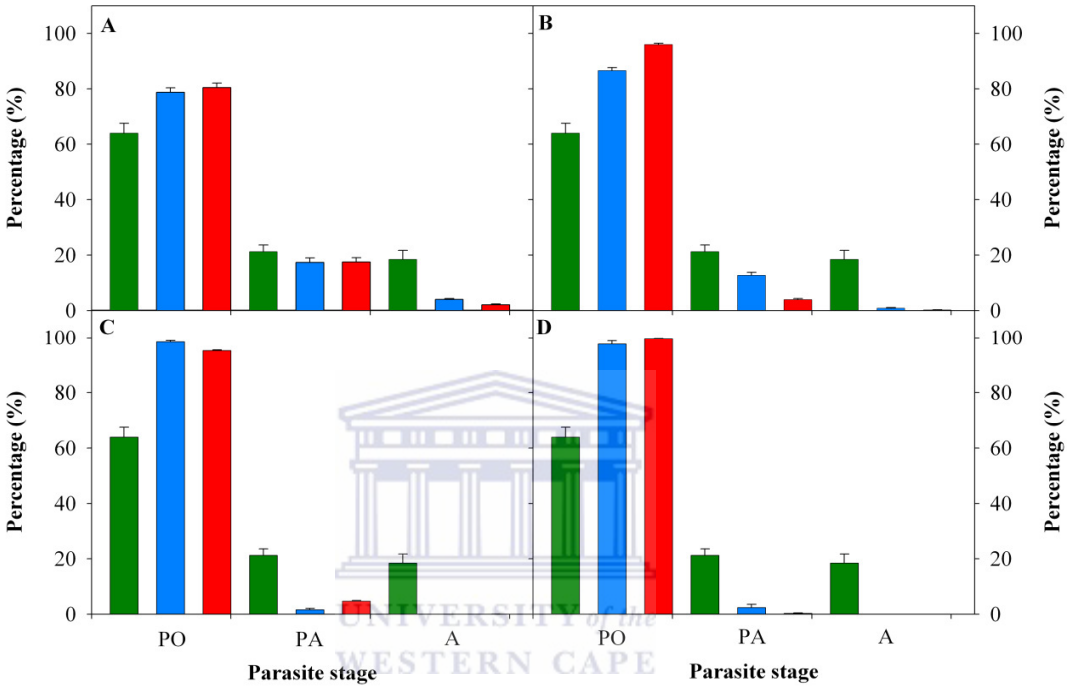

Figure 5.9: The average proportionate percentage parasite stage changes in relation to the control population: The three different parasite stages are presented by **1;** post-oncomiracidia.**2;** juveniles and **3;** egg producing adult parasites. The four graphs represents the comparisons of the population dynamic changes after treatment within the four different treatment groups and between formulations **A;** the SL treatment group. **B;** the LL treatment group. **C;** the SH treatment group. **D;** the LH treatment group. A- SL, B- LL, C-SH, D- LH. Green – Control, Blue – Solution, Red - Suspension

As demonstrated in Figure 5.9, the different treatments effected the different parasite stages in such a manner that in all of the treatments the adult parasites were the most sensitive followed by the juveniles and lastly the post-oncomiracidium stages.

When the average proportionate percentage parasite stage reduction values were compared (Table 5.18), it summarised the results for further interpretation. Comparing this data with blood glucose as a stress indicator and drug toxicity to get a better understanding of the impact of these drug treatments on the *D. oliveri* on dusky kob. This will assist one in the choice for the safest most effective treatment combination.

Table 5.18: This table represents the average proportionate percentage reduction within the parasite population of the juvenile and adult parasite stages for all the treatments and both formulations.

Treatment group	SL		LL		SH		LH	
Formulation	Sol	Susp	Sol	Susp	Sol	Susp	Sol	Susp
Juvenile (2)	20	20	42	82	93	78	89	99
Mature adults (3)	72	86	95	99	100	100	100	100

5.4.4. Evaluation of the habitus of the fish

The habitus of the fish were evaluated during and after the exposure. The observations were done hourly over the full period that the fish were exposed to their respective drug concentration and durations for both the formulations. The changes in the habitus of the fish once exposed to the drug concentrations presented a common behaviour pattern.

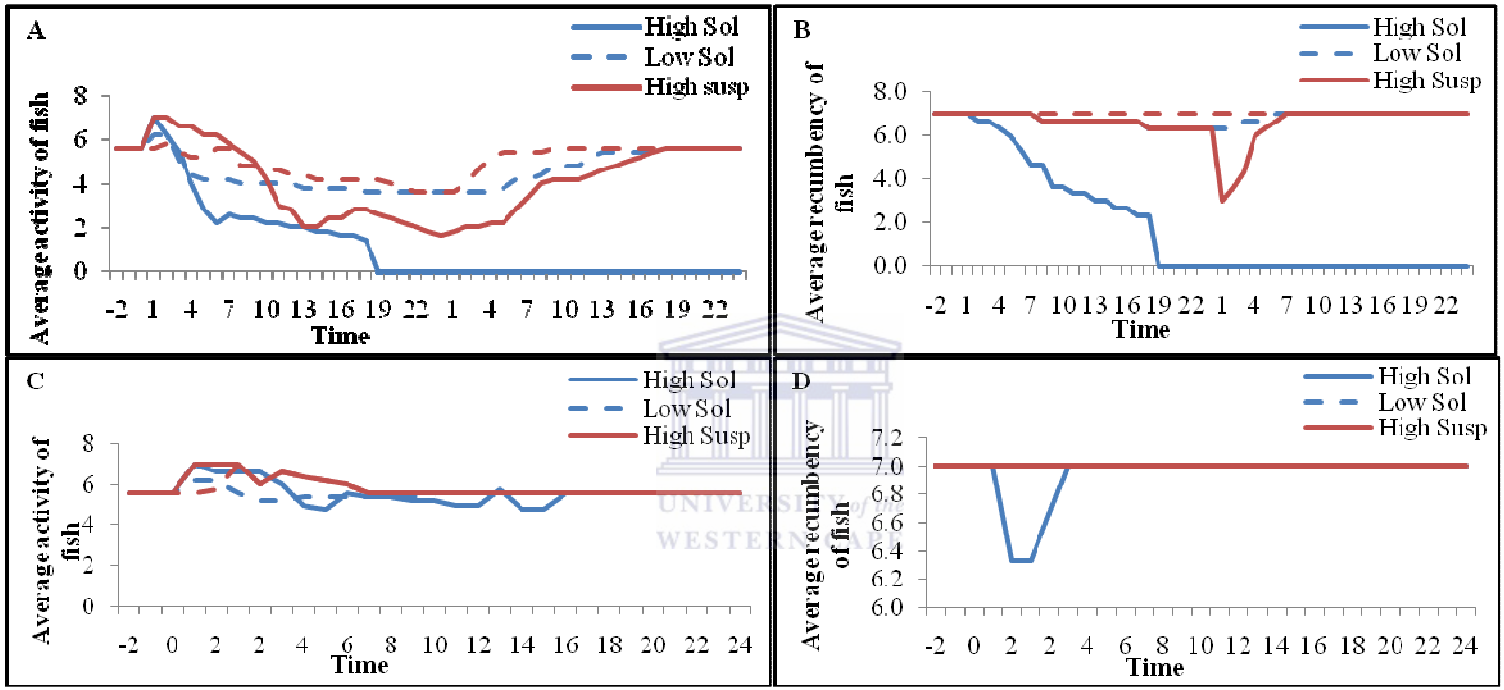

Figure 5.10:The average habitus changes, specifically concerning the activity and recumbency of fish treated with the solution and suspension formulations followed by their post treatment recovery: A; the average of the fish that displayed changes in their activity during the long exposures (24 hours), low and high concentration treatments with both formulations. **B;** average changes in the recumbency of the fish during the long exposures treatments (24 hours), low and high concentrations with both formulations. **C;** average changes in the activity of the fish during the short exposures treatments (2 hours) with both formulations**. D;** average changes in the recumbency of the fish during the short exposures treatments (2 hours) with both formulations.

5.4.4.1. Habitus changes when fish were exposed to treatments

5.4.4.1.1. Activity/movement

The fish presented a sudden onset of distress and hyper activity when exposed to the different treatments. The number of fish affected, the time of onset and rate of escalation were drug concentration related.

All the fish exposed to the 20 ppm concentration were distressed from the time exposure started. This was consistent for both formulations. This distress was followed by suppression/depressed behaviour. The central nervous system affected by the drug formulation displayed a succession of symptoms over time presented as follows: hyper activity → suppression → sluggish → very little movement. Fish presented this behaviour with both formulations. This decline from CNS stimulation followed by depression was more rapid with the solution formulation and resulted in death. The whole decline for all the fish took 18 hours. Only 85% of fish reached this state of very little movement after 24 hours when exposure to the suspension formulation. The fish exposed to the 20 ppm concentration and two hour exposure were returned to their normal aquaria after two hours.

Fish exposed to the 2 ppm concentration presented with less severe symptoms and rate of regression was also much slower. Also here the fish were more sensitive to the solution than to the suspension, where 43% of the fish presented with hyper activity at the onset compared to none of the fish with the suspension. With both the formulations only 43% reached the sluggish stage at 24 hours.

5.4.4.1.2. Recumbency

The first fish displayed lateral recumbency after two hours with the LH solution treatment. Ventral recumbency set after six hours. After nine hours, 57% of fish displayed lateral recumbency and 43% ventral recumbency in the 20 ppm solution treatment. At 16 hours all fish were ventral recumbent and died after 18 hours. In the LL solution treatment lateral recumbency set in after eight hours and only two fish were affected after 24 hours. Two fish were lateral recumbent after 2 hours with the SH solution.

The first fish displayed lateral recumbency in the 20 ppm suspension formulation after 11 hours and ventral recumbency set in at 18 hours. At 24 hours 100% of fish were in a ventral recumbent position.

5.4.4.1.3. Disorientation

In the 20 ppm solution, within one hour all fish were slightly disorientated which progressed to a moderate disorientation in two hours and severely disorientated after five hours. In the suspension treatment only after eight hours did all fish display slight disorientation which progressed to moderate at 16-hours and severe at 24 hours.

5.4.4.1.4. Head bobbing

Head bobbing were present from the onset with the 20 ppm solution and 86% displayed this behaviour after three hours from when the numbers declined due to the disorientation of the fish and altered recumbency. The first head bobbing in fish with the suspension treatment was only displayed at two hours. The numbers increased to where all the fish displayed the symptom at 10 hours. No head bobbing was present after 17 hours.

5.4.4.2. Habitus changes recovering after treatments

All the fish treated with the 20 ppm concentration and 24 hour duration solution formulation started dying at 18 hours and was removed for post mortem, so these fish will not be considered in the following discussion.

5.4.4.2.1. Activity/movement

Normal behaviour returned for all fish treated with solution formulation of LL, SH and SL combinations after 18, 14 and 10-hours respectively once returned to normal untreated water. Normal behaviour returned for all fish treated with suspension formulation of LH, LL, SH and SL combinations after 18, 9, 7 and 5-hours respectively. The recovery period for the fish took twice as long for the solution as with the suspension.

5.4.4.2.2. Recumbency

Normal dorsal recumbency was restored from lateral recumbency for the remaining LL and SH solution treatment groups after five hours and 2-hours respectively. In the suspension formulation where the LH treatment group was still alive, all fish revered from ventral to lateral recumbency after 3-hours and after six hours all were in normal dorsal recumbency.

5.4.4.2.3. Disorientation

All fish in the solution treatment groups LL, SH and SL recovered by 12, 5 and 3 hours respectively. The recovery of fish in suspension groups LH, LL and SH was completed by 13, 7 and 3 hours respectively. A comparison of the recovery rate from similar treatment groups between formulations, demonstrate that the rate of recovery from suspension was more rapid that with the solution.

5.4.4.2.4. Head bobbing

Head bobbing disappeared after two hours with the SH solution formulation. In the LH suspension treatment head bobbing seized after 10 hours.

5.4.5. Evaluate the effect of the drug formulations on blood glucose concentration of fish.

The blood glucose concentration of the fish over 24 hours within each treatment concentration were determined and averaged. An increase in plasma glucose concentration response was detected already in the first blood samples taken 2 hours into the treatment and persisted for the duration of the exposure duration. There was a direct proportional increase between the drug concentration of both formulations and the plasma glucose concentration ($mmol.L^{-1}$). Thus the behavioural changes and physiological stress that the chemotherapeutic formulations imposed on the fish does reflect in the blood chemistry. The plasma glucose concentration did prove to be a stress indicator of the drug concentration. No samples were taken to monitor the recovery once circulation was restored after 24 hours (Figure 5.11).

Comparisons between the different treatments and the control population demonstrated that a significant difference exist between both the formulations containing 15 ppm and 20 ppm. This suggested that future evaluation of these formulations may find that a 10 ppm drug concentration may be just as effective as the 20 ppm concentrations and prove to be a more suitable concentration considering the toxicity 20 ppm. (Schmahl & Mehlhorn 1985; Buchmann 1987; Szekely & Molnar 1990; Thoney & Hargis 1991).

Table 5.19: P values for plasma glucose concentrations, All Pairwise Multiple Comparison Procedures, Kruskal-Wallis One Way Analysis of Variance on Ranks, (df = 10, N = 110, H = 83.66110, p = 0.0000). Statistical level = 0.05

	Control	2 Sol	2 Susp	5 Sol	5 Susp	10 Sol	10Susp	15 Sol	15 Susp	20 Sol	20 Susp
Control											
2 Sol	1.00000										
2 Susp	1.00000	1.00000									
5 Sol	1.00000	1.00000	1.00000								
5 Susp	1.00000	1.00000	1.00000	1.00000							
10 Sol	0.386738	0.961567	1.00000	1.00000	1.00000						
10 Susp	0.319107	0.896404	1.00000	1.00000	1.00000	1.00000					
15 Sol	**0.000261**	**0.001008**	**0.00451**	**0.01105**	0.50607	1.00000	1.00000				
15 Susp	**0.000015**	**0.000069**	**0.00037**	**0.00102**	0.08453	0.40728	0.21456	1.00000			
20 Sol	**0.000000**	**0.000000**	**0.00000**	**0.00000**	**0.00058**	**0.00544**	**0.00217**	1.00000	1.00000		
20 Susp	**0.000018**	**0.000091**	**0.00056**	**0.00167**	0.17409	0.86557	0.45227	1.00000	1.0000	1.00000	

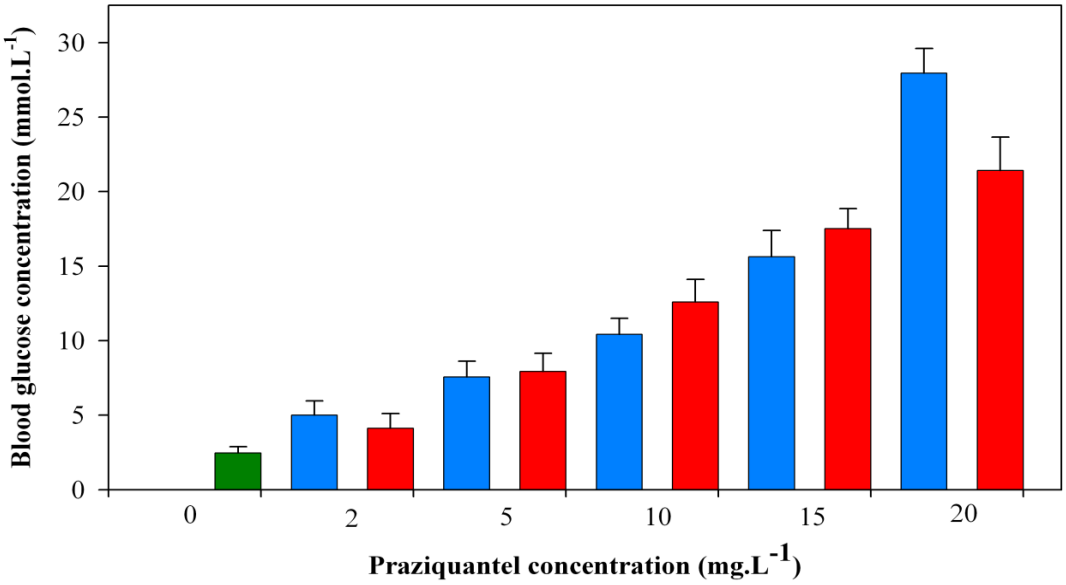

Figure 5.11: The figure display the average increase in plasma glucose concentration when groups of twelve fish were exposed to five different treatment concentrations for both formulations. A direct proportionate increase exists between plasma glucose concentration and drug concentration.

5.5. Discussion

The challenges that the relatively young and developing South African aquaculture industry faces are multi factorial. Farmers are continually developing and improving their hatching and on-growing facilities to provide the optimum conditions in system design, water quality, and nutrition and disease management. Further exploration into alternative types of production systems has lead to an expansion into floating sea-cage systems and designated industrial aquaculture zones within coastal industrial development zones.

Important aspects in any production facility are disease control which depends on the detailed knowledge of diseases, reliable methods for accurate diagnosis and effective treatment. Incorporating the correct and strategic use of chemotherapeutic drugs will secure an effective integrated parasite management

plan. These drugs can derive from following categories. Firstly, drugs registered for the treatment against specific disease causing organisms. Secondly, exciting registered drugs formulations which are effectively used against disease in other countries, but may be imported as alternative drugs for off label use under special regulations. These include the use of existing formulations, used in other species (terrestrial or aquatic) that may be effective and thus may be used as an "off label" drug. "Off label" use raises further questions about efficacy, toxicity, tissue retention of the drug and food security.

5.5.1. The efficacy of the drug treatments to reduce the parasite egg production

The average egg production of the parasites on the fish was homologous for the three pre-treatment days. This was confirmed by comparisons within each treatment groups, between treatment groups and between both formulations. This demonstrated that the daily average egg production was relative constant. This indicated that possible factors which could have influenced the parasite egg production and caused variation were limited to the experimental variables. It also demonstrated that the sediment sample evaluation proves to be an effective, reliable and repeatable method for estimating parasite component population size. The daily average egg production of the parasites post treatment was homologous within all treatment groups for the solution formulation, with no recovery of the parasites egg production for these three day post treatment period. This was also observed with the suspension formulation except in the LH treatment group. All treatment groups for both formulations displayed a decrease in parasite egg production when comparing the average pre- and post-treatment groups,

indicating that all treatments reduced the parasite egg production. When comparing the average percentage parasite egg reduction between treatment groups, the treatments with SL, LL and SH were 77.3, 99.2 and 99.8 percent effective for the solution formulation. Whereas with the suspension groups SL, SH, LL and LH were 89.7, 98.9, 99.4 and 99.9 percent effective in parasite egg production (Table 5.18).

5.5.2. The efficacy of the drug treatments to reduce the parasites on the fish

The total parasites counts demonstrated that the formulations were effective in the reduction of parasites on the fish. All treatment groups had a significant reduction in the total parasites for the solution formulation, but only a significant reduction occurred in the LL and LH treatment groups of the suspension formulation. In both formulations the LH treatment was the most effective in parasite reduction. When the reduction of the total averaged parasite counts were expressed as average percentage parasite reduction compared to the control within formulations, the order of increasing treatment efficacy for the solution formulation was as follows; LL, SL, SH and LH with 58, 62, 65 and 92 percent respectively. The order for the suspension was as follows; SL, SH, LL and LH with 35, 60, 72 and 89 percent respectively (Table 5.18).

Comparing the average percentage parasite egg reduction with average percentage parasite reduction for each treatment, a relationship was detected where the proportion percentage parasite reduction is less than the percentage egg

reduction. This implied that immature non egg producing parasite stages were still present in the post-treatment population.

5.5.3. The effect of the drug treatments on the parasite population dynamics

The comparisons of the different parasite stages as a proportionate percentage of the population demonstrated that the effect on the different stages were not homologous. Compared to the control, the adult egg producing parasites were the group most sensitive to all the different treatments and displayed biggest quantitative reduction followed by the juvenile group and least effected post oncomiracidium stages respectively. The populations of the two control groups were homologous and comparison between formulations demonstrated that all the different suspension treatments were more effective than the solution treatments in reducing the adult stages. Similar drug formulation effect was detected on the juvenile stages, with the exception of the SH group where the effect was reversed between the two formulations. The variation in response of the parasite stages to the different treatments demonstrated the variation in sensitivity of the different parasites stages, with the adults the most sensitive to treatments. This is consistent with what Chisholm & Whittington (2002) suggested that the bigger parasites are more susceptible to treatment than smaller parasites. It is clear that these formulations do not sterilise the infection of all the parasite stages, yet a 100% reduction of adult stages occurred with SH and LH treatment groups of both formulations. The LL suspension was also very effective and had a reduction of 82% and 99% on juveniles and adults respectively. The importance of this fact is

that with the knowledge of the maturing rate at which parasite matures from newly hatched oncomiracidium to egg producing adults, treatment intervals can be calculated to target every new generation of adults. If all adults are effectively removed from population the parasite egg production will be interrupted to prevent a future generation. Also by effectively removing all adult parasites, drug resistance will be limited and would extent the longevity of the drug as an effective treatment. Again, the percentage parasite egg reduction obtained from the quantitative assessment of the egg containing sediment is reflected in the percentage reduction in adult parasites, which highlight the value of this management tool.

5.5.4. The effect of the drug treatments on the habitus of the fish

The behaviour of the fish that were exposed to the drug formulations were all affected to some degree by the different drug treatments. Comparing the effects on the habitus and survival rate of the fish with the efficacy the treatments had on the reduction of parasite and parasite egg production, demonstrated the safety and the effective treatment of choice. In other words, the drug treatment of choice would be the treatment with the least detrimental effect on the fish with the most effective reduction in parasite egg production and parasites.

The SL treatment groups proved to be ineffective in the complete reduction of either the juvenile or adult parasites and can be discarded as an option. The reduction of juveniles and adults by the SL solution was 20% and 72% respectively. The reduction by the SL suspension was 20% and 86% respectively.

On the other hand, although both the LH formulations were effective in removing all the adult parasites, they were also the treatments with the most profound effect on the fish behaviour and survival rate. All the fish in the LH solution treatment died after 18 hours of exposure. In the LH suspension group 86 percent of fish displayed very little movement, with hundred percent of the fish severely disorientated and all presented in ventral recumbency after 24 hours. The stress on the fish was also demonstrated by the elevated plasma glucose concentration. Grutter & Pankhurst (2000) detected an increase of plasma glucose concentration to reach concentration of 4 mmol L^{-1} as an acute stress response, following handling of the coral reef fish *Hemigymnus melapterus* (Bloch, 1791). The plasma glucose concentration in the fish treated with the 20 ppm solution and suspension treatment were 28 and 21mmol.L^{-1} respectively compared to the 2 mmol.L^{-1} in the control. The toxicity and stress on the fish of this treatment disqualified it as a future "off label" use treatment option. Thoney & Hargis (1991) found in clupeoids and other juvenile fish that 20 ppm praziquantel for 90 minutes was too high. Signs of stress were absent at a lower concentration of 10 ppm with a treated time of three hours. The increased toxicity of the formulation might be contributed to other chemicals in the compounds which may need further investigation.

Considering the LL formulations, the disadvantages include the following. The treatments for the solution and suspension were very effective, but unable to remove all of any of the juvenile or adult parasites from the fish with an efficacy of 42% and 82% in juveniles and 95% and 99% in adults respectively. This residual parasite population that survived the treatment can increase the chance for

parasite drug resistance. From a practical point of view, the duration of exposure to this treatment is 24 hours, during which there is no circulation through the holding tanks to prevent the removal of the drug during treatment. The water quality will deteriorate. In intensive culture systems, stock density can boarder crowding with the maximum space density limit (fish weight/unit water). One of the important factors in calculating stock density is based on sufficient water circulation and filtration to maintain water quality by constantly removing toxic metabolites like ammonia, carbon dioxide and faecal matter (Wedemeyer 1996). Advantage of the low concentration treatments over the high concentration treatments are that a smaller volume of drug is needed which has a financial implication. Also less fish displayed hyper activity and disorientation with the low concentrations. In the LL solution group 43%, 29% and 43% displayed normal, hyper activity and suppressed movement respectively and only 29% was slight disorientated. In the LL suspension only 14 percent of the fish displayed hyper activity and none of any of the other changes. The fish were less stressed with plasma glucose concentrations of 5 and 4 $mmol.L^{-1}$ plasma glucose for solution and suspension formulations respectively.

Both the SH solution and suspension were effective and reduced juvenile and adult parasites with an efficacy of 93% and 100% for the solution and 78% and 100% for the suspension respectively. The average percentage of fish that displayed habitus changes with the SH solution were; hyper activity (86%), suppressed movement (14%), lateral recumbency (29%), moderately distressed (86%), severely distressed (14%) and head bobbing (71%) after 2 hours. The SH suspension had much less effect on the fish. Although all fish displayed hyper

activity, 14 percent slight disorientation, none of the other more advanced habitus changes occurred. All the fish recovered completely after seven hours without mortalities during the following three days.

Considering all the drug treatments tested, it appeared that the SH (20 ppm for 2 hours) suspension treatment proved to be the treatment of choice. When compared with the other three treatments (SH-sol, LH-sol and LH-susp) which also had a hundred percent reduction of adult parasites, it was the treatment with the least habitus changes and best survival rate. In practice, the short treatment will also reduce the exposure of fish to a decline in water quality. A disadvantage was that fish did display hyper activity and stress induced hyperglycaemia of 28 and 21 mmol.L^{-1} with the solution and suspension respectively. The high concentration would also have higher financial input cost.

5.6. Conclusion

The immediate short term option for a treatment solution was pursued to evaluate the use of praziquantel as an "off label" drug use against *D. oliveri* on dusky kob and proved to be effective and successful. The short exposure/high concentration suspension treatment suggested being the treatment of choice, compared to the other treatments. This study demonstrated the affect of four treatment combinations with results varying from the inability to remove parasites to being toxic to the fish. It will be of interest to determine if a reduced intermediate concentration of less than the maximum of 20 ppm will have the same favourable results in the reduction of parasites and with fewer side effects on

the fish. This study provided a recommendation treatment dosage with the suspension of 20 ppm (praziquantel) in bath treatment with duration of 2 hours.

CHAPTER 6: General discussion

The aquaculture industry in South Africa has come a long way over the past three decades. The approach and experience generated in this sector was mainly through trial and error. The future success of this fast growing aquaculture industry strongly relies on the development of the support sector providing more tolerant government legislation, sponsorship, participation of the pharmaceutical industry with research and development of aquatic organism health management. Remarkable advances have been made with regard to the farming of dusky kob and silver kob with interest in future species like white stumpnose and yellowtail. On-shore, reticulating farms have made the most significant advances to date. The industry will never be without challenges. The increasing awareness of food safety, environmental conservation and pollution cause more pressures on farmers to produce better quality and "safe" food. Furthermore fresh water culture will always be challenged by the fact that South Africa is a water scarce country. Water conservation and pollution will always be a priority. Marine sea cage culture will be challenged by factors like the development of robust cages to withstand conditions of a high energy coast line, seals and other predators. The industry is also constrained by new challenges of emerging pathogens, their unknown epidemiology and the limited number of approved chemotherapeutic agents to treat or control infections (Schnick *et al.* 2005).

The lack of drugs for disease control in aquaculture globally and South Africa can force local fish farmers to use medication out of desperation, without authorisation, with very little guidance on efficacy, toxicity, residue retention in tissue and drug resistance in their attempt to sustain fish health and securing future

production (Buchmann 1998). Due to the cost of drug development and the size of the industry, it is still unlikely that pharmaceutical companies will invest significantly in the research and development of new anthelmintics (Buchmann 1998). Therefore, the identification and testing of existing formulations globally from the aquaculture, veterinary and medical fields that might be effective in treatment of diseases locally needs to be pursued further. These drugs can then be evaluated for their efficacy, toxicity, tissue residue and possible environmental impact for their "off label" use. Legislation does exist for the import of registered drugs from overseas, but not registered in South Africa as well as the raw compounds to compound the drugs locally. The "off label" use and compounding of drugs can relieve the short term need, while better long term solutions are pursued. Accommodating legislation needs to be implemented to facilitate controlled drug evaluation at an accredited research facility. It is therefore important, firstly to identify and determine the extent of the disease or parasite infection at hand. Based on this impact, the challenge can be characterised and prioritised. Once this is done, the search for a treatment is pursued to alleviate the lack of available effective drugs.

Diplectanum oliveri, a monogenean gill parasite has been confirmed as an emerging threat and is becoming more prevalent on dusky kob and silver kob in the developing aquaculture industry of South Africa. *Diplectanum aequans*, which is one of the most important ectoparasites in sea bass causing pathology and significant mortalities (Cecchini *et al.* 1998), emphasises the potential importance of *D. oliveri* as a pathogen. *D. oliveri* causes significant tissue destruction of the secondary lamellae due to aggressive feeding, but with much less impact at the

attachment site, as in the case of *D. aequans*. The pathology caused by *D. oliveri* provided evidence for concern. This ever increasing importance of monogeneans as parasites causing pathology should not be under estimated and early effective diagnostic practices and treatment possibilities need to be developed to anticipate the challenge which can ultimately lead to stock losses if unattended.

The assessment of the monogenean infection necessitated the development of appropriate methods to accurately diagnose parasite intensity qualitatively and quantitatively in cultured animals. Understanding processes like transmission, infection dynamics, egg production and egg-laying rhythms that are strongly impacted by the parasite's reproduction history, it was possible to apply the acquired knowledge to evaluate and develop an elementary but effective method. The eggs produced by the parasites on the host were successfully isolated from the sediment and counted. The egg counts were compared with the number of parasites present on the gill tissue of the fish. This approach to qualify and quantify monogenean infections on fish, by counting the eggs produced by infra-populations of *D. oliveri* over a 24-hour period, proved to be a reliable non-invasive/non-destructive method to quantify the intensity of an individual infra-population of parasites on a single host. This non-invasive method of testing was successfully utilised to test and evaluate existing drug formulations, potentially effective against *D. oliveri* infection for "off label" use. This method can also be used for early diagnosis of parasite infection, repeated on-going monitoring of the infection intensity of the parasite on the host and assessing treatment success of drugs on parasites. The knowledge of parasite egg production, together with the advantages and limitations of this method can now be used to further develop a

more accurate and specific sampling technique. The technique can be adapted and implemented as a practical diagnostic non invasive tool in future to determine the prevalence of *D. oliveri* in aquaculture production facilities. The method can also possibly be expanded to include additional parasite species for a more comprehensive application in the field. This will improve fish health management and better integrated parasite control.

This available immediate short term option for a treatment solution was pursued to evaluate the use of praziquantel as an effective "off label" drug against *D. oliveri* on dusky kob. Four treatment combinations of the two formulations were compared. The formulations were found to be more effective in removing adult stages than the juveniles, which will have an important bearing on the treatment regime. This is consistent with what Chisholm & Whittington (2002) found, that due to the smaller size of the juveniles, they might be more protected by the lamellae in the interlamellar spaces. The efficacy of the drugs in removing the parasites varied from very limited to being toxic to the fish on the other hand, which clearly demonstrated the dosage boundaries of these formulations as an "off label" application. Although praziquantel proved to be effective at concentrations as low as 2 ppm it did not eliminate the juvenile or adult stages completely. The advantages of the low concentrations were that there were less behavioural changes with lower levels of stress and lower toxicity. The 2-hour/2 ppm treatments were the least effective on the parasites. The high concentrations (20 ppm) were effective in removing all adult stages, but caused severe stress, behavioural changes and proved to be lethal in the case of the solution formulation after 18 hours. This study also demonstrated that although the same concentration

of 20 ppm praziquantel of the two formulations were used, there efficacy and toxicity differed due to possibly the other compounds in the formulation. Therefore caution should be taken to presume that different formulations with the same active ingredient will have the same result. The 2-hour/20 ppm treatments also effectively removed all adult parasites, but here again the solution formulation proved to be more stressful and toxic. The data from this study demonstrate that a exposure/concentration (2-hour/20 ppm suspension treatment) proved to be the treatment of choice, compared to the other treatments. Schmahl & Mehlhorn (1985) demonstrated that a praziquantel treatment of 3-hour/10 ppm, to be effective in removing *Dactylogyrus eztensus, D. vastus, Diplozoon paradoxum*. This treatment was also effectively used by Thoney & Hargis (1991) to eliminated *Gyrodactylus* spp. from *Rana catesbiana*. Szekely & Molnar (1990) demonstrated significantly reduced numbers of *A. vistulensis* on European catfish with a 5-hour bath at 10 ppm. Further investigation into effective drug concentration might prove that a reduced intermediate concentration of less than the maximum of 20 ppm will have the same favourable results in the reduction of parasites and with fewer side effects on the fish.

Cecchini *et al.* (1998) demonstrated that egg hatching of *Diplectanum aequans* starts on day four and was completed by day six at 20°C. Post-larval stage reached maturity after 15 and 25 days at temperatures of 26°C and 20°C respectively. Christison (2005) demonstrated hatching of *D. oliveri* eggs after 4 days and oncomiracidium development to mature egg production adults 14 days later at 25°C. With hatching and maturing periods very close for these two species at a similar temperature, a projection of 25 days for *D. oliveri* to reach maturity at

20°C is suggested. Thus, this treatment may be repeated after 29-31 days to target the next generation adult parasite before they go into egg production. It would have been ideal if the juvenile stages were more sensitive to the treatments than the adult, because then the immature non egg producing juveniles could be targeted and eliminated. The interruption of the parasite life cycle at the juvenile stage level would prevent the development of the next generation adult egg production.

The next step will be to determine tissue residues and depletion rate of the drug in fish treated with suggested dosage. It is important to remember that the two formulations also contain other chemicals that were added to enhance taste and improve drug suspension characteristics for their original application, which may also be present as a residue (Buchmann 1998). Kim *et al.* (2003) utilised praziquantel on cultured rockfish, *Sebastes schlegeli* Hilgendorf, 1880 as an in feed treatment at concentrations of 200 $mg.Kg^{-1}$ and 400 $mg.Kg^{-1}$ bodyweight respectively. Three consecutive in feed treatments were fed at 24-hour intervals. Post treatment tissue samples revealed that with the 200 $mg.Kg^{-1}$ treatment, praziquantel was still detected until one day post-treatment in muscle and three days post-treatment in the skin. With the 400 $mg.Kg^{-1}$praziquantel was detected up to five days post-treatment in muscle and 6 days post-treatment in the skin with a consistent decline in tissue residue with time lapsed.

Through the initiative taken by Marine and Coastal Management (MCM) of the Department of Environmental Affairs and Tourism to grant funds through a Frontier Programme to address Mariculture Research and Development and together with the cooperation of a pharmaceutical company committed to sponsor

laboratory tests during the drug trials and to provide two veterinary drug formulations, it was demonstrated that relevant research was possible with promising results.

The promising results of this pilot study proved that it was possible to identify, test and evaluate existing approved drugs for off label use in aquaculture. This successful drug evaluation may encourage the future evaluation of other potentially effective drugs for an extra-label use against *D. oliveri* and other immerging parasites. Future drugs may prove to be effective against other stages in the parasites life cycle and pursuing and evaluating in feed treatments as alternatives. An increase in number of drugs available creates the opportunity and option to use drugs in an alternating succession to develop a better parasite management regime and to reduce parasite drug resistance. In a combined effort by all parties in the support sector to diligently cooperate and solve immerging challenges in drug treatments, it will prevent the aquaculture industry from being overwhelmed by the challenges. There was never a better time than now for universities, pharmaceutical companies, private research laboratories and government research facilities to sponsor and start gathering relevant information on pathogens, parasite biology, infection dynamics and epidemiology to support the evaluation and testing of drugs for the aquaculture industry.

CHAPTER 7: References

1. American Fisheries Society Fish Culture Section (2011) Guide to Using Drugs, Biologics, and Other Chemicals in Aquaculture.

2. AVMA. Minor Use and Minor Species Health Act. American Veterinary Medical Association Web site. Available at: http://www.avma.org/science/mums/default.asp.

3. Becker B., Mehlhorn H., Andrews P., Thomas H., and Eckert J. (1980). Light and electron microscopic studies on the effect of praziquantel on *Schistosoma mansoni, Dicrocoelium dendriticum* and *Fasciola hepatica* (trematoda) in vitro. *Z. Parasitenkd* **63:** 113 – 128.

4. Beugnet F., Polack B., and Dang H. (2008) *Atlas of coproscopy*. Kalianxis, Davout, Paris, France, 277pgs.(ISBN 978-2-9157-5815-3).

5. Bouloux C., Langlais M., and Silan P. (1998) A marine host-parasite model with direct biological cycle and age structure. Ecological Modelling **107:** 73 – 86.

6. Buchmann K. (1987). Effects of praziquantel on the monogenean gill parasite *Pseudodactylogyrus bini.* Acta Veterinaria Scandinavica. **28**: 447 – 450.

7. Buchmann K. (1993). Screening of 22 anthelmintic for their efficacy against the gill-parasitizing monogeneans *Pseudodactylogyrus* spp. from the European eel (*Anguilla anguillae).* Bulletin of the Scandinavian Society for Parasitology **1:** 11 – 19.

8. Buchmann K. (1997) Infection biology of gill parasitic monogeneans with

special reference to the congeners *Pseudodactylogyrus bini* and *P. anguillae* (Monogenea: Platyhelminthes) from the European eel. Dissertation, Royal Veterinary and Agricultural University, Frederiksberg, Denmark.

9. Buchmann K. (1998)a Chemotherapy of monogenean infections in fish. IX International Congress of Parasitology. Makuhari Messe Chiba, Japan, pgs 251 – 259.

10. Buchmann, K. (1998)b. Chemotherapy of monogenean infections in fish. *Symposium (C) / Parasitology international 47 (suppl.) (1998) 71 – 95*

11. Buchmann K., and Bresciani J. (2006) Monogenea (Phylum Platyhelminthes). In Woo P.T.K. Fish Diseases and Disorders , Volume 1: Protozoan and Metazoan Infections, 2nd Edition, CABI, CABI Head office, Nosworthy Way, Wallingford, Oxfordshire OX 10 8DE, pgs 297 – 344.

12. Campbell W.C. (1986). The chemotherapy of parasite infections. *Journal of Parasitology* **72:** 45 – 61.

13. Cecchini S. (1994) Influence of temperature on the hatching of eggs of *Diplectanum aequans*, a parasite of sea bass. *Aquaculture International* **2:** 249 – 253.

14. Cecchini S., Saroglia M., Berni P., and Cognetti-Varriale M. A. (1998) Influence of temperature on the life cycle of *Diplectanum aequans* (Monogenea: Diplectanidae), parasitic on sea bass, *Dicentrarchus labrax*. *Journal of Fish Diseases* **21:** 73 – 75.

15. Chambers C.B., and Ernst I. (2005) Dispersal of skin fluke *Benedenia seriolae* (Monogenea: Capsalidae) by tidal currents and implications for sea-cage farming of *Seriola* spp. *Aquaculture* **250:** 60 – 69.

16. Chisholm L.A., and Whittington I.D. (1996). Descriptions of the larvae of six species of monocotylid monogeneans from *Himanturafai* (Dasyatididae) and *Rhinobatos typus* (Rhinobatidae) from Heron Island, Great Barrier Reef, Australia. *Systematic Parasitology* **35:** 145 **-** 156.

17. Chisholm L.A., and Whittington I.D. (2002). Efficacy of praziquantel bath treatments for monogenean infection of the *Rhinobatos typus. Journal of Aquatic Animal Health* **14:** 230 – 234.

18. Christison K.W. (2005) *Diplectanum* infections in cultured kob (*Argyrosomus japonicus*) in South Africa. Proceedings of the 5th International Symposium on Monogenea held in Guangzhou, China, pgs 67.

19. Christison K.W., and Mouton A. (2007) Qualitative Disease Risk Assessment with respect to Irvin & Johnson's proposed sea-cage aquaculture project in Mossel Bay, pgs 36.

20. Cone D.K. (1995) Monogenea (Phylum Platyhelminthes). In: Woo PTK (ed) Fish diseases and disorders: protozoan and metazoan infections. CAB international, Wallingford, pgs. 289 - 327.

21. Cringoli G., Rinaldi L., Veneziano V., Capelli G., and Scala A. (2004) The influence of flotation solution, sample dilution and the choice of McMaster slide area (volume) on the reliability of the McMaster technique in estimating the faecal egg counts of gastrointestinal strongyles and *Dicrocoelium* in sheep.

Veterinary Parasitology **123:** 121 – 131.

22. Department of Water Affairs and Forestry (DWAF). 1995. South African Water Quality Guidelines for Coastal Marine Waters. Volume **4**:2-1 Mariculture.

23. Deveney M.R., Chisholm L.A., and Wittington I.D. (2001) First published record of the pathogenic monogenean parasite *Neobenedenia melleni* (Capsalidae) from Australia. *Diseases of Aquatic Organisms* **46:** 79 – 82.

24. Dezfuli B.S., Giari L., Simoni E., Menegatti R., Shinn A.P., and Manera M. (2007) Gill histology of cultured European sea bass, *Dicentrarchus labrax* (L.), infected with *Diplectanum aequans* (Wagner 1857) Diesing 1958 (Diplectanidae: Monogenea). *Parasitology Research* **100:** 707 – 713.

25. Dryden M.W., Payne P.A., Ridley R., and Smith V. (2005) Comparison co common fecal flotation techniques for the recovery of parasite eggs and oocysts. *Veterinary Therapeutics* **6(1):** 15 – 28.

26. Ernst I., Whittington I.D., Corneillie S., and Talbot C. (2002) Monogenean parasites in sea-cage aquaculture. *Austasia Aquaculture* **16:** 46 – 48.

27. Ernst I., Whittington I.D., Corneillie S., and Talbot C. (2005) Effects of temperature, salinity, desiccation and chemical treatments on egg embryonation and hatching success of *Benedenia seriolae* (Monogenea: Capsalidae), a parasite of farmed *Seriolae* spp. *Journal of Fish Diseases* **28:** 157 – 164.

28. Ferguson H. (2006) *Systemic Pathology of Fish: A text and atlas of normal tissue responses in teleosts, and their responses in disease.* Scotian Press; London, United Kingdom, pgs 366 (ISBN - 978-0-9553037-0-8).

29. Fetterer R.H., Pax R.A., and Bennett J.L. (1980). Praziquantel, potassium and 2,4-dinitrophenol: analysis of their action on the musculature of *Schistosoma mansoni. European Journal of Pharmacology* **64:** 31 – 38.

30. Gannicott A.M., and Tinsley R.C. (1997) Egg hatching in the monogenean gill parasite *Discocotyle sagittata* from the rainbow trout (*Oncorhynchus mykiss*). *Parasitology* **114:** 569 – 579.

31. Genten F., Terwinghe E., and Dunguy A. (2009) *Atlas of Fish Histology.* Science Publishers, 234 May Street, Post Office Box 699, Enfield, New Hampshire 03748, United States of America, pgs 215 (ISBN 978-1-57808-544-6).

32. Gonzales-Lanza C., Alvarez-Pellitero P., and Sitja-Bobadilla A. (1991) Diplectanidae (Monogenea) infestations of sea bass, *Dicentrarchus labrax* (L.) from the Spanish Mediterranean area. *Parasitology Research* **77:** 307 – 314.

33. Griffiths M.H. (1996). Life history of the dusky kob *Argyrosomus japonicus* (Sciaenidae) off the east coast of South Africa. *South African Journal of Marine Science* **17:** 135 – 154.

34. Grutter A.S., and Pankhurst N.W. (2000). The effects of capture, handling, confinement and ectoparasite load on plasma levels of cortisol, glucose and lactate in coral reef fish *Hemigymnus melapterus*. *Journal of Fish Biology* **57:** 391-401.

35. Harnett W. and Kusel J.R. (1986). Increased exposure of parasite antigens at the surface of adult male *Schistosoma mansoni* exposed to praziquantel in vitro. *Parasitology* **93:** 401 – 405.

36. Hayat M.A. (1993). *Stains and Cytochemical Methods*. Plenum Press, New York, USA, pgs. 63-64.

37. Hecht T., and Britz P. J. (1992). The current status, future prospects and environmental implications of mariculture in South Africa. *Suid Afrikaanse Tydskrif vir Wetenskap* ***88*****:** 335**-**336.

38. Hecht T., Britz P. J., and Uys W. (1992). Aquaculture in South Africa. *World aquaculture* ***23*****(1)**, 8.

39. Heemstra P., and Heemstra E. (2004) *Coastal Fishes of South Africa,* National Inquiry Service Centre (NISC), Grahamstown, South Africa, pgs 488. (ISBN1-920033-01-7).

40. Hendrix S. (2004) Some aspects of the biology and life history of *Bothitrema bothi* (Monogenea: Bothitrematidae) from the flounder, *Scophthalmus aquosus* (Bothidae), from New Jersey, USA. *Folia Parasitologica* **51:** 229 – 237.

41. Hinrichsen E., and Brink D. (2004) Clarification of the Department of Water Affairs and Forestry's Requirements for Regulating the Utilisation of Water for Aquaculture Purposes and Development of Relevant Protocol. The Department of Water Affairs and Forestry, Inception report, Project number (June 2004) 2003 – 325.

42. Ivona M. (2006) Check list of the parasitofauna in the Adriatic Sea cage-

reared fish. Acta Veterinaria (Beograd), Volume **56**, Number 2-3, pgs 285 – 292.

43. JSA. United States Joint Subcommittee on Aquaculture Web site. Available at: http://ag.ansc.purdu.edu/aquanic/jsa/.

44. Kearn G.C. (1968) The development of the adhesive organs of some diplectanid, tetraonchid and dactylogyrid gill parasites (Monogenea). *Parasitology* **58:** 149 - 163.

45. Kearn G.C. (1973) An endogenous circadian hatching rhythm in the monogenean skin parasite Entobdella soleae and its relationship to the activity rhythm of the host (Solea solea). *Parasitology* **66:** 101 – 122.

46. Kearn G.C. (1985) Observation on egg production in the monogenean *Entobdella soleae. International Journal for Parasitology* **31 (8):** 815-821.

47. Kearn G.C. (1986) The eggs of monogeneans. *Advanced Parasitology* **25:** 175 – 273.

48. Kearn G.C. (2002) *Entobdella soleae* – pointers to the future. *International Journal for Parasitology* **32:** 367 – 372.

49. Kearn G.C. (2004) *Leeches, Lice and Lampreys: A natural history of skin and gill parasites of fishes*. Springer, Dordrecht, The Netherlands, pgs 432.(ISBN 1-4020-2925-X).

50. Kim C.O., Cho J.B., Ahn K.J., Lee J.I., and Kim K.H. (2003) Depletion of praziquantel in muscle tissue and skin of cultured rockfish (*Sebastes schlegeli*) under the commercial culture conditions. *Aquaculture* **219:** 1 – 7.

51. Lewbart G.A. (1998). Self-*assessment Colour Review of Ornamental Fish.* Manson, Corringham Road, London, pgs. 192.(ISBN: 1-874545-81-2).

52. Llewellyn L., and Simmons J.E. (1984) The attachment of the monogenean parasite *Callorhyncicola multitesticulatus* to the gills of its holocephalan host *Callorhynchus millii. International Journal for Parasitology* **14:** 191 – 196.

53. Macdonald S., and Jones A. (1978) Egg-laying and hatching rhythm in the monogenean *Diplozoon homoion gracile* from the southern barbell (*Barbus meridionalis*). *Journal of Helminthology* **52:** 23 – 28.

54. Macdonald S., and Llewellyn J. (1980) Reproduction in *Acanthocotyle greeni* N sp. (Monogenea) from the skin of *Raia* spp. At Plymouth. Journal of Marine Biology Association. United Kingdom **60:** 81 – 88.

55. Mansell B., Powell M.D., Ernst I., and Nowak B.F. (2005) Effects of the gill monogenean *Zeuxapta seriolae* (Meserve,1938) and treatment with hydrogen peroxide on pathophysiology of kingfish, *Seriola lalandi* Valenciennes,1833. *Journal of Fish Diseases* **28:** 253-262.

56. Mes T.H.M. (2003) Technical variability and required sample size of helminth egg isolation procedures. *Veterinary Parasitology* **115:** 311 – 320.

57. Mooney A.J., Ernst I., and Whittington I.D. (2006) An egg-laying rhythm in *Zeuxapta seriolae* (Monogenea: Heteraxinidae), a gill parasite of yellowtail kingfish (*Seriolae lalandi*). *Aquaculture* **253:** 10 – 16.

58. Margolis L., Esch G.W., Holmes J.C., Kuris A.M., and Schad G.A. (1982) The use of ecological terms in parasitology (report of an ad hoc committee of

the American Society of Parasitology). *Journal of Parasitology* **68**(1)**:** 131 - 133.

59. NEPAD (2005) ZAMBIA: Support to NEPAD-CAADP Implementation, TCP/ZAM/2906 (1) (NEPAD Ref. 04/01 E), pgs. 17.
60. Ogawa K. (1994) *Anoplodiscus tai* sp. nov. (Monogenea: Anoplodiscidae) from cultured red sea bream *Pagrus major. Fish Pathology* **29:** 5 – 10.
61. Ogawa K. (1997) Copulation and egg production of the monogenean *Heterobothrium okamotoi*, a gill parasite of cultured tiger puffer (*Takifugu rubripres*). *Fish Parasitology* **32:** 219 – 223.
62. Ogawa K. (2002) Impacts of diclidophorid monogenean infections on fisheries in Japan. *International Journal for Parasitology* **32:** 373 – 380.
63. Olson K.R. (1991) *Journal of Electron Microscopy Technique* **19:** 389 – 405.
64. Ostrander G.K. (2000) *The Handbook for Experimental Animals: The Laboratory Fish.* Academic Press, A Harcourt Science and Technology Company, Harcourt Place, London, pgs 678 (ISBN 0-12-529650-9).
65. Paling J.E. (1966) The attachment of the monogenean *Diplectanum aequans* (Wagener) Diesing to the gills of *Morone labrax* L. *Parasitology* **56:**493 – 503.
66. Paperna I. (1996) Parasites, infections and diseases of fish in Africa - An update Rome, FAO. *CIFA Technical Paper* No. **31**, pgs. 220. (ISBN 92-5-103772-8).
67. Paperna I., Colorni A., Gorgin H., and Kissil G.W. (1977) Diseases of *Sparus aurata* in marine culture at Elat. *Aquaculture* **10:** 195-213.

68. Paperna I., Diamant A., and Overstreet R.M. (1984) Monogenean infestations and mortality in wild and cultured red sea fishes. *Helgoländer Meeres-Untersuchungen* **37:** 445 – 462.

69. Rajendran K.V., Thirunavukkarasu A.R., and Santiago T.C. (2000) Mortality of sea bass, *Lates calcarifer* (Bloch) due to monogenetic parasite, *Diplectanum latesi* (Tripathi, 1957). *Journal of Aquaculture in the Tropics* **15(3):** 119 - 206.

70. Reno P. W. (1998) Factors involved in the dissemination of disease in fish populations. *Journal of Aquatic Animal Health*, **10:** 160-171.

71. Rohde K. (2005) *Marine Parasitology.* Csiro publishing, 150 Oxford Street, Collingwood, Victoria, Australia, pgs 565. (ISBN 0 643 09025 8).

72. Roubal F.R. (1994) Observations on the egg and fecundity of dactylogyrid and diplectanid monogeneans from the Australian marine sparid fish, *Acanthopagrus australis. Folia Parasitologica* **41:** 220 – 222.

73. Schmahl G., and Mehlhorn H. (1985). Treatment of fish parasites. 1. Praziquantel effective against monogenea (*Dactylogyrus vastator, Dactylogyrus extensus, Diplozoon paradoxum). Zeitschrift fur Parasitenkunde* **71:** 727 -737.

74. Schnick R. A., Alderman D. J., Armstrong R., Le Gouvello R., Ishihara S., Lacierda E. C., Percival S., and Roth M. (2005) World Aquaculture Drug and Vaccine Progress. *Worldwide aquaculture drug and vaccine registration progress.* JSA. (United States Joint Subcommittee on Aquaculture).

75. Scholz T. (1999) Parasites in cultured and feral fish. *Veterinary Parasitology* **84:** 317-335.

76. Sharp N.J., Diggles B.K., Poortenaar D.W., and Willis, T.J. (2004). Efficacy of Aqui-S, formalin and praziquantel against the monogeneans, *Benedenia seriolae* and *Zeuxapta seriolae,* infecting yellowtail kingfish *Seriola lalandi lalandi* in New Zealand. *Aquaculture* **236** (2004)**:** 67 - 83.

77. Smyth J.D., and Halton D.W. (1983) *The Physiology of Trematodes*, 2nd edition, Cambridge University Press, Cambridge.

78. South African Veterinary Council (SAVC). (1998) *News from your Council* **23:** 2.

79. Stephens F.J., Cleary J.J., Jenkins G., Jones J.B., Raidal S.R., and Thomas J.B. (2001). Treatments to control *Haliotrema abaddon* in the West Australian dhufish, *Gloucosoma hebraicum. Aquaculture* **215 (2003):** 1 - 10.

80. Storey S. (2005) Challenges with the development and approval or pharmaceuticals for fish. *The AAPS Journal:* **7**(2) Article 32, 335-343.

81. Stuttgart H.K.D. (2005) Evaluation of compounds and strategies for controlling aquatic animal disease. United States of America Agricultural Research Service www.ars.usda.gov/research/projects/projects.htm//ACCN_NO=409106&fy+2005#page 1. Accessed April 25, 2007.

82. Swan G.E., Sykes R.D., Banoo S., and Naidoo. V (2004) *Veterinary drug control and management for the practising veterinarian in South Africa.* LAKATO (pty) limited.

83. Sykes R.D. (2007) Pers. comm.

84. Szekely C., and Molnar K. (1990). Treatment of *Ancylodiscoides vistulensis* monogenean infestations of the European catfish (*Silurus glanus*). *Bulletin of the European Association of Fish Pathology* **10:** 74 - 77.

85. Thienpont D., Rochette F., and Vanparijs J.F.O. (1979) *Diagnosing helminthiasis through coprological examination.* Janssen Research Foundation, Beerse, Belgium, pgs. 187. (361/521401).

86. Thoney D.A. (1989). The effects of various chemical on mongeneans parasitizing the skin of elasmobranches. American Association of Zoological Parks and Aquariums, Annual Conference Proceedings, Pittsburgh, PA, September 24 -28: pgs. 217 – 221.

87. Thoney D.A. (1990). The effects of trichlorfon, praziquantel and copper sulphate on various stages of the monogenean *Benedeniella Posterocolpa,*a skin parasite of the cownose ray, *Rhinoptera Bonasus. Journal of Fish Diseases* **13:** 385 – 389.

88. Thoney D.A., and Hargis W.J. (1991). Monogenea (Platyhelminthes) as hazards for fish in confinement. *Annual Review of Fish Diseases* **3:** 133 – 153.

89. Tinsley R.C. (2004) Platyhelminth parasite reproduction: some general principles derived from monogeneans. Canadian *Journal of Zoology* **82:** 270 – 291.

90. Tubbs L.A., Poortenaar C.W., Sewell M.A., and Biggles B.K. (2005) Effects of temperature on fecundity in vitro, egg hatching and productive development of *Benedenia seriolae* and *Zeuxapta seriolae* (Monogenea) parasitic on yellowtail kingfish *Seriola lalandi*. International *Journal for Parasitology* **35:**

315 – 327.

91. Van Gass (2007) Newsletter of Aquaculture Association of South Africa & Aquaculture Institute of South Africa **4: (02)** 15 – 16.

92. Ward M.P., Lyndal-Murphy M., and Baldock F.C. (1997) Evaluation of a composite method for counting helminth eggs in cattle faeces. *Veterinary Parasitology* **73:** 181 – 187.

93. Wedemeyer G.A. (1996) *Physiology of fish in intensive culture systems.* Chapman & Hall, 115 Fifth Avenue, New York, pgs. 232. (ISBN 0-412-07801-5).

94. Wedemeyer G.A., Barton B.A., and McLeay D.J. (1990). Stress and acclimation. C. Schreck and P. Moyle (eds.), Methods of Fish Biology, American Fisheries Society, Bethesda, Maryland. pgs 451-489.

95. West G.P. (1988) *Black's Veterinary Dictionary*, sixteenth edition, A&C Black's, 35 Bedford Row, London, pgs. 703. (ISNB 0-7136-3004-3).

96. Whittington I.D. (1998). Diversity down under: monogeneans in the antipodes Australia with a prediction of monogenean biodiversity worldwide. *International Journal of Parasitology* **28:** 1481 -1491.

97. Whittington I., Ernst I., Corneillie S., and Talbot C. (2001) Sushi, fish & parasites. *Australasian Science* (April) pgs. 33 – 36.

98. Whittington I.D., and Ernst I. (2002). Migration, site-specificity abd development of *Benedenia lutjani* (Monogenea: Capsalidae) on the surface of its host, *Lutjanus Carponotus* (Pisces: Lutjanidae). *Parasitology* **124:** 423 – 434.