MANAGING *ICHTHYOPHONUS* IN MULTI-SPECIES EXHIBITS AT THE TWO OCEANS AQUARIUM

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Declaration

I declare that this is my own work, that **Managing *Ichthyophonus* in multi-species exhibits at the Two Oceans Aquarium** 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.

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ABSTRACT

Ichthyophonus hoferi has been diagnosed in multiple species at the Two Oceans Aquarium, this study focuses on *Rhabdosargus globiceps* (White stumpnose). *I. hoferi* is a mesomycetozoan parasite that multiplies in blood rich organs in the fish hosts causing a wide range of clinical signs resulting in organ dysfunction. *I. hoferi* can be diagnosed from microscopic examination of tissue squash preparations, culture, polymerase chain reaction (PCR) and histopathology. In the literature only lethal methods of diagnosis are described. The development of a non-lethal diagnostic tool for disease monitoring is vital for collections where euthanasia of specimens is not possible. Liver biopsies were obtained from potentially infected *R. globiceps* (n=30) comparing two surgical methods; coeliotomy (n=15) and coelioscopy (n=15). A control group (n=10) did not undergo any surgical procedure. In this study, coeliotomy refers to an incision which was made through the body wall from which the liver was exteriorised and biopsied. Coelioscopy was performed with the use of an endoscope. Two small holes were made in the body wall, one hole for the biopsy forceps and the other for the endoscope. The liver was located with the endoscope and biopsied with the forceps. Biopsy material for each fish was divided into three sections for three methods of diagnostic testing including; tissue squash preparation, PCR and culture. Post-surgical monitoring was also conducted for 6 weeks on all fish, these included photographs of the surgical site to monitor healing over time, blood sampling to provide an indication of health and also a physical check of the fish for any post-surgical complications. The blood parameters measured were packed cell volume (PCV), glucose, total proteins and white blood cell counts. All fish were monitored for 6 weeks post-surgery and blood samples drawn at weeks 0, 1, 2, 5 and 6. After 6 weeks the fish were euthanised for full post mortem examination. The liver, kidney, spleen and heart were examined for *I. hoferi* using tissue squash preparation and histopathology. The liver was further tested at post mortem using culture and two PCR methods (conventional and nested) to detect *I. hoferi*. The diagnostic gold standard, was determined by positive results in two or more tests at post mortem, this allowed correct assignment to one of two groups; *I. hoferi* infected fish and non-infected fish. The tissue squash preparation at post mortem provided insight into which other organs were infected with *I. hoferi*. Of the 40 fish, 32 fish were found to be positive for *I. hoferi* by tissue squash preparation of the liver, spleen, kidney and heart at post mortem. *I. hoferi* was identified in the spleen in 94% of infected fish (30 out of 32), in the liver in 78% of infected fish (25 out of 32), in the kidney in 72% of infected fish (23 out of 32) and in the heart in 69% of infected fish (22 out of 32 fish). The post-surgical blood parameters provided an

indication of how well the fish recovered and healed over the six weeks post-surgery. The control group did not undergo any surgical procedures but underwent weekly blood sampling. At week 2, in the surgical groups, there was an initial decrease in PCV, an increase in glucose and an increase white blood cell counts compared with the control group. At week 6, when compared to the control group; PCV, glucose and white blood cell counts showed no significant difference and all fish displayed similar health profiles. This suggests that after six weeks the fish had healed and returned to normal physiological functioning after both surgical procedures, with some fish being infected and others non-infected. The sensitivity of tissue squash preparation, culture, C-PCR, N-PCR and histology for the diagnosis of *I. hoferi* on liver biopsies were low at 50%, 30%, 0% and 10%, respectively, for biopsies obtained by coeliotomy and 64%, 21%, 0% and 0%, respectively, for biopsies obtained by coelioscopy. When the surgical groups were combined to determine the overall sensitivities of the biopsy diagnostics of tissue squash preparation, culture, conventional PCR and nested PCR the percentages were, 58%, 25%, 0% and 4%. Tissue squash preparation and culture proved to be better tests at diagnosing *I. hoferi* in liver biopsies when compared to PCR and histology. The surgical approach of choice would be coelioscopy. PCR was the least sensitive diagnostic test.

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CONTENTS

CHAPTER 2 – Materials and Methods

CHAPTER 3 – Results: Surgery, post-surgical health and haematology

CHAPTER 1

Introduction

1.1 Background

Ichthyophonus hoferi Plehn and Muslow (1911) is a fungal or fungal-like parasite that affects marine bony fishes, is easily transmitted and shows a high infectivity between hosts (von Hofer, 1893). It has a widespread geographical distribution occurring in cold and temperate oceans in the Northern and Southern hemisphere (McVicar, 1982). Infections of *I. hoferi* in isolated freshwater environments have occurred due to fish being fed *I. hoferi* infected marine fish (Slocombe, 1980). Since 1916, *I. hoferi* has been responsible for a number of epizootics worldwide in wild and cultured fish resulting in large fish mortalities and economic losses (Franco-Sierra *et al.*, 1997; Mellergaard and Spanggaard, 1997; Rahimian and Thulin, 1996; Slocombe, 1980). It has been documented in more than 80 species of fishes since its discovery (Spanggaard *et al.*, 1994). There are historical records suggesting that reptiles, elasmobranches and piscivorous birds have been infected (McVicar, 1999; Mikaelian *et al.*, 2000). Despite historical reports there is no scientific evidence to support the diagnoses in birds, reptiles and elasmobranchs, however Mikaelian *et al.* (2000) provide evidence of *Ichthyophonus*-like infections in amphibians. The infection in amphibians was found to be different to that of fish, whereby only the skeletal muscle, and no other organ, was infected (Mikaelian *et al.*, 2000). Mammals, including humans, are not susceptible to *I. hoferi* infections and there have been no reported cases to suggest otherwise (McVicar, 1999).

1.2 Taxonomy

I. hoferi was first discovered over a century ago by Bruno von Hofer in 1893 (Hofer, 1893). *I. hoferi* is a eukaryote and belongs to the supergroup Opisthokonta. In this supergroup are the true fungi and the Mesomycetozoea (Gozlan *et al.*, 2014). Two orders exist within the Mesomycetozoea namely Dermocystida and Ichthyophonida, with *I. hoferi* belonging to Ichthyophonida (Gozlan *et al.*, 2014). Until recently the genus *Icthyhophonus* has been considered to comprise of only one species, *I. hoferi*. A second species or possibly a new

isolate, *Ichthyophonous irregularis* has been isolated from *Limanda ferruginea* (yellowtail flounder) off the Nova Scotia shelf (Rand *et al.*, 2000).

1.3 Morphology and life cycle

A wide variation exists regarding the size and shape of the same developmental stages of *I. hoferi* (McVicar, 2011). Varying nutritional and physiological factors may influence these morphological variations between hosts and even within different parts of the same host (McVicar *et al.*, 1985). The most common form that can be seen from a fresh tissue squash preparation is the presence of the spherical resting stages or schizonts which range between 10 to 250µm in diameter (McVicar, 2011). Two studies have described the effect of pH on morphology; when maintaining a low pH or when altering the pH levels, different morphological and developmental forms exist (Okamoto *et al.*, 1985; Spanggaard *et al.*, 1995). These varying morphologies were all documented using *in vitro* culture which provided a useful tool for describing the morphology *in vivo*. Depending on the life stage of the parasite these developmental forms include amoeboblasts, amoeboid bodies, plasmodium-like bodies, spherical uni- or binucleate bodies, thick-walled spherical multinucleate bodies or schizonts, and spherical multinucleate hyphal terminal bodies. Amoeboblasts and amoeboid bodies have been shown to be present in the blood after ingestion of infected tissue (Kocan *et al.*, 2013). These cells circulating in the blood stream are small enough to pass through many of the smaller blood vessels to enter into the blood rich organs. *I. hoferi* is not a true fungus, so for the purpose of this thesis and in accordance with the literature, hyphal bodies or hyphae-like structures will be termed germination tubes. With a reduction in pH from 7 to 3.5 *in vitro* germination tubes are developed rapidly from the schizonts (Spanggaard *et al.*, 1994). This occurs by cytoplasmic contents flowing up the germination tubes and accumulating in the tips. This happens within four to five hours after the pH is lowered. The germination tubes start to branch off and after three days the tips start to round up to spherical tubular terminal bodies also known as schizonts. An *in vitro* study demonstrated that by day seven the tips had rounded up to schizonts with no further development after day seven (Spanggaard *et al.*, 1994). When the pH was altered back to 7 from 3.5, it was observed that there were two methods by which cells multiplied; the first was after 24 hours the ends of the germination tubes would rupture, and the second was the multinucleate schizonts split into uni-nucleate cells which then developed into multinucleate cells within 3 days. The stomach of a fish has a low pH and it was suggested

that these germination tubes would start to develop once the infected tissue is in the host's stomach (Spanggaard *et al.*, 1994). This study describes the successional changes within the developmental forms in culture *in vitro* and these changes give a guideline on how the parasite may change or develop in response to differing pH levels. However this is not always a true reflection of what may happen *in vivo*. No germination tubes formed in the stomachs of the sculpin and rainbow trout that were examined in an experiment where *Leptocottus armatus* (Pacific staghorn sculpin) and *Oncorhynchus mykiss* (rainbow trout) were fed infected tissue (Kocan *et al.*, 2013). Similarly, Spanggaard *et al.* (1995) found that the multinucleate schizonts transitioned in the stomach to smaller uni-nucleate cells. Germination tubes were found in the stomachs of dead fish and may be the method of dispersal of infectious cells into the environment post death. This method of transmission may include a possible necrophagic intermediate host (Spanggaard *et al.*, 1995).

A large amount of ambiguity and uncertainty exists with regards to the life cycle of *I. hoferi* and a summary of plausible models have been compiled to attempt to explain observations reported in the literature and explain transmissions in wild populations (Kocan, 2019). There are several direct and indirect life cycle models. Kocan *et al.* (2013) suggests a direct life cycle in piscivorous fish whereby a cryptic amoeboid cell invades the host stomach following ingestion of infected tissue. The parasite will then be transported throughout the body via the blood stream. These cells then increase in size by asexual nuclear division and form schizonts in the host's organs. The host's body may respond and form a granuloma around the multinucleate schizonts and will try and prevent further spread of the parasite. These encapsulated granulomas will remain in the organs until the fish dies and is scavenged by another fish or is eaten by a carnivorous fish to repeat the life cycle (Kocan *et al.*, 2013). The route of transmission is the low pH allowing for *I. hoferi* to infect the fish through the stomach wall as mentioned previously. These small uninucleate cells may also be small enough to pass through the capillary beds of the gills and infect pelagic planktivirous species such as *Clupea harengus* (herring) (Kocan *et al.*, 2013). Another direct method of transmission is whereby a reservoir infection is maintained in a population or environment and the parasite is transmitted to another population that comes into contact with the maintaining population or environment (Haydon *et al.*, 2002). The reservoir infection is suggested to be maintained by a demersal piscivorous species inshore and when the pelagic planktivorous species migrate inshore and ingest waterborne cells that have been shed by the piscivorous species (Kocan, 2019). Two

indirect life cycle models are suggested by Kocan (2019) which include a proposed intermediate host and a free-living stage as part of the indirect life cycle. The intermediate host has been proposed as a copepod which acts as a carrier for the parasite until it is consumed by a planktivorous fish. This has been proposed but there is no clear evidence of a direct transmission of the parasite between hosts and both models need to be investigated further. Epidermal ulcers or papules described by McVicar (2011) provide some insight into a proposed life cycle of *I. hoferi* in both planktivorous and piscivorous fish species. Papules on the skin form ulcers which have been shown to release schizonts and it has been suggested that they might be consumed by new hosts (Kocan *et al.*, 2010). However, this has not been definitively demonstrated experimentally, and a study placing naïve *Clupea pallasii* with ulcer bearing conspecifics and attempting to infect the naïve fish was unsuccessful (Gregg *et al.*, 2012). In the wild, predation on fish is generally performed by consuming prey whole allowing the ulcer to be in direct contact with the stomach wall, exposed to hydrochloric acid and pepsin. This potentially provides a more rapid penetration of schizonts into the host as opposed to a prey item with no ulcers. If an infected host with no ulcers is consumed the schizonts could potentially be embedded in the bolus and pass through to an elevated pH in the intestine and ultimately passing through the whole digestive tract without infecting the host (Kocan *et al.*, 2013). This suggests these ulcers and papules provide a valuable link in the life cycle of *I. hoferi*.

1.4 Pathogenesis and clinical signs

I. hoferi has been documented to have a low parasite to host specificity in fish (Gozlan *et al.*, 2014; McVicar, 2011). The pathogenesis of *I. hoferi* infections are due to the disruption of infected tissue caused by the development and reproduction of schizonts in the organs. *I. hoferi* causes a systemic granulomatous inflammatory response in the host. In the advanced stages of growth of the parasite, when granulomatous inflammation and necrosis occur in response to the parasite, or if the parasite mass is large enough to disrupt the organ, the organ cannot continue to perform its normal function (Rahimian, 1998). In some advanced cases the parasite can be distributed to the brain where normal neurological function will be compromised (McVicar, 1982; Okamoto *et al.*, 1985; Paperna, 1986; Rahimian, 1998). *I. hoferi* spreads through the host via the blood stream or lymphatic system and lodges in the capillary beds. The blood rich organs are typically the more common locations of infection (McVicar, 1999). It is

found in the organs as spherical, thick-walled multinucleate cells which are commonly referred to as resting spores or schizonts (Mendoza *et al.*, 2002). *I. hoferi* can present itself as ulcerations on the skin caused by the tissue reaction to schizonts (Kocan *et al.*, 1999). In herring this is a common clinical sign and is often referred to as 'sandpaper skin' in the literature. *I. hoferi* multinucleate cells in these ulcerations are initially present in papules under the skin, which break the skin, forming ulcers and the epithelium starts sloughing (Kocan *et al.*, 2010). The schizonts move from the skin into the mucous layer and finally into the aquatic environment as viable infectious cells (Kocan *et al.*, 2010).

Infection occurs in most if not all of the visceral organs in fish but is often more prevalent in certain organs depending on the species of fish. In *Salmo trutta* (Brown trout) sampled in Tasmania and a *Mugilidae* sp. from a lagoon in Southern Africa *I. hoferi* was found to have a higher infection intensity in the liver, kidney and spleen (Slocombe, 1980; Paperna, 1986). In herring species (*C. harengus & C. pallasi*) *I. hoferi* had a high infection intensity in the heart when compared to other organs (Kocan *et al.*, 1999; Rahimian, 1998). In Chinook salmon *Oncorhynchus tshawytscha* the heart was the organ infected more heavily than the other organs (Zuray *et al.*, 2012). In wild and cultured sea bass (*Dicentrarchus labrax*) in the Spanish Mediterranean Sea, *I. hoferi* was most prevalent in the kidney (Sitja-Bobadilla and Alvarez-Pellitero, 1990). These varying organ susceptibilities could be linked to the fishes' behaviour and life history patterns (Zuray *et al.*, 2012).

1.5 Treatment and control

I. hoferi is considered a terminal disease therefore, rather than finding a cure, emphasis has been placed on its prevention (McVicar, 1999). This is also largely due to the fact that it has serious implications on populations of fish and is so easily transmitted between individuals. There have been recommendations of potential treatments but none have been tested and found to cure an animal infected with *I. hoferi* (McVicar, 2011). One study demonstrated that ketoconazole delayed the onset of infection but once a fish was infected it could not be cured (Hontoria *et al.*, 2013). Infections have been diagnosed in aquaculture facilities as a consequence of the feeding of fresh marine fish (Okamoto *et al.*, 1985). This can be avoided by feeding a pelleted feed and avoiding using fresh marine fish as a source of food. In a study examining varying temperatures, pH and salinities on organism viability, growth was observed

at a temperature range of 0-25°C and a pH range of 3-7 (Spanggaard and Huss, 1996). There was no growth or survival at 30°C and a 10% NaCl concentration (Spanggaard and Huss, 1996). A heat treatment of 40°C for three minutes has been shown to be effective at treating *I. hoferi* in feeds (Spanggaard and Huss, 1996). This is often not possible for many facilities and a temperature of -20°C is considered a viable alternative (Athanassopoulou, 1992). The schizonts of *I. hoferi* have been shown to be very resilient to varying physical conditions which are a problem in the control and treatment of the disease. The schizonts can remain viable in seawater for up to two years and in sterile seawater for up to six months (Spanggaard and Huss, 1996). Chlorine and Iodine solutions have been tested to sterilize *I. hoferi* schizonts *in vitro*. In an experiment varying concentrations of disinfectant were tested at intervals from 1-60 minutes. Schizonts used in the experiments were obtained from the heart tissues of *Clupea pallasii* (Pacific herring) that were positive for *I. hoferi* and were grown in culture. The schizonts were cycled through a high-low-high pH regime to promote the growth of germination tubes and resporulation (Hershberger *et al.*, 2008a). The study concluded that sea water effluent should be treated with >6ppm total chlorine for a minimum time of one hour, this will ensure an inactivation of 90% of *I. hoferi* schizonts. Freshwater effluent contact time can be reduced to five minutes at the same concentration. It was suggested that any laboratory instruments or nets that cannot be autoclaved, should be immersed in freshwater solutions containing ≥6ppm total halogen for 5-15 minutes to ensure complete inactivation of schizonts (Hershberger *et al.*, 2008a).

1.6 Epidemiology

Epidemiology refers to investigations of incidence, transmittance to other animals and the control of a disease (Margolis *et al.*, 1982). It is the study of the disease as it pertains to a whole population of animals rather than that of an individual animal (Margolis *et al.*, 1982). An important feature of any epidemiological study is the prevalence and the incidence of the disease. Prevalence is the number of host species that are infected with a parasite divided by the number of total individuals in a population that are examined for the parasite at one point in time (Margolis *et al.*, 1982). *I. hoferi* causes very different immune responses in different species of fish and clinical signs can vary between species and between individuals (McVicar *et al.*, 1985). *I. hoferi* is not distributed uniformly between different areas and within populations making it very difficult to determine true prevalence and incidence of disease in a

population in wild stocks. A low prevalence of *I. hoferi* does not necessarily mean a low incidence (McVicar, 2011). Incidence is the rate of new infections of the parasite by individuals within the host population and is described by the number of new hosts that have been infected in a certain time period divided by the number of uninfected hosts at the start of the experiment/time interval (Bush *et al.*, 1997; Margolis *et al.*, 1982).

I. hoferi has been shown to cause serious epizootics in the wild in species such as *C. harengus* (herring), *Oncorhynchus tshawytscha* (chinook salmon) and *Hippoglossus stenolepis* (halibut) but does not always cause a serious epizootic in all species (Sindermann and Scattergood, 1954; Mellergaard and Spanggaard, 1997; Zuray *et al.*, 2012; Dykstra *et al.*, 2013). The prevalence of *I. hoferi* can also be attributed to certain environmental conditions or cultured conditions. In *Sparus aurata* (Gilthead sea bream) and *Dicentrarchus labrax* (Sea bass) there was a higher prevalence in closed system culture conditions compared with open and semi-intensive systems. There have been no recorded reports of farmed stocks being infected with *I. hoferi* from wild populations despite their proximity to many of the wild epizootics that have occurred in European and Canadian waters (McVicar, 1999). These epizootics have created more interest and epizootiological research into the disease. It can be hypothesized that epizootics caused by fish parasites are not common in the wild, not because they do not occur but because they are not easily found or observed due to the difficulty in monitoring or observing fish in the ocean (McVicar, 2011). Scientific infrastructure may also not be available in the respective area, country or coastline. When mass fish mortalities occur, unless these fish are netted, floating or wash up before being eaten or decomposed they will not be found. *I. hoferi* is thus very different to any other parasite as there have been a number of epizootics in wild populations due to this infection that have been recorded (McVicar, 2011). In wild stocks there have been surveillance programmes implemented to monitor the disease. After epizootics occurred off the Norwegian coast affecting *Clupea pallasii* (North Sea herring), a programme was implemented to try and quantify the extent of the disease by doing fish surveys and looking for infected fish. Due to the effect *I. hoferi* has on many commercial species, it has been of scientific interest since its discovery (Hjeltnes and Skagen, 1992).

1.7 Significance as a pathogen

I. hoferi has been found to adapt very well to host environments and is now widely recognised as a potential problem for wild populations of pelagic fish, cultured fish and aquaria (Hershberger *et al.*, 2008b).

1.7.1 Significance as a pathogen at the Two Oceans Aquarium

I. hoferi is known to cause disease and death of fish species in both wild and in captive environments. If introduced or attained in a captive environment like an aquarium it poses a risk to valuable individual fish species in a collection. Once it is diagnosed from an exhibit it is difficult to prevent other fish contracting the disease and can be a risk to all other animals housed in that exhibit and in other exhibits. Caution needs to be exercised with regards to feeding, cleaning and any movement of animals once it has been isolated from exhibit. It is difficult to know which species may be more susceptible than others and the necessary precautions with regards to biosecurity need to be implemented.

Currently the Two Oceans Aquarium houses a number of large, mature valuable teleost fish which are currently endangered and are red listed according to the SASSI guidelines (Cowley *et al.*, 2007). In the aquarium *I. hoferi* has been detected in marine fish from various exhibits and in a preliminary study of juvenile fish in one exhibit the prevalence was found to be 88.8% (G.Cole Pers comm)[1]. Observations included; ulcerations on the caudal peduncle, neurological symptoms such as whirling/spinning and redness in the eyes. In a preliminary study where five species of fish, were removed from another exhibit it was found that eight of the ten individuals were positively diagnosed for *I. hoferi* (G.Cole Pers comm)[1].

1.8 Diagnosis of the pathogen

Infection with *I. hoferi* can cause behavioural changes such as lethargy and nervous disorders (McVicar, 2011). External physical signs that could occur in some species are: roughening of the skin, ulcerations, lesions, papules, ulcers, and haemorrhaging of the skin. It may also result in other physical changes such as emaciation, colour anomalies and fluid accumulation

[1] Dr. Georgina Cole, the veterinarian at the Two Oceans Aquarium first identified *I. hoferi* (Unpublished data).

(McVicar, 2011). It should be cautioned that diagnosis cannot be made by observing behaviour and physical signs alone as these may mimic other diseases. Gross lesions visible in organs can be used to aid diagnosis of *I. hoferi* and have been used when making a diagnosis in large numbers of fish from wild populations (Rahimian and Thulin, 1996). Using gross pathology to diagnose *I. hoferi* accurately will ultimately be dependent on the level of infection and pathogenicity in the host, as well as the tissue observed (Kocan *et al.*, 2011). Macroscopic identification of white nodules in the heart in wild herring has been used to identify *I. hoferi* (Rahimian, 1998; Rahimian and Thulin, 1996). As stated, gross lesions will vary between species and individuals, and many infected individuals have no obvious lesions, therefore macroscopic diagnosis is unreliable. Macroscopic and microscopic visualization of tissue samples, polymerase chain reaction (PCR), culturing of tissue samples, histology and blood sampling are all established methods of diagnosing *I. hoferi* (Criscione *et al.*, 2002; Jones and Dawe, 2002; Kocan *et al.*, 2013; Rahimian and Thulin, 1996; Spanggaard *et al.*, 1994). Many of these methods are described in the literature with varying diagnostic and analytical sensitivities and specificities (Kocan *et al.*, 2011). In one study, macroscopic visualization of *Clupea harengus* hearts detected a lower infection prevalence than microscopic tissue squash preparations (Rahimian and Thulin, 1996). Microscopic visualization of organs and tissues has been used with great success and is considered the easiest and most cost effective diagnostic method (Kocan *et al.*, 2011). There are, however, few distinguishing features when observing these schizonts under magnification and caution should be exercised when diagnosing the disease, particularly when small numbers of the parasite are present in the host tissue (McVicar, 2011).

It has become common practice for researchers using microscopic techniques to support their diagnoses with PCR and culture. A study looked at the accuracy of PCR compared to histology and culture to determine the prevalence of *I. hoferi* in three areas of the Yukon in *O. tshawytscha* (Whipps *et al.*, 2005). Heart, kidney and muscle tissues were sampled and used in the diagnostic tests. The PCR test was highly sensitive for the diagnosis of *I. hoferi* when compared to the histology and culture. PCR was highly sensitive when performed on heart tissue of fish that were considered to have a heavy infection, however in fish with a low level infection the PCR test was less sensitive as *I. hoferi* is not uniformly distributed within each organ and within each fish (Whipps *et al.*, 2005).

Pathogen free laboratory reared *C. pallasii* were exposed to *I. hoferi* and used in a study to compare *in vitro* culture and histology (Kocan *et al.*, 1999). Of the 30 fish examined, 70% tested positive using culture methods and 7% were positive using histology (Kocan *et al.*, 1999). In a different study *Sebastes emphaeus* (Puget Sound rockfish) were tested for *I. hoferi* using *in vitro* culture, histology and gross lesions. They found that 11% (33/302) were positive by culture, 1% (3/302) by histology and 0% exhibited visible lesions (Halos *et al.*, 2005). A study on the sensitivity of culture was performed on *Oncorhynchus mykiss* (rainbow trout) where fish were exposed to *I. hoferi* and the hearts cultured *in vitro* (Kocan *et al.*, 2011). It was found that 98.7% (75/76) of the fish were positive for *I. hoferi.* In the same study a sample of 104 *O. tshawytscha* were tested for *I. hoferi* using histological evaluation. It was found that the histology evaluation detected 74% (77/104) of fish to be positive for *I. hoferi*. It has been shown that in clupeids, salmonids and cottids the heart is the organ infected most frequently and is often the only organ that is affected (Rand and Cone, 1990). A study looked at a potential amoeboid-like infective stage of *I. hoferi* found in the stomach bolus of *L. armatus* (pacific staghorn sculpin) and *Oncorhynchus mykiss* (rainbow trout) (Kocan *et al.*, 2013). These fish were all pathogen free and were fed infected herring tissue homogenate. Contrary to what was initially suggested by previous studies, germination tubes were not present in the stomach in this study (Kocan *et al.*, 2013; Spanggaard *et al.*, 1994). The amoeboid-like cells were found in the tunica propria (stomach wall) post ingestion of the infected tissue. Aortic blood was sampled and 30% (14/46) of the sculpins tested positive for *I. hoferi* using blood culture in the first six days post-exposure (Kocan *et al.*, 2013). The first positive blood sample using culture was after six hours post-exposure. The trout showed 31% (5/16) of the fish to be positive between 24 and 96 hours post-exposure using blood culture (Kocan *et al.*, 2013). After six days in both species the parasite was found sporadically in the blood (Kocan *et al.*, 2013). The use of blood in PCR testing had a very low sensitivity where only 8.7% (2/23) were positive by this method (Whipps *et al.*, 2005). Taking a blood sample and testing it for *I. hoferi* would be an ideal non-lethal way to obtain a diagnosis, but with the poor sensitivity suggested by this study it may not be an effective diagnostic tool.

It is clear that some techniques, when testing different organs, tissue or blood, prove to be more sensitive than others. It is largely recognised that the hierarchy of sensitivity is; *in vitro* culture, tissue squash preparations, histology and then gross lesions (Kocan, 2019). PCR is not included due to its inability to only detect live *I. hoferi* but can be used as a screening tool or method in

conjunction with another diagnostic method (Kocan, 2019). Traditionally *I. hoferi* has been diagnosed from tissue and organs from dead or freshly sacrificed fish. Scientific work into the diagnosis of the parasite in living animals is limited or non-existent and remains a novel concept. Many of the fish in the aquarium are endangered or threatened and therefore cannot simply be restocked from collecting wild stock. These animals require accurate diagnosis to enable them to be managed as individuals and as groups to determine their future in the aquarium. It is therefore vital for us to investigate possible methodologies to determining if it is possible to obtain adequate samples of an organ for diagnosis without sacrificing the animal. Diagnosis of disease in fish without using lethal methods often relies on external diagnostic techniques, for example sampling the gills, skin or fins for parasites. Sub-sampling a population and using necropsy to diagnose is often preferred if there is a fish or fish to sacrifice, however, when there are few or one valuable individual this is not acceptable or practical. There have been several studies that identified *I. hoferi* in the liver of various species of fish such as *L. armatus, Plecoglossus altivelis* (ayu), *C. harengus* in *Sprattus sprattus* (Sprat), and in *Pleuronectes flesus* (Flounder) *Scopelogadus beanii* (Günther), *O. tshawytscha* and *Oncorhynchus kisutch* (Coho salmon) (Gartner and Zwerner, 1988; Gavryuseva, 2007; Kocan *et al.*, 2004; Miyazaki and Jo, 1985; Olson, 1986; Rahimian, 1998). The liver is a large organ that could potentially be biopsied with minimal impact on fish health and used to determine if *I. hoferi* could be diagnosed from a liver biopsy.

Procedures such as ovarian and kidney biopsies can be performed on a fish and can provide valuable information. Ovarian biopsies are used to determine developmental stages of eggs. These can be performed by inserting a catheter tube into the oviduct and collecting ova via capillary action (Noga, 2011). Kidney biopsies can be used to diagnose bacterial and viral diseases in fish. The kidney in most fish is positioned behind the branchial chamber which allows access for needle aspiration (Noga, 2011). With the fish anaesthetized and by opening the operculum a needle can be inserted dorsally and then dorso-caudally to the last branchial arch (Noga, 2011). Another method of obtaining a biopsy sample is using a 'Tru-Cut' biopsy needle. A study published in 2014 proposed a needle core biopsy method to obtain liver tissue to use for histopathological research in fish (Tresise *et al.*, 2014). They performed the biopsies on *Clarias gariepenus* (Sharptooth catfish). This method uses a single entry approach and a sample of liver is cored out and the needle removed. This method is done blind and the position

of the liver needs to be determined on a necropsy of a dead sample of the same species before attempting in a live fish (Tresise *et al.*, 2014).

In preliminary sampling of fish infected with *I. hoferi* at the Two Oceans Aquarium, it was found that the liver and spleen showed a relatively high level of infection intensity when compared to other organs in *Rhabdosargus globiceps* (White Stumpnose). Choosing the spleen for the biopsy in *R. globiceps* would be technically difficult in comparison to the liver. The spleen is small in size compared to the liver and more challenging to access. The liver is also one of the few organs that can allow for some tissue loss and still function normally and this is the reasoning contributing to the selection of the liver as the organ to biopsy. To obtain a liver biopsy sample, two surgical methods will be compared, coelioscopy and coeliotomy. There has been some research into various aspects of fish surgery such as position of incisions, different suture materials, fish anaesthesia, wound healing, and biopsy methods (Murray, 2002). A priority would be that the fish heals normally and regains normal behaviour and functionality post-surgery. A non-lethal sampling method will be an invaluable tool for the future of valuable teleost fish at the Two Oceans Aquarium and any other facility that may need to diagnose a disease such as *I. hoferi*. When taking a biopsy to diagnose a disease, a balance must be established between sampling enough tissue for a diagnosis whilst refraining from sampling too much tissue which could be detrimental to the fish's health or result in death. Information on the size of liver tissue needed from a biopsy to successfully diagnose *I. hoferi* is not currently known and will need to be determined. This will also vary, corresponding to the level of infection in the animal. Another factor to consider would be where within the liver the parasite is located which may vary between individuals and in species.

1.9 Surgery

In the past, surgical procedures on fish, were often performed in water with the fish submerged. Surgery underwater poses many challenges such as wound contamination, viewing the surgical area and suturing (Stoskopf, 1993). Once fish anaesthetics became available and surgical practices progressed, it became common practice to perform surgery on fish out of the water. A pump with a pipe is used to keep oxygenated water containing the anaesthetic agent flowing over the gills to allow the fish to breathe and it can be kept in the position the practitioner needs to perform the surgery. Fish anatomy exhibits a similar pattern to vertebrate anatomy however

the placement of the organs and viscera in the peritoneal cavity differ vastly among taxa. Due to this the practitioner will need to gain a good understanding and knowledge of the specific anatomy of the fish they will be performing surgery on. This can be done by obtaining a specimen of that species and observing where all organs are placed in the peritoneal cavity (Murray, 2002). Surgery in fish is not novel and has been used to remove cutaneous masses from many freshwater fish. This is well documented by Harms and Lewbart (2000) who are seen as the pioneers in fish surgery along with Noga (2011) and Stopskof (1993).

Two surgical methods were tested on channel catfish *Ictalurus punctatus* (channel catfish) where 30 fish were assigned to a control, coeliotomy and coelioscopy group (Boone *et al.*, 2008). Pre- and post-surgical blood parameters were tested and the quality of biopsy specimens were also assessed. Similar to Boone *et al.* (2008), in this study, all surgical sites were monitored over time by means of photography and visual observation to obtain an indication of postsurgical healing and recovery.

1.9.1 Coeliotomy

Coeliotomy requires a vet with appropriate surgical skills and does not facilitate the need for expensive equipment. This approach may be better suited to wild sampling as access to electricity during field sampling may be limited (Boone *et al.*, 2008). A larger biopsy can be sampled using coeliotomy as the practitioner has access to more of the organ by exteriorization. If haemorrhage occurs post biopsy sampling, the practitioner has access to the site to terminate the bleeding. The surgical site healing process is expected to be longer in coeliotomy due to a larger incision being made and it being a more invasive procedure (Boone *et al.*, 2008).

1.9.2 Coelioscopy

Coelioscopy is the use of an endoscope housed in a sheath integrated with fibre optic light that can be inserted into the coelomic cavity of a fish. The endoscope has a camera that relays a video image onto a monitor to allow the practitioner to see where in the coelomic cavity the endoscope is positioned. Another incision needs to be made for the biopsy forceps and the user needs to triangulate the camera and the forceps to obtain a biopsy. An alternate method is by only making a single incision for entry and a biopsy sheath is used that allows for optical biopsy

forceps to be inserted along the sheath to biopsy directly in front of the camera. For the purpose of this study optical biopsy forceps were not available thus two incisions were made to obtain the biopsies. In conjunction with the monitor, most endoscopes are equipped with an insufflator. Insufflation expands the coelom with carbon dioxide (CO_2) providing better viewing of the relevant viscera in the coelom. There have been practitioners that use saline for insufflation and it is suggested that it reduces risk of post-surgical buoyancy problems that can arise in fish (Divers *et al.*, 2013). However, if using CO_2 this can be mitigated after surgery by ensuring all the CO_2 is removed from the coelom before the fish is recovered from anaesthetic.

It has been demonstrated that non-lethal liver biopsies can be successfully obtained from two species of sturgeon *Scaphirhynchus platorynchus* and *Scaphirhynchus albus* on the banks of the Mississippi using endoscopic equipment (Divers *et al.*, 2013). Non-lethal biopsy methods are becoming increasingly important and necessary as more fish become endangered and lethal sampling is becoming more restricted. In the case of the sturgeon the liver biopsies were needed for toxicology and laboratory analysis as these two species are affected by organic and inorganic chemical contaminants that end up in the river (Divers *et al.*, 2013). The main advantage of coelioscopy is the ability to gain a better view of the viscera *in situ* within the coelomic cavity, afforded by the telescope. Coelioscopy also allows for a minimally invasive approach with small incision sizes (Boone *et al.*, 2008). Some of the disadvantages would be the cost of the equipment required which also requires more skill, practice and training to operate. The size of the biopsy is limited by the size of the endoscopic biopsy forceps. If haemorrhaging does occur the surgeon does not have access to the site of the biopsy to attempt to stop blood loss. The overall recovery and healing process is expected to be shorter due to the smaller size of the surgical site and less invasive procedure, resulting in a reduced level of stress on the individual (Boone *et al.*, 2008).

1.10 Haematology and post-surgical health

Blood sampling would be performed at varying intervals post-surgery to provide an insight into the overall recovery of each surgical group compared to a control group that did not undergo surgery. Although the control group did not undergo surgery these fish may or may not have been infected with *I. hoferi* and depending on their infection levels could have influenced haematology results. Assessing the overall physical health, documenting healing

and monitoring the wound would provide a good measure of health and healing (Boone *et al.*, 2008). By including blood parameters and the change or stability thereof will provide valuable quantitative information on the physiology of the fish. The blood samples were used to test the following parameters, packed cell volume (PCV) (%), blood glucose (mmol/L), total proteins (TP) (g/dL^{-1}) and blood smears made for white blood cell counts.

Packed cell volume is used for many animals as an indicator of physiological and overall health; with a lower PCV most commonly indicating anaemia or bleeding and a higher PCV possibly indicating dehydration (Weiss and Wardrop, 2011). There are no current PCV reference values for *R. globiceps*, however, five sparid species in three separate studies were sampled and reference values for these species were determined (Mozanzadeh *et al.*, 2015; Shen *et al.*, 2011; Yildiz, 2009). The average PCV for farmed *Rhabdosargus sarba* in a toxicology study in Hong Kong was 30.8% (Shen *et al.*, 2011). Similarly, the average PCV values for *Lithognathus mormyrus* was 28.87%, *Dentex dentex* was 34.02% and *Sparus aurata* was 29.23% (Yildiz, 2009). In *Sparidentex hasta*, the average PCV was 29.6% (Mozanzadeh *et al.*, 2015).

Blood glucose is a non-enzymatic and non-protein component of fish serum and can be a good indicator of stress in fish (Morgan and Iwama, 2011). Stoskopf (1993) showed that glucose increased in fish that had severe bacterial infections for 24 hours before it decreases precipitously.

Total proteins have been shown to be an indicator of health, stress, presence of infection and nutritional condition (Mozanzadeh *et al.*, 2015). Total protein consists of albumin and globulin which are components of blood. It has been shown that total protein levels decreased where carp had septicaemia from *Aeromonas* sp infections due to a decrease in circulating immunoglobins (Evenberg *et al.*, 1986). These infections can result in elevated glucose levels initially, but then decrease with lowered PCV and total erythrocyte counts.

Fish blood comprises of erythrocytes, leukocytes and thrombocytes. The leukocytes, or white blood cells, are the most morphologically and functionally diverse of all these three major groups in fish blood. Within the leukocytes there are heterophils, eosinophils, basophil, monocytes, lymphocytes and plasma cells (Campbell, 2015). White blood cell counts can range

from 10100 cells/µl in *Crassius auratus* to 282 000 cells/µl in *Pleuronectes americanus* (Weiss and Wardrop, 2011). It has been suggested that individuals with high white blood cell counts are fighting infection. This is due to a direct correlation between white blood cell counts and immune responses (Weiss and Wardrop, 2011).

1.11 Research problem

The Two Oceans Aquarium houses a number of valuable and threatened fish species that are of adult size. The kelp forest exhibit holds roughly 800 000 litres of water and exhibits live kelp (*Ecklonia maxima)* that is replaced on a regular basis. The temperature throughout the year varies with a range of 12°C-16°C. All the fish in this exhibit are indigenous to the South African coast. Fish such as; *Lithognathus lithognathus* (White steenbras), *Chrysoblephus cristiceps* (Dageraad), *Dichistius capensis* (Galjoen), *Pestrus rupestris* (Red steenbras), *Chrysoblephus gibbiceps* (Red stumpnose), *Cymatoceps nasutus* (Black musselcracker), *Sparadon durbanensis* (White musselcracker), and *Chrysoblephus laticeps* (Red roman), are some of the valuable fish housed in this exhibit and many of them have been in this exhibit since 1995. From 2013 to 2015 *I. hoferi* has been diagnosed in a number of mortalities that have occurred in this exhibit. Another exhibit that receives the effluent water from the Kelp exhibit is the Seal Pool which also houses numerous indigenous South African fish species. Like the Kelp Forest exhibit, *I. hoferi* has been identified in mortalities that have occurred in the Seal Pool. Lethal sampling has also taken place in the Seal Pool and *I. hoferi* has been identified in the majority of sampled fish. Diagnosis of *I. hoferi* in the literature has been performed using lethal sampling techniques on wild and cultured fish and involves the sacrifice of the fish. An accurate diagnosis allows continued surveillance and provides key information that is needed for management decisions. A non-lethal tool used to diagnose *I. hoferi* would be beneficial because it would enable the detection of *I. hoferi* in many of the valuable fish species and allow for informed decisions to be made for the wellbeing of all fish in the exhibit. Not only will this research help and aid the Two Oceans Aquarium but will provide a basis for other facilities, aquaculture or aquariums to use the same diagnostic tools to diagnose *I. hoferi*.

1.12 Aim

- Develop a non-lethal diagnostic tool for the diagnosis of *I. hoferi*

1.13 Objectives

- Obtain liver biopsies from a group of fish, comparing two methods of surgery used for obtaining the biopsies: coeliotomy and coelioscopy
- Use PCR, culture and microscopic tissue squash preparations as diagnostic tools on the liver biopsies to diagnose *I. hoferi*
- Monitor the healing post-surgery using blood parameters and analysis of the wounds through visual observation comparing coeliotomy and coelioscopy
- Obtain a complete diagnosis using, PCR, culture microscopic tissue squash preparations and histology by means of a full post mortem on each fish in the coelioscopy, coeliotomy and control groups

This study used a population of *R. globiceps* naturally infected with *I. hoferi* from two separate exhibits. Our objectives were to obtain the liver biopsies using two surgical methods and then compare three different methods of detecting the parasite in the obtained tissue samples. To quantify these diagnoses and correctly identify these fish as positive or negative for *I. hoferi*, euthanasia and full examination of the fish at the end of the study was required. In the full examination the three diagnostic methods, with the addition of histology, was performed on the liver tissue samples. As a result we will have a non-lethal diagnostic tool. All the fish in this study were euthanised by the attending veterinarian as part of a disease control measure which was approved by the Two Oceans Aquarium ethics committee.

CHAPTER 2

Materials and Methods

2.1 General

All fish used in the study were from the Two Oceans Aquarium Kelp Forest exhibit or the Seal Pool exhibit. They were all housed in the quarantine facility in pools at the Two Oceans Aquarium during acclimation, surgery and post-operative observation. The molecular work was completed at the Sea Point Research Aquarium, Department of Agriculture, Forestry and Fisheries, Cape Town, South Africa. Surgical procedures were performed by the staff Veterinarian at Two Oceans Aquarium. Ethical clearance was granted and approved through the ethics committee at the Two Oceans Aquarium. All research conducted met all standards required and was within all ethical guidelines of the Two Oceans Aquarium.

2.2 Pilot study

The purpose of this study was to determine if it was possible to diagnose *I. hoferi* by microscopic examination of wet preparation squash mounts from a liver biopsy. Ten fish (Three *Rhabdosargus globiceps*, two *Pachymetopon blochii*, three *Boopsoidea incornata*, one *Diplodus hottentotus* and one *Spondyliosoma emarginatum*) from a population known to be infected with of *I. hoferi* were euthanised, liver biopsies were taken for microscopic examination. Of the ten fish, two were negative for all sampling and did not have *I. hoferi*. The staff veterinarian and two researchers would have to confirm observing *I. hoferi* microscopically to make a diagnosis. To determine if the method was sensitive in the diagnosis of *I. hoferi*, a post mortem exam was carried out on all the fish. The heart, liver (a larger sample), kidney and spleen were examined microscopically in all fish to determine if the fish were infected or not. The liver biopsy sensitivity was 87% for the ten fish. The sensitivities for the heart, larger liver sample, kidney and spleen were 87%, 100%, 100% and 100%, respectively. It was a small sample size, however, it did provide for the confidence to investigate the techniques and options for non-lethal sampling of a liver biopsy.

2.3 Fish acquisition and acclimatization

Forty *R. globiceps* (White stumpnose) were removed from the Seal Pool (n=25) and Kelp Forest (n=15). The fish were randomly assigned to either the coelioscopy group (n=15), coeliotomy group (n=15) or the control group (n=10) to allow for a representative sample and equal number of fish from each exhibit.

The fish were netted by divers and staff using a beach seine net and hand nets to remove them from the exhibits. Fish were immediately placed into 70 litre plastic rectangular bins containing seawater with a low concentration of 2-Phenoxy-ethanol (2-PE) (Acti-Chem SA [Pty] Ltd, Westville, Kwa-Zulu Natal, 3630 South Africa) at 0.05ml/litre to reduce any injury to the fish and mitigate stress. A Fivestar FBX-B 12mm microchip (Fivestar ID Pet Microchips, Farm Hartbeesfontein, Ellisras, Limpopo, 555), used to identify each individual fish for the entire study, was inserted into the muscle on the left side just below the dorsal fin on each fish. Weights and measurements of the fish were also recorded before the fish were moved into the 12000l holding pool where they would remain for the entirety of the study. An acclimatization period of one week was given prior to surgery. Water quality was monitored and recorded daily at approximately the same time. Temperature (°C), pH, dissolved oxygen (mg/l) and salinity (ppt) were measured using a YSI Professional plus multi-parameter water quality meter (YSI Incorporated, 1700/1725 Brannum Lane, Yellow Springs, Ohio 45387-1107 USA). Non-ionized ammonia (mg/l) was measured using a Palintest photometer 7100 (Palintest HQ, Palintest House, Kingsway, Team Valley, Gateshead, Tyne & Wear NE11 0NS, England). Fish were fed *Pyura stolonifera* (Red bait), *Todaropsis eblanae* (squid), *Donax serra* (White mussel) and *Litopenaeus vannamei* (Prawn) four times a week. All food that was fed was weighed and recorded. Natural seawater pumped in from the harbour was filtered through seven large sand filters before being pumped to the pool. Additional filtration included a dedicated sand filter that filtered the pool water by means of recirculation. Temperature ranged from 12-18°C according to naturally fluctuating local sea temperatures. Food was withheld from all fish for 48 hours prior to anaesthesia and surgical procedures to allow for complete gut evacuation.

2.4 Anaesthesia protocol

Anaesthesia was carried out as described below, for all surgical procedures and blood sampling to ensure adequate fish immobilization, and for the reduction of any physical damage and to mitigate stress. For each procedure, initially 0.05ml/l 2-PE was added to the main holding pool to provide light sedation. The individual fish were identified with a Virbac microchip reader (Backhome®Virbac V800 scanner, 38 Landmarks Avenue, Samrand business park, Centurion 0157) and placed into a 300 litre tank next to the main pool (Fig. 2.1) as required. Once all of the required fish were removed, the incoming water for the main pool was opened and the anaesthetic was flushed out to allow the remaining fish to recover. A third and fourth tank of 100l capacity containing a dose of 0.1ml/l and 0.25ml/l of 2-PE, respectively were then used to deepen the plane of anaesthesia for surgical purposes and later blood sampling (Fig. 2.1). Once the fish were anaesthetised they were taken to the veterinary clinic and placed onto the fish anaesthetic rebreathing system (FARS).

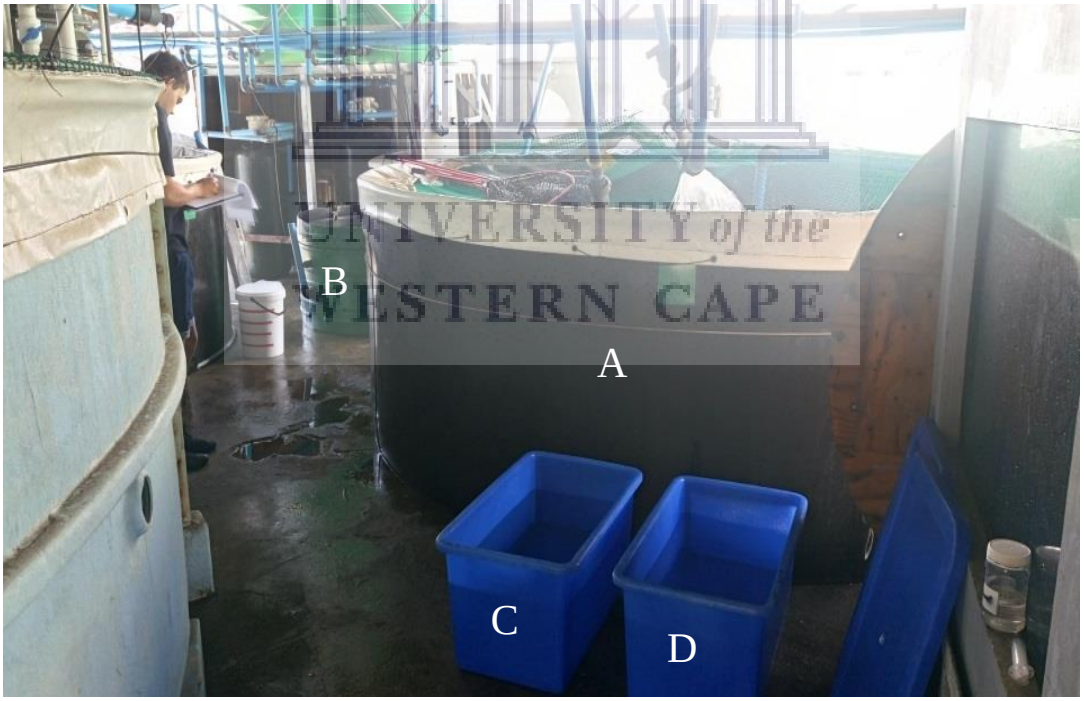

Figure 2.1: Holding pool and bin/tank arrangement for anaesthesia. A) Main holding pool for all fish, 2-PE anaesthetic concentration of 0.05ml/l. B) Temporary holding pool C) Blue bin used to induce a deeper level of anaesthesia at 2-PE anaesthetic concentration of 0.1ml/l. D) Fourth blue bin to induce to the level of anaesthesia required for surgery, 2-PE anaesthetic concentration of 0.25ml/l.

The FARS was fabricated to keep the fish stable, anaesthetised and allow for oxygen uptake over the gills whilst immobile during surgery. The FARS system was built at the Two Oceans Aquarium by the author of this research. It consists of two 50 litre cylindrical sumps that hold the anaesthetic water at the desired concentration (in this case 0.25ml/l 2-PE) in one sump and clean sea water containing no anaesthetic in the second sump, connected in the middle by an external pump (Lifetech aquarium pump, Hmax: 4.2m, Qmax: 4800l/hr, 50Hz, 145W). The pump connected to a 32mm delivery pipe which splits into three differently sized delivery pipes (5mm, 10mm and a 25mm) with valves to regulate flow (Fig. 2.1). The 10mm delivery pipe was used in all fish surgeries. A series of small outlet holes were placed towards the end of the silicone tube with the very end of the pipe being sealed, allowing water to be delivered into the gill arches laterally, and preventing water from entering the oesophagus. Water drains through the holes in the grid (Fig. 2.2B), into the trough (Fig. 2.2C) and into the relevant sump to be oxygenated and reused again (Fig. 2.2 D and E). The volume of water flowing out of the opercular openings was constantly monitored by ensuring the gill chambers were flooded at all times keeping the fish oxygenated and anaesthetised. A flow rate of between 4-6 l/min was maintained in all fish during surgery. A ceramic air stone connected to a medical oxygen cylinder was used to supply oxygen into the sump that was being used. The FARS was setup in the veterinary clinic on a stainless steel table to allow for the correct height at which surgical procedures could be performed comfortably (Fig. 2.3).

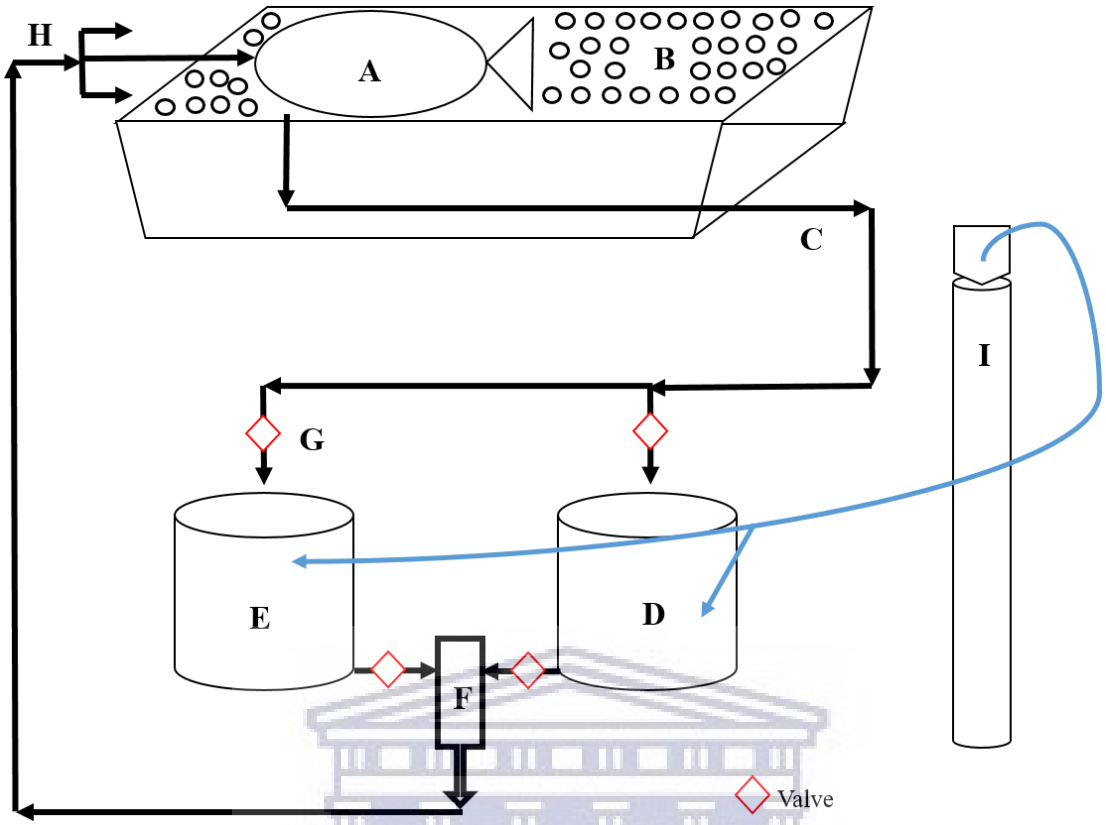

Figure 2.2: Schematic diagram of the Fish Anaesthetic Rebreathing System

(A) Fish on top of the trough receiving oxygenated water

(B) Grid to allow water to drain through to the respective sump below

(C) Excess water draining out of the trough and being gravity fed back to the respective sump

(D) Sump to store non-anaesthetised water. Oxygen supplemented into sump via ceramic air stone

(E) Sump to store anaesthetised water. Oxygen supplemented into sump via ceramic air stone

(F) Pump used to pump water up to fish at (A)

(G) Valves used to isolate/close the sump not being used and open the sump that is being used

(H) Three sizes of pipes for different fish according to their mouth size

(I) Oxygen being supplied to the sumps

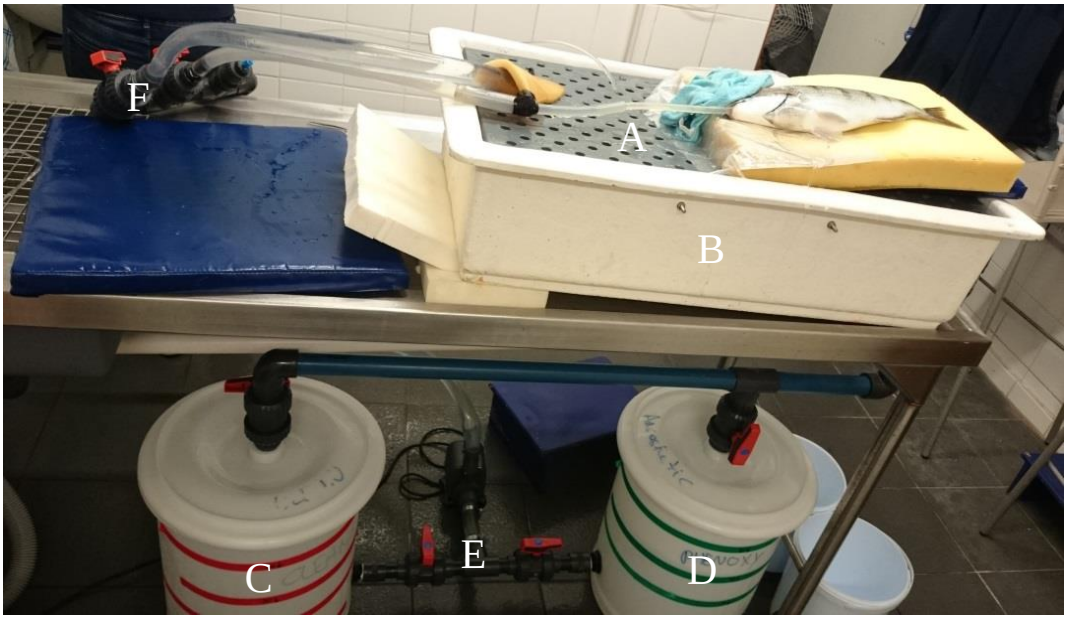

Figure 2.3: Fish Anaesthetic Rebreathing System (FARS) pictured with anaesthetised fish

(A) Grid used for fish placement and to allow water to drain into the trough

(B) Trough used to catch excess and used water

(C) Seawater containing no anaesthetic for recovery post-surgery

(D) Seawater containing anaesthetic agent at required dosage

(E) Pump used to deliver required water from the sumps (C) or (D) to (A)

(F) Three varying sizes of delivery pipes

2.5 Surgical procedure

A liver biopsy was obtained either by coeliotomy (n=15) or by coelioscopy (n=15). It should be noted that five of the coeliotomy fish had their surgical procedures done a few months prior to the start of the main study and did not have all the required blood samples taken from them. Post-surgical blood sampling was therefore performed on fifteen fish in the coelioscopy group, ten fish in the coeliotomy group and ten fish in the control group. Before the start of each surgical procedure, the fish would be weighed (g), measured (cm) and then placed onto the FARS. All fish were weighed during the study to monitor any weight changes over time and to determine the condition factor of the fish. Once positioned correctly with oxygenated anaesthetic water flowing suitably over the gills, the blood would be sampled from the caudal peduncle of the fish and processed accordingly. Fish were only weighed and measured on the day of surgery and only weighed again at the end of the study at post mortem.

All surgical equipment was cold sterilised in Cidex ® OPA (Active ingredient: *ortho*-phthalaldehyde 0.55%, Medos International Sarl, Chemin-Blanc 38, CH2400 Le Locle, Switzerland) following the manufacturer's instructions, prior to each surgical procedure. It is recommended that when performing several surgeries the surgical instruments be kept in sterilization solution rather than disinfectant (Harms, 2005).

2.5.1 Coeliotomy

The surgical site, and surrounding area, was rinsed with sterile saline solution to reduce contamination. A plastic drape was used to cover the area around the site for sterility and to keep the fish moist. All fish were placed in right lateral recumbency with the pectoral fin held forward towards the head, preventing obstruction of the surgical site. The point of incision was at the surgeon's discretion, just caudal to the pectoral fin, directly above the caudal edge of the left liver lobe and not too far dorsally (to avoid risk of rupturing the swim bladder). The distance of the incision from the pectoral fin was dependant on the size of the fish, as a larger fish would have a proportionally larger liver. The depth was also taken into consideration; the larger fish had a much deeper muscle layer to dissect through. A few scales were removed where necessary prior to making the incision. The incision was made through the skin and then the muscle tissue until the coelom could be visualised. Care was taken not to cut or rupture any viscera. All of the incisions were made in the intercostal space, ensuring no injury to the ribs. Once access to the coelom was achieved, a pair of Gelpi retractors was used to hold the incision site open for improved access to the liver. After the tip of the left liver lobe was exteriorised it was gently grasped with mosquito forceps and a small piece of the liver was bluntly dissected whilst ensuring no major vessels were cut in the liver. If any haemorrhage was observed the site was cauterized using a Jorgensen Laboratory high temperature cautery power handle (Jorgensen Laboratories, Loveland, Co. Ref: Del1-JORG. Mfg. Lot: 1214-3). Once the biopsy was obtained, the Gelpi retractors were removed (Fig. 2.4A-D).

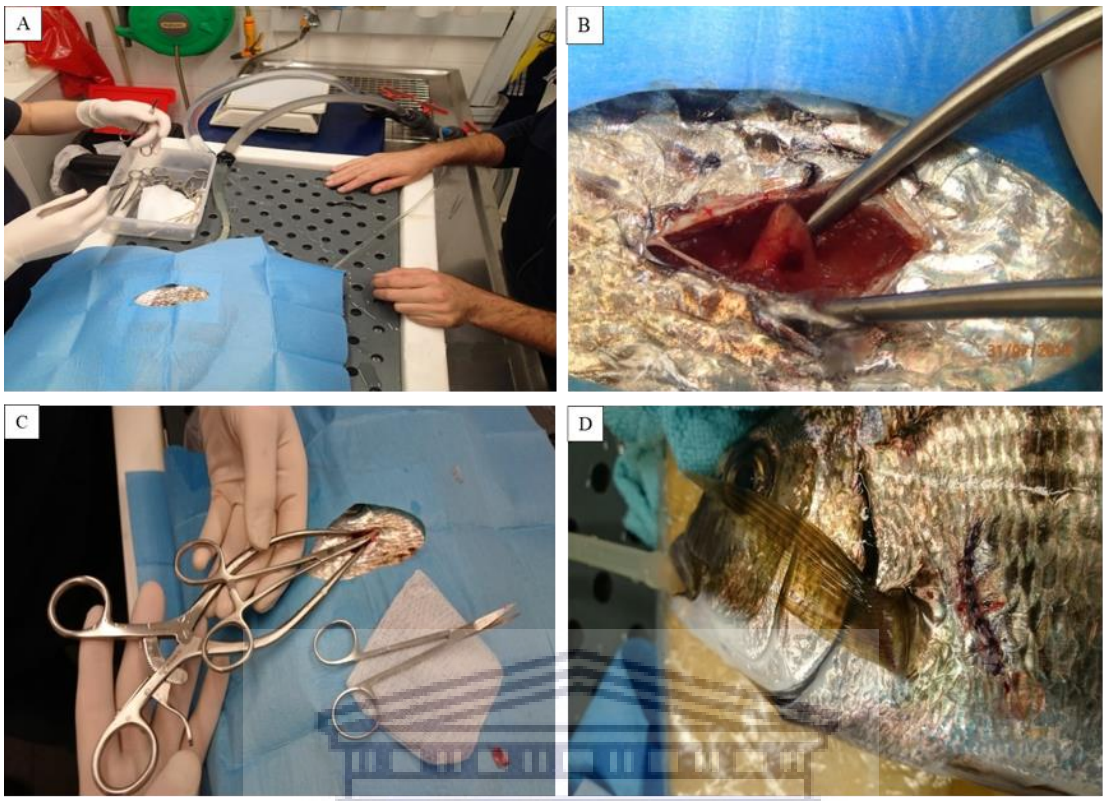

Figure 2.4: Liver biopsy obtained by coeliotomy. A) Sterile drape is placed over the fish. B) Exteriorized liver lobe from surgical incision. C) Gelpi retractors used to open surgical site, mosquito forceps used to hold liver and minimize haemorrhage once biopsy is sampled. D) Surgical incision suture closed.

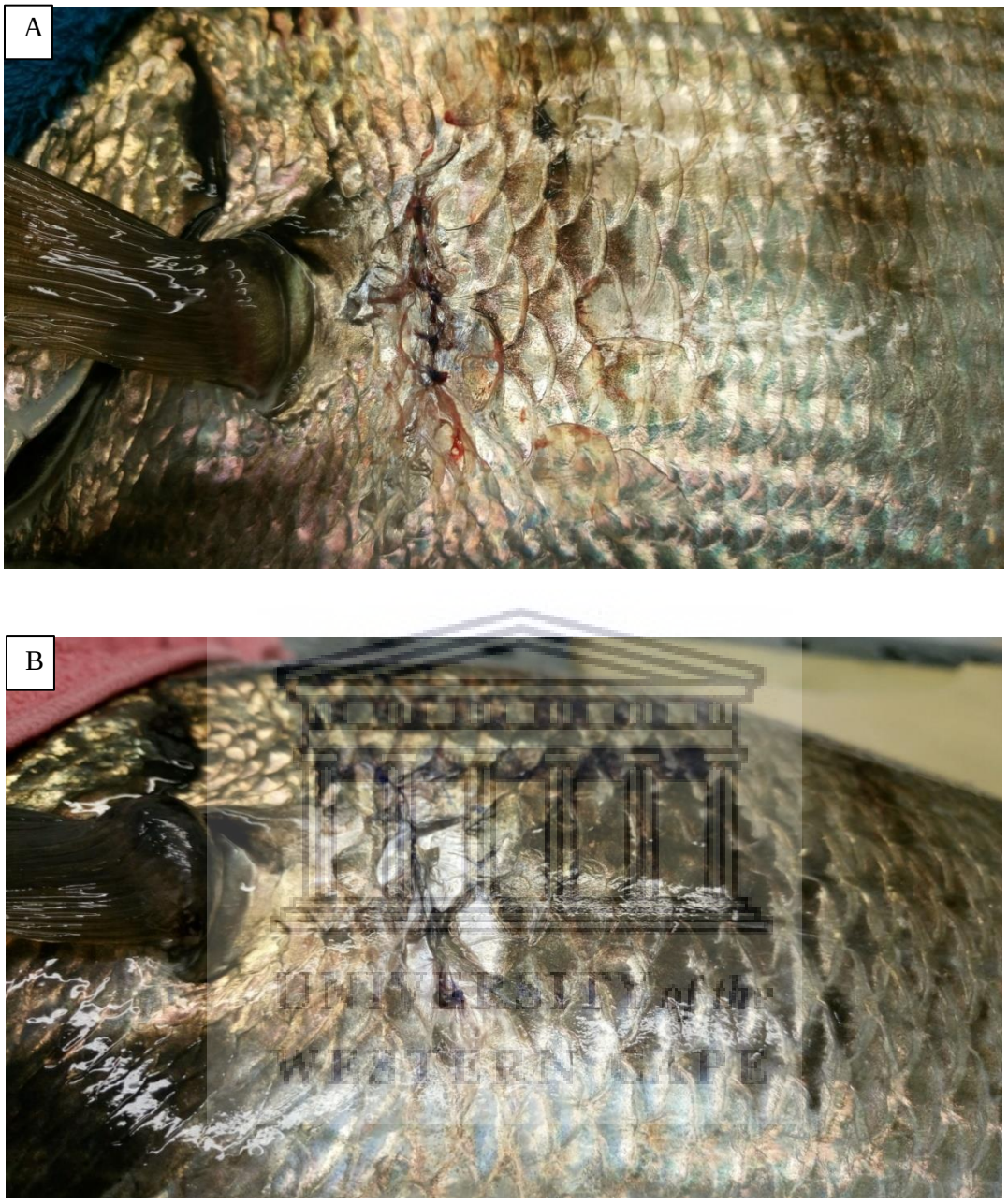

Figure 2.5: Suture techniques used to close the body wall after coeliotomy A) Simple interrupted with exposed knots. B) Buried suture method.

To close the incision, violet monofilament synthetic poly (P-Dioxanone) absorbable suture material (CliniSolv (Pty) Ltd, 25 Harrower road, Kensington, Port Elizabeth, 6001) of varying thicknesses (3/0 and 4/0) was used depending on the size of the fish. The coelomic cavity was closed by means of an exposed or buried suture method (Fig. 2.5A&B). The buried suture methods ensure that there are no exposed knots at the suture site and conversely the exposed suture techniques had exterior suture knots, with ends of suture material protruding from the

skin. These varying techniques were used to observe the healing over time (weeks 1, 2, 5 and 6) and observe any adverse trauma that may have been caused from the technique and/or suture material. The suture technique was determined and trauma was classified as either no trauma, mild or moderate. No severe trauma was observed so it was not used in the classification. Once the incision was sutured closed a thin layer of Betadine (1g contains: Povidone-iodine 100mg equivalent to 10mg available iodine. Mundipharma (Pty) Ltd, P.O.Box 23162, Claremont, 7735, South Africa) was placed over the sutures as a precautionary measure. The sump was changed on the FARS to provide the fish with oxygenated water containing no anaesthetic to begin recovery. When the fish showed signs of movement by starting to lift the caudal fin it was transferred back to the recovery pool with fresh seawater where it was then allowed to recover fully.

2.5.2 Coelioscopy

Fish were anaesthetised in the same manner as previously described, however, the fish were positioned in right lateral recumbency at a 45° angle to the trough surface with more of the ventral surface of the fish exposed (Fig. 2.6B). A Karl Storz Hopkins Endoscope 2.9mm 0° with a 9 Fr examination sheath and a telepack vet X 69045020 video camera head and video endoscope with 50W Hi-Lux light source and digital processing module was used. A 5mm incision was made on the ventral midline a few centimetres caudal to the base of the pectoral fins to insert the examination sheath. A second incision, also approximately 5mm in length was made 4-6cm caudal to the first incision and this was used to insert the biopsy forceps.

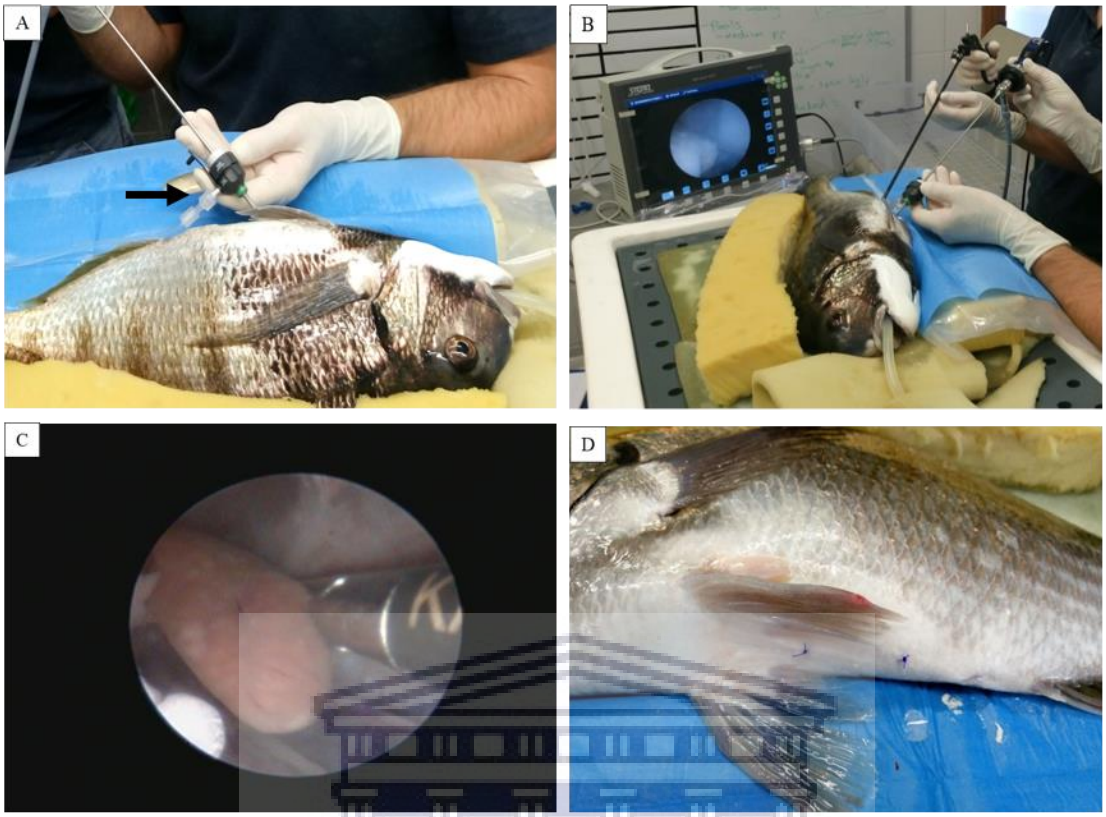

Figure 2.6. Liver biopsy obtained by coelioscopy. A) An anaesthetised fish with the optical examination sheath in the coelom. The black arrow indicates the insufflation attachment to the examination sheath. B) The examination sheath and biopsy forceps in the coelom visualised through the endoscopy camera and screen. C) A biopsy being taken from the caudal edge of the liver. D) The two incision sites sutured post-surgery.

The endoscope was inserted into the sheath allowing visualisation of the coelomic contents. Saline in a one litre bag was connected to the examination sheath via an insufflation port and flow was regulated using the giving set attached to the saline bag (Fig. 2.6A). Insufflation was achieved by allowing the saline to flow into the coelom and once there was good separation of all viscera the flow was turned off. The liver was located using the fibre optic telescope (Fig. 2.6B&C). The biopsy forceps (Fig. 2.7) were inserted into the second incision and were positioned to be visible on screen. The forceps were then moved over the area of interest on the liver and the jaws closed to obtain the biopsy. The forceps were held in place for 30-60 seconds to allow haemostasis to occur and were then removed from the coelom.

The camera was held in place for a minute to observe the biopsy site and to check for any significant bleeding. Saline was allowed to drain out of the examination sheath once the endoscope was removed. A small amount of pressure was placed on the body wall to stimulate passive saline drainage. Once the saline had stopped flowing the examination sheath was removed. The two incisions were sutured closed using violet monofilament synthetic poly-dioxanone absorbable suture (CliniSolv (Pty) Ltd, 25 Harrower road, Kensington, Port Elizabeth, 6001) with a single simple interrupted suture each (Fig. 2.6D). The method of recovery from anaesthesia was the same as described above for the fish in the coeliotomy group. All fish that underwent surgery (coeliotomy and coelioscopy groups) were injected with butorphanol (Kyron Prescriptions, Johannesburg, Benrose 2094, South Africa (10mg/ml)) at 0.4mg/kg, prior to surgery, for pain relief (Harms, 2005). Ceftazidime (1g Ceftazidime Pentahydrate. Brimpharm SA (Pty) Ltd, 215 Main Road, Claremont, Cape Town, South Africa) at 20mg/kg was injected into the epaxial muscle as antibiotic prophylaxis immediately after surgery and at weeks 1 and 2.

Figure 2.7: Karl storz biopsy forceps 33121.

2.6 Postoperative care, blood sampling, surgical site healing and blood processing

Blood was drawn from all fish, in each surgical group and the control group, from the caudal vein. Blood was drawn for the purpose of obtaining a baseline of overall health on the day of surgery and then monitoring of the fish post-surgery. Blood samples were taken at week 0, 1, 2, 5 and 6. This equated to days 0, 7, 14, 35 and 46 for the coeliotomy and the coelioscopy groups, however due to practical reasons the control group was sampled at days 0, 12, 19, 40

and 50. The blood parameters that were tested were the packed cell volume (PCV) (%), blood glucose (mmol/L) and total proteins (g/dL). Blood smears were made for white blood cell counts and differential counts. Prior to sampling, one millilitre of Heparin sodium Fresenius 5000 I.U./1 ml (Fresenius Kabi Manufacturing SA (Pty) Ltd, 6 Gibaud Road, Korsten, Port Elizabeth, South Africa, 6020) was drawn into a syringe with 50ml saline solution to create a 1:50ml dilution. A small amount of this dilution was then drawn up into the syringe prior to blood sampling and then expressed leaving residual dilute heparin in the syringe to prevent any blood coagulation. Three millilitre syringes and 23Gx1", 21Gx1" and 21Gx1 ½" needles were used. A blood volume of approximately 0.5ml was drawn and placed into a lithium heparin (LH) microtainer tube. A single sodium-heparinized 80 µl micorhaematocrit capillary tube was also filled. A blood smear was made, blood glucose was measured using an Accu-Chek Performa Nano glucometer. The capillary tubes were spun for five minutes at 5000 x g in the centrifuge. PCV was calculated and total plasma solids measured using a handheld refractometer. White blood cell counts and differential counts were performed on all blood smears. All differential counts were performed by the author and a veterinarian and all processing was overseen by the veterinarian. The smears were viewed on a Leica Galen 3 light microscope using a 50X oil immersion objective lens. The recommended laboratory technique is the use of a 40X objective lens (Sheldon *et al.*, 2016). Due to the difficulty in observing the cells at the recommended 40X objective lens a 50X oil objective lens was used for easier cell identification. Ten replicate counts in ten different fields of the monolayer were performed. These results then averaged with the use of the following equation (Sheldon *et al.*, 2016):

$$WBC/\mu l = (\text{average no. of cells per field}) \times (\text{objective power})^2$$

All fish were blood sampled and then euthanised on day 43 or 44 for a full post mortem to obtain a diagnosis (infected or not infected with *I. hoferi*), to examine surgical healing and overall health by documenting all internal and external lesions.

2.7 Processing the biopsy

All biopsies were weighed to the nearest 0.01g. The biopsy sample was then divided into three sections and weighed. One section was placed onto a slide, covered with a cover slip (squash preparation) and examined microscopically for schizonts and germination tubes. A second

piece of the biopsy was placed directly into culture media in a sterile manner. The third section of biopsy was placed in 99.9% ethanol and preserved for PCR.

2.8 Diagnostic tests

The diagnostics tests that were performed on the liver biopsies were; microscopic examinations of a squash preparation, culture, conventional PCR, and a nested PCR. A biopsy sample was deemed positive only if two or more tests independently yielded a positive result for that sample. At post mortem, a larger liver sample was obtained for each diagnostic test. Diagnostic tests performed on liver samples obtained at post mortem were; microscopic examinations of squash preparations of the liver, spleen, kidney and heart, culture of the liver, conventional PCR of the liver, nested PCR of the liver, and histology of the heart, liver, kidney and spleen. There is no recommended gold standard test for the diagnosis of *I. hoferi*, so a fish was classified positive if it tested positive at post mortem in two or more of the previously listed diagnostic tests. According to the World Organisation for Animal Health (OIE) standards for diagnostic testing, a minimum of two tests are needed to diagnose an animal with or without a disease or pathogen (OIE, 2016).

2.8.1 Microscopic squash preparation examination

For liver biopsies: the biopsy was placed on a slide, pressed down and squashed underneath a cover slip with moderate force. The squash preparation was viewed initially using a 10X objective lens and the presence of *I. hoferi* confirmed by using the 40X objective lens. The *I. hoferi* structures of life stages noted were; germination tubes, granulomatous tissue with schizonts present, and single or clustered schizonts. Each biopsy was viewed independently by three examiners and recorded as positive or negative for the presence of *I. hoferi*. The results were then reviewed by all examiners and if there was a discrepancy regarding a result the relevant slide would be viewed again until a consensus between examiners was reached. For post mortem examination, squash preparations (prepared as described for liver biopsies) were made of the liver, spleen, kidney and heart, were examined using light microscopy by three examiners following the same procedure described above. These were diagnosed as positive or negative by observing any of the following structures namely: germination tubes, granulomatous tissue with schizonts present and single or clustered schizonts.

2.8.2 Culture

A culture media was prepared in advance using a protocol taken from Spanggaard *et al.* (1994) and Hershberger *et al.* (2008b). Culture media was prepared by using the Lonza BE17-737E HEPES Buffer ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)), 1M stock in normal saline and adding it to the Minimum Essential Medium working stock (MEMS) using Sigma-Alrdich #M0268. The culture media was then autoclaved and inoculated with streptomycin and penicillin to inhibit bacterial growth (Spanggaard *et al.*, 1994). 5ml of the culture media containing the HEPES, MEMS and antibiotics was transferred to 15ml sterile culture tubes. The tubes were supplemented with 5% foetal bovine serum (FBS) (Hershberger *et al*, 2008b; Spanggaard *et al.*, 1994). The tubes containing the growth media were stored at 4°C. Once biopsies were taken they were placed in the growth media and incubated at 15°C. All cultures were checked for growth either at 7, 8 or 9 days and then again at either 14, 15 or 16 days. To check for growth each tube would be hand homogenised and vortexed before each count. Homogenization had to be done to allow for the most accurate count of *I. hoferi* within in each tube and because the biopsy was not homogenised when it was originally placed into the tube on the day of surgery. Homogenisation took place at day 7 and day 14 when the samples were counted and checked for growth. A 100µl sample was pipetted out of the tube and placed on a slide and covered with a 22x40mm cover slip. Three replicates from each individual culture were examined. All samples were examined microscopically using a 40X objective lens with a 400X total magnification. Schizonts and hyphae were counted and a clicker counter was used to count the cells per 100 µl. Only living anatomical structures were counted, granulomatous tissue or dead schizonts, germination tubes or scatter were not considered. The colour and contents of schizonts and germination tubes was used to determine if individual *I. hoferi* were alive or dead. At the final necropsy, a larger section of the liver was cultured using the method described above.

2.8.3 PCR

All biopsies were stored in 99.9% ethanol. DNA was extracted using the Qiagen DNeasy Tissue extraction kit **(**QIAGEN, Lake Constance GmbH**,** Jacques-Schiesser-Str. 3, 78333 Stockach, Germany) according to the manufacturer's instructions. A conventional PCR was used to analyse the liver biopsy samples obtained from the two surgical methods. The protocol used was as follows:

PCR was performed on the biopsy samples using ICH1F and ICH4R primers (Criscione *et al.*, 2002). PCR was cycled at 95 °C for five minutes, 94 °C for 45 seconds, 51.7 °C for 1 minute, 72 °C for 90 seconds, 72 °C for seven minutes for 35 cycles. A conventional PCR was performed on the liver samples obtained at post mortem which were homogenised after being placed into the tubes. Following diluting products 10^{-2} for the nested step primer set BW2F and BW2R were used (Bret Wurdeman pers. comm). This was cycled at 95 °C for five minutes, 94 °C for 45 seconds, 51.7 °C for one minute, 72 °C for 90 seconds, 72 °C for seven minutes through a total of 35 cycles (Bret Wurdeman pers. Comm). *I. hoferi* DNA was used as PCR positive control. All of the PCR reactions were performed in duplicate with the final volume of 25 µl consisting of 50 ng of liver DNA, KAPA SYBR fast qPCR master mix and 10 µM of PCR grade water. A non-template control was included in all PCR assays. The products were amplified and sequenced in both directions for confirmation. The protocol used for this PCR was obtained from Bret Wurdemann (Bret Wurdeman pers. comm)[2].

Nested PCR is considered a more sensitive test than conventional PCR as it uses two pairs of primers in two consecutive runs. The product obtained initially is used as a template for the second run which allows for a more sensitive test (Fernández *et al.*, 2008). This method of PCR also requires less initial target DNA and in this case is useful as minimal tissue was available due to the size of the sample. Nested PCR was used in this case when conventional PCR for the biopsy samples resulted in a low number of positives and optimization of the PCR method was required.

2.9 Final diagnosis by post mortem

At the end of the post-operative monitoring, blood sampling and observation period the fish were euthanised. A post mortem examination was performed to determine the status of *I. hoferi* infection in the fish. Once blood was sampled, the fish were placed in a pool containing 1ml/litre 2-PE with oxygen. Fish were classified as deceased at least ten minutes after the cessation of observable opercular movements (Leary *et al.*, 2013). Fish microchips were read using the Virbac scanner, the fish were weighed and placed on the post mortem examination table. Photographs were taken of any external abnormalities or damage that the fish may have incurred while in holding. A small incision was made 1cm anterior to the cloaca with a scalpel.

[2] Bret Wurdeman optimised various methods of PCR on *I.hoferi* and one of the protocols was used in this study.

Scissors were used to cut from the incision towards the head, through the pectoral girdle. The scissors were then used to cut along the body cavity in a half circle towards the cranial aspect of the base of the pectoral fin. Care was taken to avoid perforating the swim bladder. While cutting, observations were made and photographs taken if any organs were adhered to the inside of the surgical site. The surgical site was examined both externally and internally and notes made. All other internal organs were examined for abnormalities or physiological inconsistencies, for example; lesions or papules on the skin, granulomas in any of the above mentioned organs or exudate in the swim bladder. These would be examined grossly and microscopically before being preserved in ethanol and formalin. For all the tissue squash preparations a small section of liver was cut out and placed onto a slide, a cover slip was then used to squash the tissue until a thin layer was obtained that could easily be viewed microscopically. A small section of liver was weighed and placed in a culture tube in a sterile manner, an additional sample was placed in ethanol for PCR. This was used as a final liver diagnosis using the culture method and the PCR method. The heart, liver, kidney and spleen were sampled and placed in neutral buffered formalin. These were processed into haematoxylin and eosin stained slides for histological exam by Ana Herrero and examined for the presence of schizonts or hyphae by the Fish Vet Group registered veterinary pathologists[3].

2.10 Statistical analysis

All statistical analyses were completed using the Statistica data analysis software system, (StatSoft, Inc, version 8.0, www.statsoft.com, 2008) and Microsoft Excel (Microsoft® Excel ® 2013 Part of Microsoft Office Professional Plus 2013). All data was tested for normality using the Shapiro-Wilks test Statistica. The surgery and post-surgical healing was documented by means of photographs, notes and categorising the healing and abrasions into groups (Chapter 3). The overall condition of the fish was assessed by using the Fultons condition factor. This was analysed using paired T-tests for dependant samples within each group with a statistical significance of $p \leq 0.05$ to compare the initial and final condition of the fish in all three groups (Chapter 3). Independent T-tests for independent samples between each group were used with a statistical significance set at $p \leq 0.05$ to analyse the surgical times and recovery times with the statistical significance set at $p \leq 0.05$ (Chapter 3).

[3] Ana Herrero of the Fish Vet Group prepared the histology slides of the heart, liver, kidney and spleen from the final post mortem of all fish and completed the analysis of the prepared slides.

A Shapiro-Wilks test was used to test for normality, which indicated that the data was normally distributed. A repeated measures ANOVA (RMANOVA) was used to examine differences in the blood parameters of the groups at the various weeks post-surgery. A Tukeys post-hoc test was used to check for significant differences in p-value ($p \leq 0.05$) between weeks 0, 1, 2, 5 and 6 within a surgical group either coeliotomy, coelioscopy or the control (Chapter 3). The biopsy weights (g) for each surgical group were tested using an independent T-Test with the statistical significance set at $p \leq 0.05$ (Chapter 4). Summary tables and 2x2 contingency tables were used to analyse the ability of the tests performed on the liver biopsies to correctly identify fish as infected or not infected with *I. hoferi*. 2x2 contingency tables (Table 2.1) were used for all sensitivity and specificity data analysis (Sauerbrei and Blettner, 2009) (Chapter 4). The sensitivity is the ability of the test to correctly identify the fish as positive for *I. hoferi* and the specificity is the ability of the test to correctly identify the fish as negative for *I. hoferi*. For a fish to be classified as positive for *I. hoferi* it had to be positive in two or more tests. The apparent prevalence is the proportion of positive individuals in the group of fish that were sampled (Lalkhen and McCluskey, 2008).

Table 2.1: The sensitivities and specificities of each diagnostic test (Glas *et al.*, 2003).

	DISEASE STATUS	
	I. hoferi positive	*I. hoferi* negative
TEST RESULT		
Positive	True positive TP	False positive FN
Negative	False negative FN	True negative TN
	Sensitivity = TP/(TP + FN)	Specificity = TN/(FP + TN)

CHAPTER 3

I. hoferi is a mesomycetozoan parasite of teleosts, has been previously isolated from various species of fish in exhibits at the Two Oceans Aquarium (G. Cole per. comm)[4]. *I. hoferi* proliferates and migrates throughout the hosts' body and is not restricted to a specific organ (McVicar, 1999). The host responds to the parasite by formation of granulomas, which cause a variety of clinical signs, including neurological abnormalities and skin lesions (McVicar, 2011). Diagnosis can be achieved via microscopic examination of tissue squash preparations, culture, polymerase chain reaction (PCR) and histopathology (Kocan, *et al.*, 2011; Spanggaard *et al.*, 1994; Whipps *et al.*, 2005). Despite no current successful treatment for the parasite, a non-lethal approach to diagnosis would benefit aquaria. The affected individuals could be quarantined from *I. hoferi* free individuals, eliminating risk of parasite transmission.

Liver biopsies of *Rhabdosargus globiceps* (White stumpnose) were obtained by two surgical techniques, coeliotomy (n=10) and coelioscopy (n=15). A total of 15 fish did undergo coeliotomy, however, five of these fish underwent surgery early on in the study but did not have surgical, blood sampling or any post-health data collected from them and unfortunately could not be used as part of the data analysis.

Results: Surgery

3.1 Surgical site healing

Photographs of the surgical sites were taken when the fish were anaesthetized for blood sampling. Initial surgical site photographs were taken on the day of surgery at week 0 for the coeliotomy group and at week 1 for the coelioscopy group, this was due to time constraints post-procedure. There were varying suture styles used in the coeliotomy group to better understand which suture technique might be better for the surgical procedure performed and for the post-surgical healing.

[4] Dr. Georgina Cole, the veterinarian at the Two Oceans Aquarium first identified *I. hoferi* (Unpublished data).

3.1.1 Coeliotomy

Table 3.1: Categorization of suture techniques used on the day of surgery, suture removal post-surgery, level of abrasion trauma and the healing at weeks 1, 2, 5 and 6 post-surgery.

Fish Number	1572733	1573388	1573888	1575929	1573142	1573280	1572616	1572245	1573857	1574998
Suture technique: Knots exposed or buried.	Exposed	Exposed	Exposed	Exposed	Exposed	Exposed	Buried	Buried	Buried	Buried
Sutures removed	Not Removed	Week 5	Not Removed	Week 2	Week 2	Not Removed	Not Removed	Not Removed	Week 2	Not Removed
Abrasion trauma on the pectoral fins from sutures (week 0-6)	Mild	Moderate	Moderate	Moderate	Mild	Mild	None	Mild	Mild	Mild
Healing at week 1	Fair	Fair	Fair	Fair	Poor	Poor	Poor	Fair	Fair	Poor
Healing at week 2	Fair	Fair	Fair	Fair	Fair	Poor	Fair	Good	Fair	Fair
Healing at week 5	Fair	Fair	Fair	Fair	Fair	Fair	Good	Fair	Good	Fair
Healing at week 6	Good	Good	Good	Good	Good	Good	Good	Good	Good	Good

To close the surgical incision, suture patterns using either buried or exposed knots were used (Table 3.1). Sutures were removed at either week 2, 5 or not removed at all post-surgery (Table 3.1). The healing and trauma caused by the sutures will be discussed, using a few individual fish as examples. If present, abrasions of the medial aspect of the pectoral fin, due to friction from the incision or scale disruption, will be described as 'mild' or 'moderate' abrasions (Table 3.1). Mild abrasions were classified as minor white lesions on the fins where moderate abrasion were thicker larger lesions on the pectoral fins. Healing of the suture incision was subjectively classified as 'good', 'fair' or 'poor' based on suture/skin integrity, erythema (redness) along the wound and the presence of excess mucus (Table 3.1). Poor would be described as severe redness or erythema on the surgical site, poor suture/skin integrity with excess mucous. Fair would be moderate erythema and trauma to the surgical site with some mucous present. Good healing was classified as no erythema, good skin/suture integrity and minimal mucus coating on the surgical site.

The buried suture technique was performed on four of the ten fish (Fish numbers: 1572616, 1572245, 1574998 and 1573857). Fish 1572616 developed no abrasions on the pectoral fin (Fig. 3.1A-D). The other three (1572245, 1574998 and 1573857) fish developed mild abrasions (Fig. 3.2C, Fig. 3.3C and Fig. 3.4C). At week 2 there was good healing of the surgical incision in fish 1572245 (Fig. 3.2C), whereas the healing of fish 1574998 (Fig. 3.3C) and fish 1573857 (Fig 3.4C) was classified as fair. Due to scale disruption during surgery, resulting in fin abrasion and subsequent damage to the surgical site, fish 1573857 presented fair healing at week 2 (Fig. 3.4C). By week 6, the four fish with buried suture knots had healed completely with a faint line from the surgical site remaining. (Fig. 3.1D, Fig. 3.2D, Fig 3.3D and Fig. 3.4D).

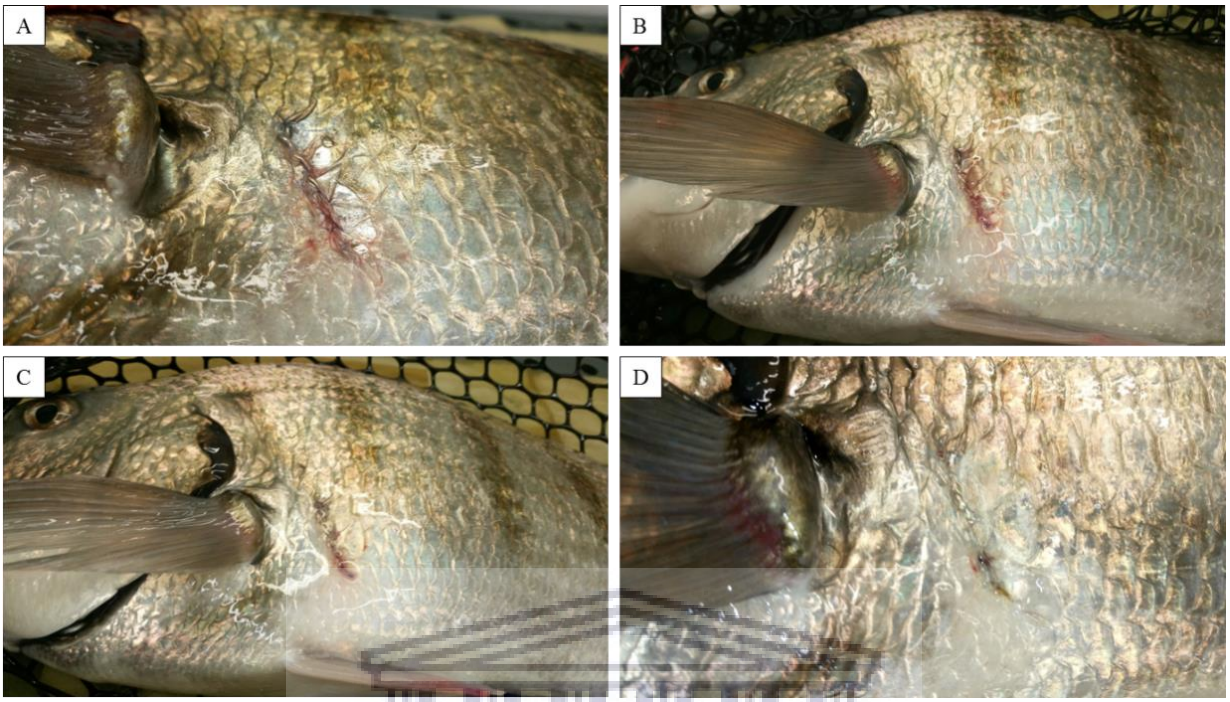

Figure 3.1: Wound healing after coeliotomy, buried suture technique, with no abrasions on the pectoral fin. Fish 1572616: A: week 0, B: week 1, C: week 2, D: week 6.

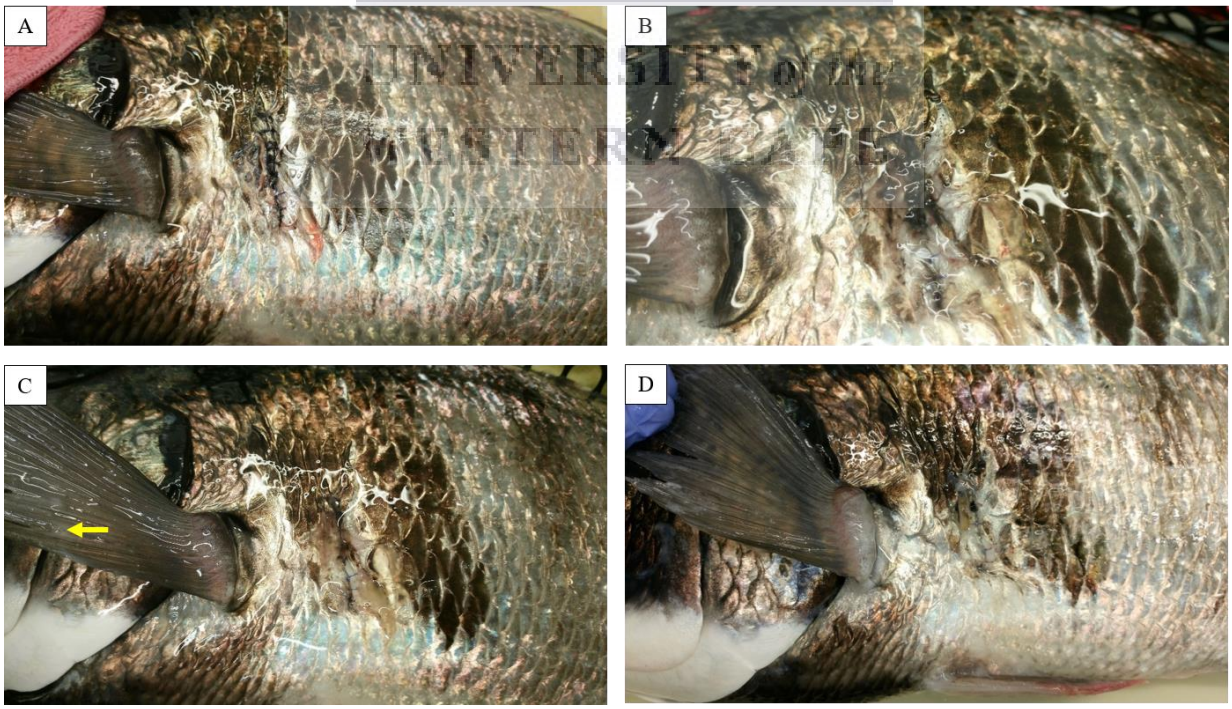

Figure 3.2: Wound healing after coeliotomy, suture technique with buried suture knots. Fish 1572245: A: week 0, B: week 1, C: week 2, D: week 6. Yellow arrow denotes mild pectoral fin abrasions.

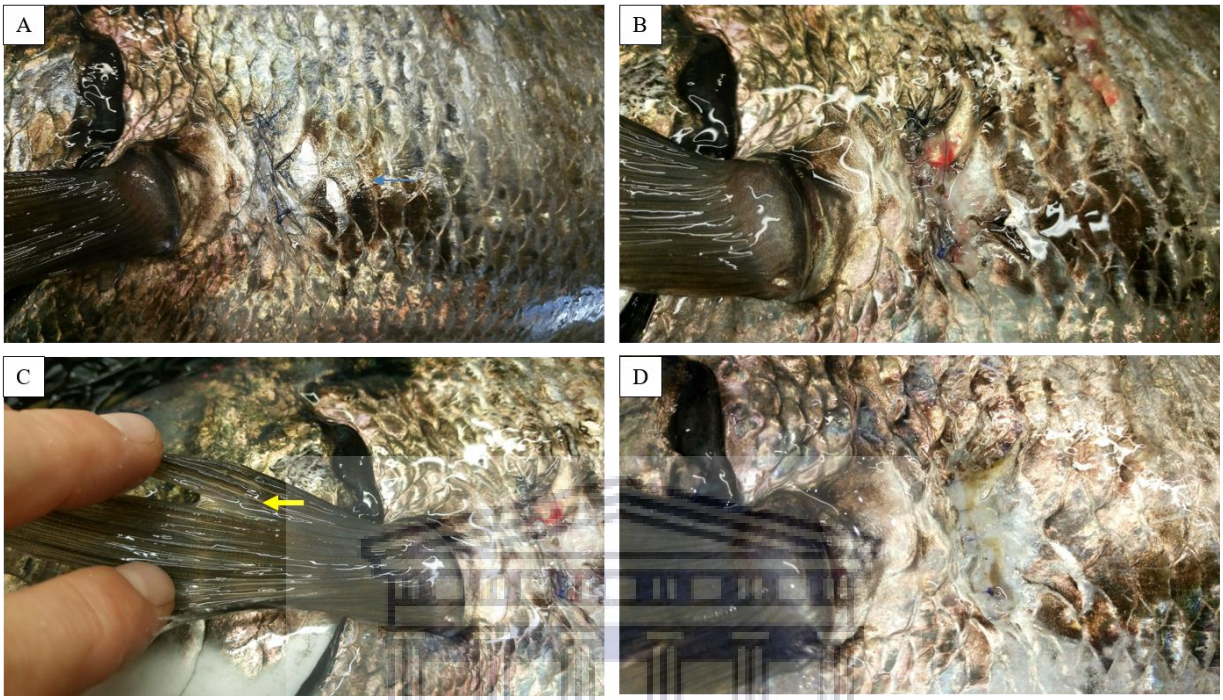

Figure 3.3: Wound healing after coeliotomy, buried suture technique. Fish 1574998: A: week 0, B: week 1, C: week 2, D: week 6. Yellow arrow denotes mild pectoral fin abrasions.

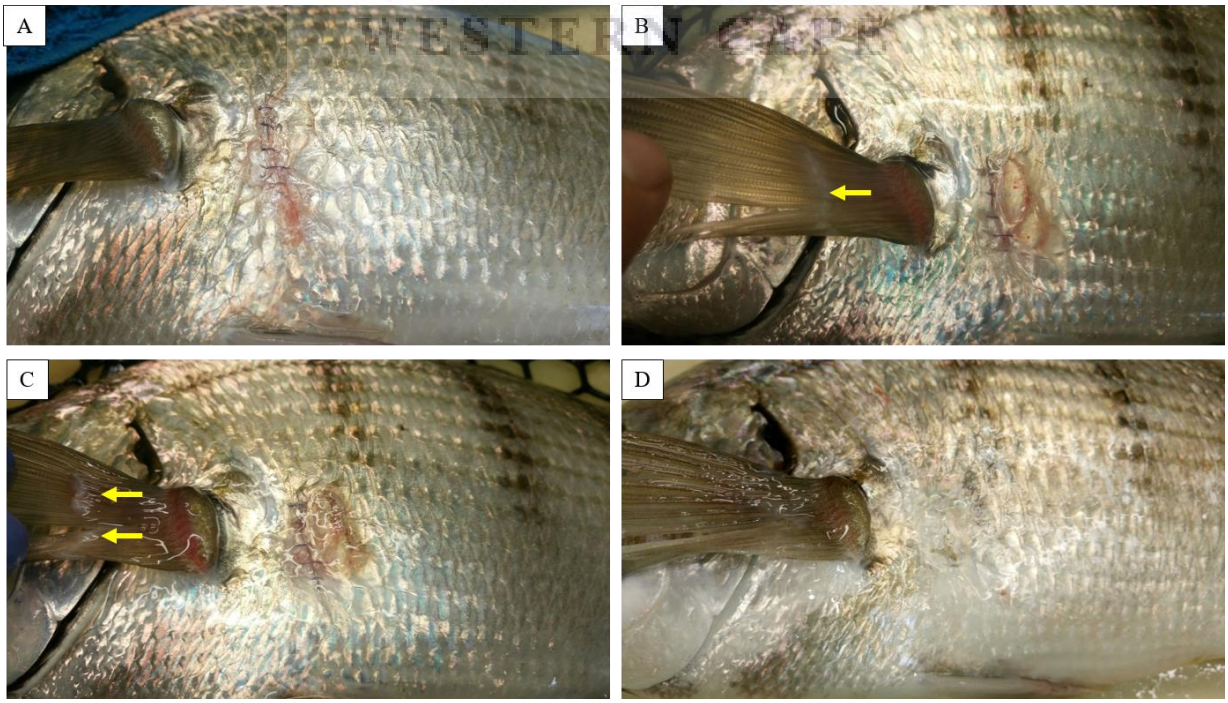

Figure 3.4: Wound healing after coeliotomy, suture technique with buried knots. Fish 1573857: A: week 0, B: week 1, C: week 2 (sutures removed), D: week 6. Yellow arrows denote mild pectoral fin abrasions.

Suture methods involving exposed suture knots were used in the remaining six fish. The exposed sutures are shown in figure 3.5A and 3.6A. Three of the six fish developed mild abrasions and the other three developed moderate abrasions. Two examples are shown here, fish 1573388 and 1575929 had moderate abrasions seen on the inside of the pectoral fin by week 1 (Fig. 3.5B and C, Fig. 3.6B and C). The ends on each knot were cut shorter to minimise the trauma on week 1 for fish when it was observed (Fig.3.5B). The remaining suture material was removed at week 5 for any fish showing a fair healing response. Between week 2 and 5 the incisions all showed a fair to good healing response (Fig 3.6B and C). By week 6 the wound had contracted leaving a faint line; good healing had occurred for all fish (Fig. 3.5D and Fig. 3.6D). All wounds healed satisfactorily and there were no wound infections or wound breakdown in any of the fish in the coeliotomy group.

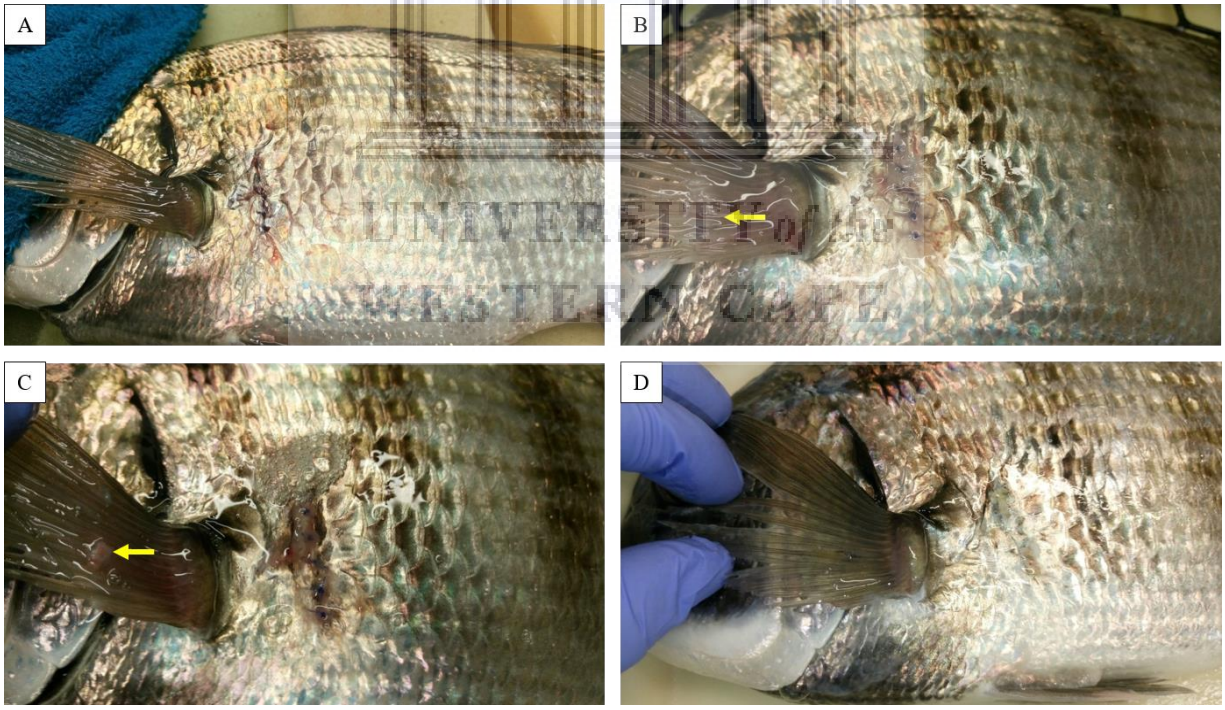

Figure 3.5: Wound healing after coeliotomy, simple interrupted, exposed suture technique. Fish 1573388: A: week 0, B: week 1, C: week 5 (sutures removed), D: week 6. Yellow arrows denote moderate pectoral fin abrasions.

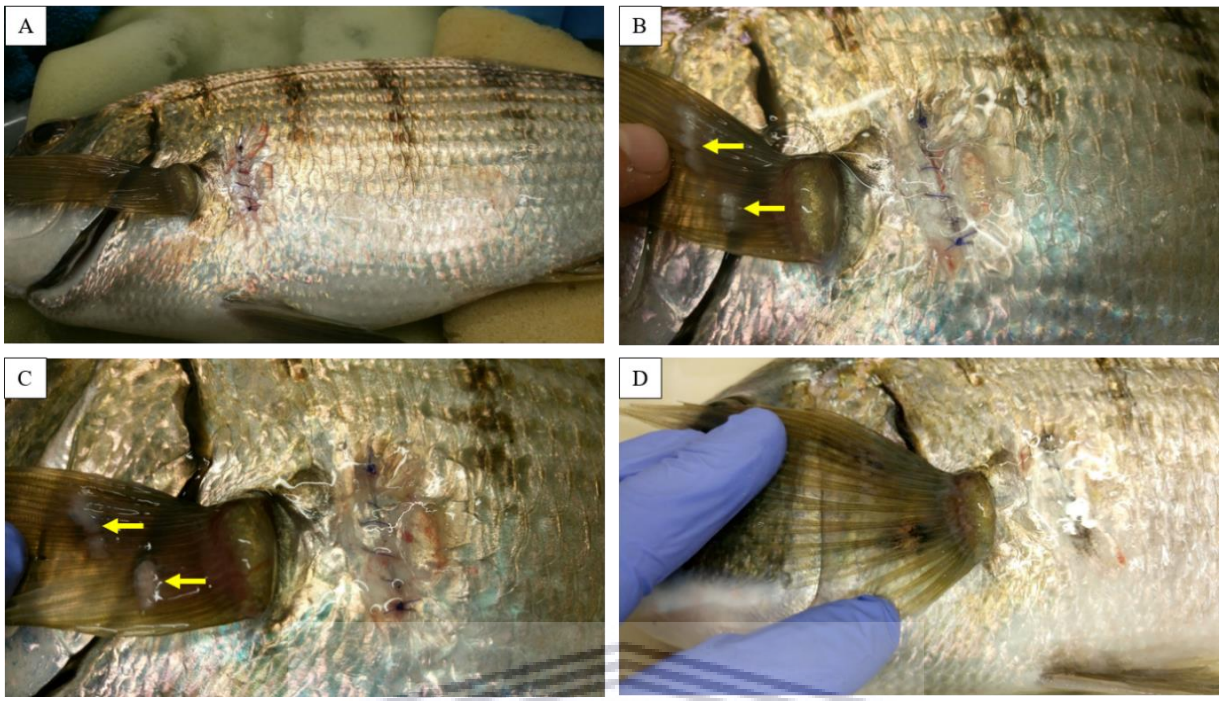

Figure 3.6: Wound healing after coeliotomy, exposed suture technique. Fish 1575929: A: week 0, B: week 1, C: week 5 (sutures removed), D: week 6. Yellow arrows denote moderate pectoral fin abrasions.

3.1.2 Coelioscopy

Table 3.2: Categorization of the suture removal post-surgery, level of abrasion trauma and the healing at weeks 1, 2, 5 and 6 post-surgery.

Fish number	1565431	1572982	1573226	1573288	1573519	1573720	1565492	1565700	1572800	1572075	1572522	1573493	1574302	1574552	1575884
Sutures removed	Not Removed	Not Removed	Week 5	Week 5	Not Removed	Week 5	Week 5	Week 5	Week 5	Week 5	Week 5	Not Removed	Week 5	Week 5	Week 5
Abrasion trauma on pelvic fins from sutures (week 0-6)	None	Mild	Moderate	Mild	Mild	Mild	Mild	Mild	Moderate	Moderate	Mild	Moderate	Mild	Mild	Mild
Healing at week 1	Good	Fair	Fair	Fair	Fair	Poor	Good	Fair	Fair	Fair	Fair	Poor	Fair	Poor	Fair
Healing at week 2	Good	Poor	Fair	Poor	Poor	Poor	Fair	Fair	Poor	Good	Poor	Good	Fair	Poor	Fair
Healing at week 5	Fair	Poor	Good	Poor	Fair	Fair	Poor	Fair	Fair	Poor	Poor	Good	Fair	Fair	Poor
Healing at week 6	Fair	Good	Good	Good	Good	Good	Good	Good	Good	Good	Good	Good	Good	Good	Good

For all coelioscopy surgical procedures there were two small incisions made to allow for the entry of the biopsy forceps and the endoscope (Table 3.2). These were sutured closed using one cruciate suture for each incision. The knot for each suture was not buried (left exposed). As for the coeliotomy group, if present, abrasions of the medial aspect of the pelvic fin, due to friction from the incision or scale disruption, are described as 'mild' or 'moderate' abrasions. Fish were examined to determine the level of healing and general state of the wound. Healing of the suture incision was subjectively classified as 'good', 'fair' or 'poor' based on suture/skin integrity, erythema (redness) along the wound and the presence of excess mucous. If poor or fair healing was observed, the sutures were reviewed (Table 3.2). If the fair or poor healing could be attributed to friction between the suture and pelvic fins, the sutures were removed, thereby reducing further trauma to the fish. There were 11 fish that had their sutures removed at week 5. These fish all showed a good healing response after suture removal. There were four fish that did not have their sutures removed at week 5, three exhibited good healing at week 6, with one fish showing a fair healing response. One fish developed no abrasions on the pelvic fins, ten fish developed mild abrasions and four fish developed moderate abrasions. Four fish are used as representative examples of the healing responses (Fig. 3.7A-D; 3.8A-D; 3.9A-D and 3.10A-D). An example of good healing with a mild abrasion to the left pectoral fin at week 5 is shown in fish 1573226 (Fig. 3.7C). The incision site of the coelioscopy fish may have become more traumatised than in the coeliotomy group due to contact with the base of the pool; the placement of the incision was more ventral and the fish often swam close to the bottom of the pool. The severity of the abrasions appeared to be affected by the length of suture material ends and knots left in the surgical sites and also on the size of the fish. The size of the fish is important as incisions that were performed more caudally in larger fish (Fig. 3.8A and B) allowed for a good healing response. The more caudally orientated wounds did not result in abrasions as the pelvic fin did not extend as far caudally as the wound. With the more cranially positioned suture sites there were abrasions due to friction resulting in poor healing between week 2 and 5, from the pelvic fins moving over the incision sites (Fig.3.9B). Similarly, this is seen in fish 1572008, where the suture of the cranial incision caused abrasions to the caudal edge of the left pelvic fin and the friction caused by the pelvic fin resulted in erythema of the incision site (Fig. 3.10 A and B). However, the caudal incision showed a good healing response. Once the sutures were removed, moderate abrasions healed with no further abrasions seen after removal (Fig. 3.10 D).

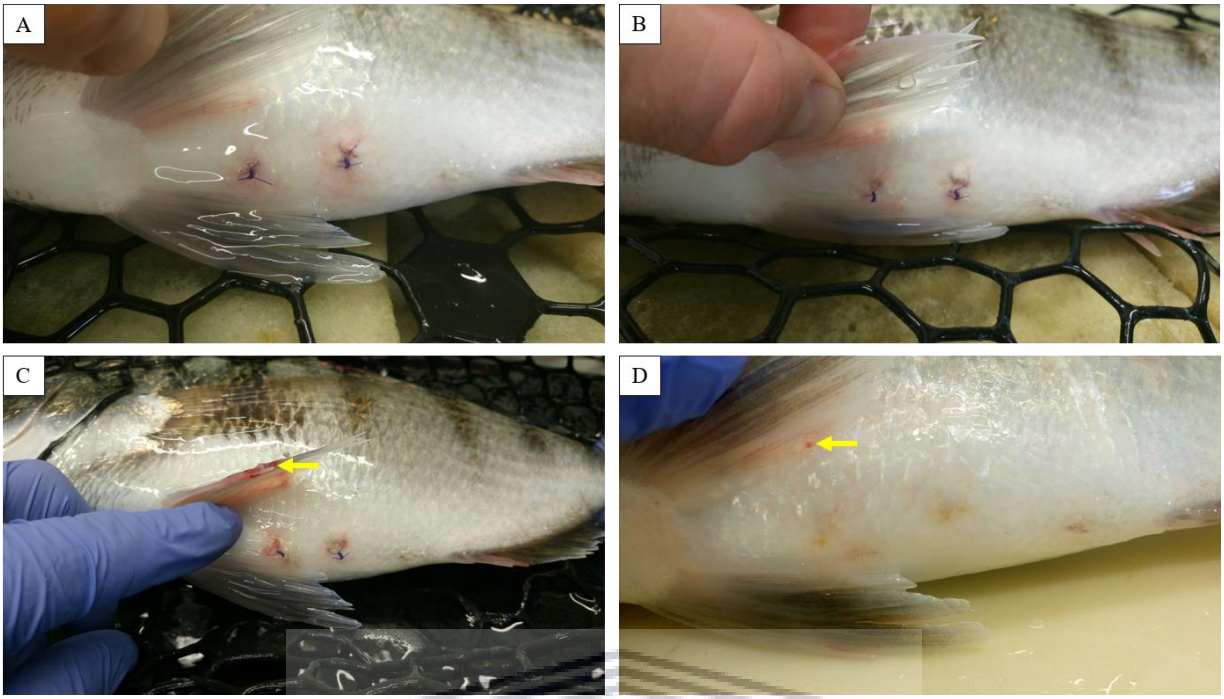

Figure 3.7: Wound healing after coelioscopy. Fish 1573226: A: week 1, B: week 2, C: week 5 (sutures removed), D: week 6. Yellow arrows indicating mild abrasions.

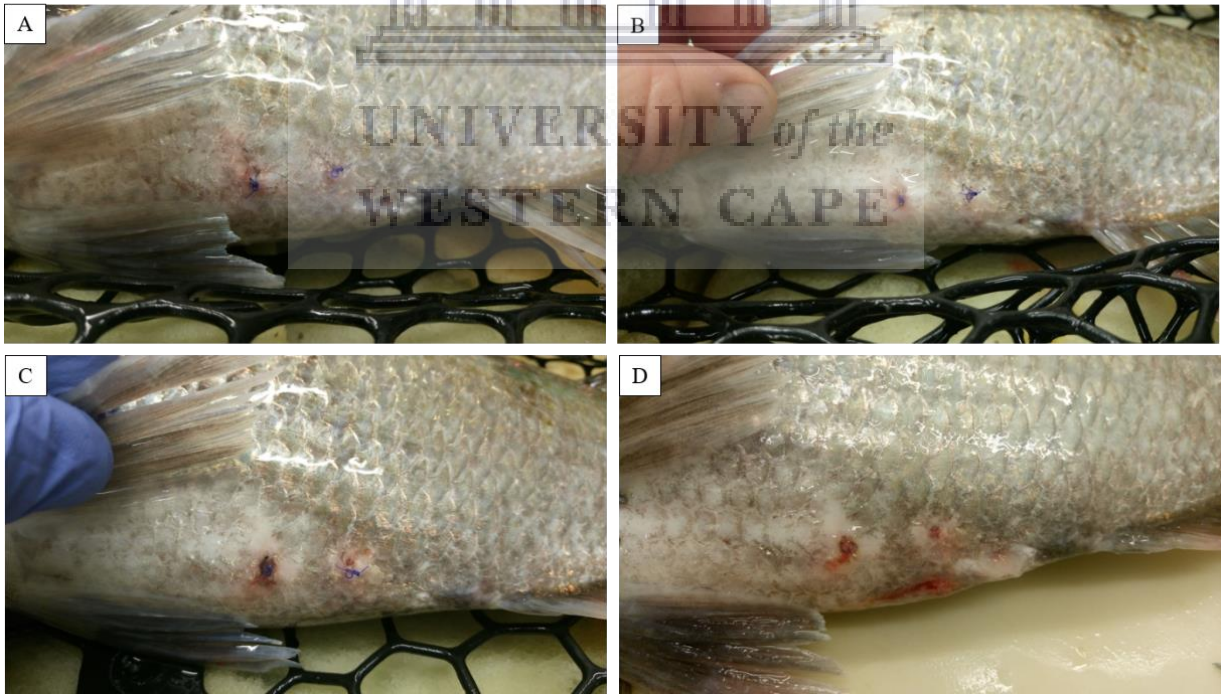

Figure 3.8: Wound healing after coelioscopy. Fish 1565431: A: week 1, B: week 2, C: week 5, D: week 6.

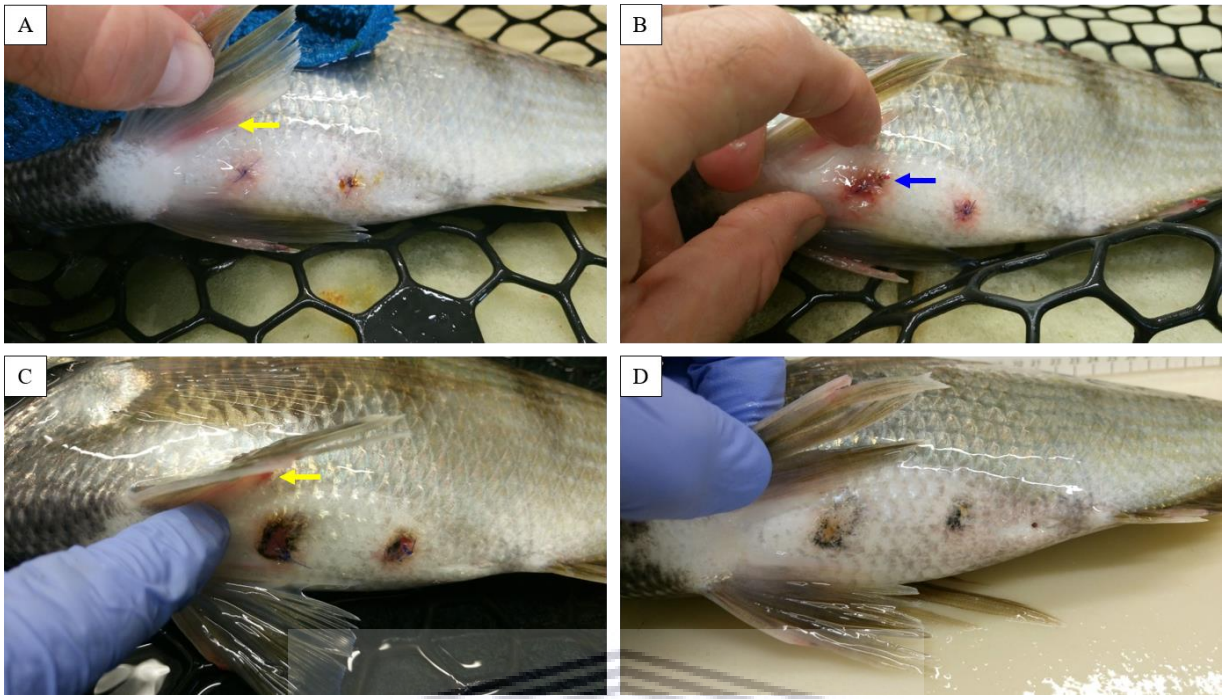

Figure 3.9: Wound healing after coelioscopy. Fish 1573288: A: week 1, B: week 2, C: week 5 (sutures removed), D: week 6. Yellow arrows denote mild abrasions to the pelvic fins. Blue arrow illustrates poor healing at the cranial surgical site.

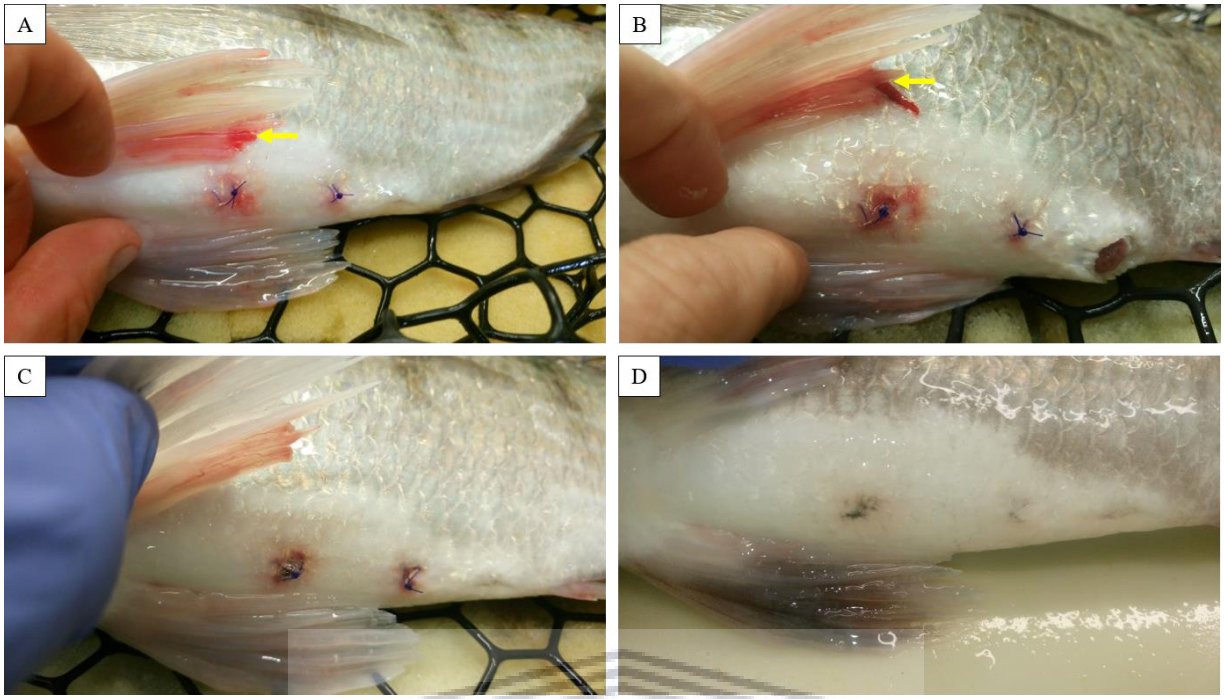

Figure 3.10: Wound healing after coelioscopy. Fish 1572008: A: week 1, B: week 2, C: week 5 (sutures removed), D: week 6. Yellow arrows denote the moderate abrasions to the pelvic fins.

3.2 Post mortem biopsy observations

It was hypothesised that a larger biopsy sample might provide a more sensitive test for *I. hoferi*. From the results obtained in this study, it was shown to be the way in which *I. hoferi* presents itself in the liver. The dispersal of the parasite and how it manifests itself in the organs of fish is still ambiguous and has not been researched yet. In the coeliotomy group the biopsies were sampled from the tip of the left liver lobe as this was the part of the liver that could be exteriorised (Fig. 3.11). In the coelioscopy group the biopsies were all sampled more dorso-cranially from the caudal edge of the liver lobe (Fig. 3.12).

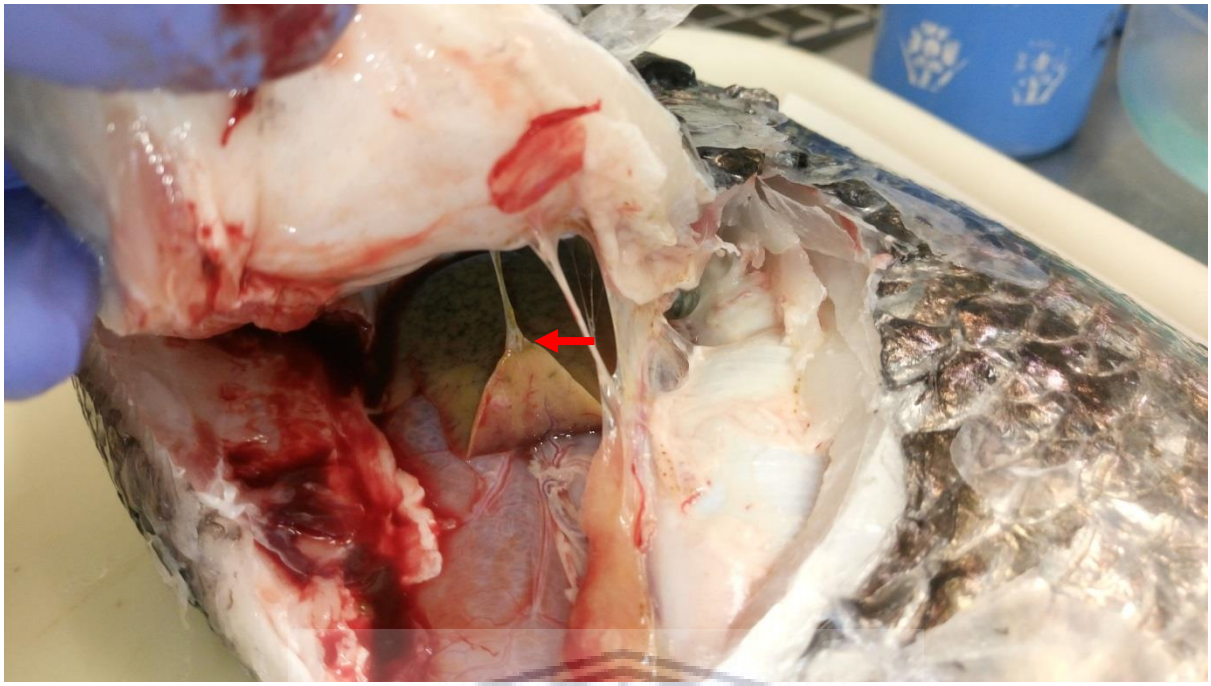

Figure 3.11: Red arrow indicating the biopsy site of the liver and adhesion to the body wall on fish 1572245 that underwent coeliotomy.

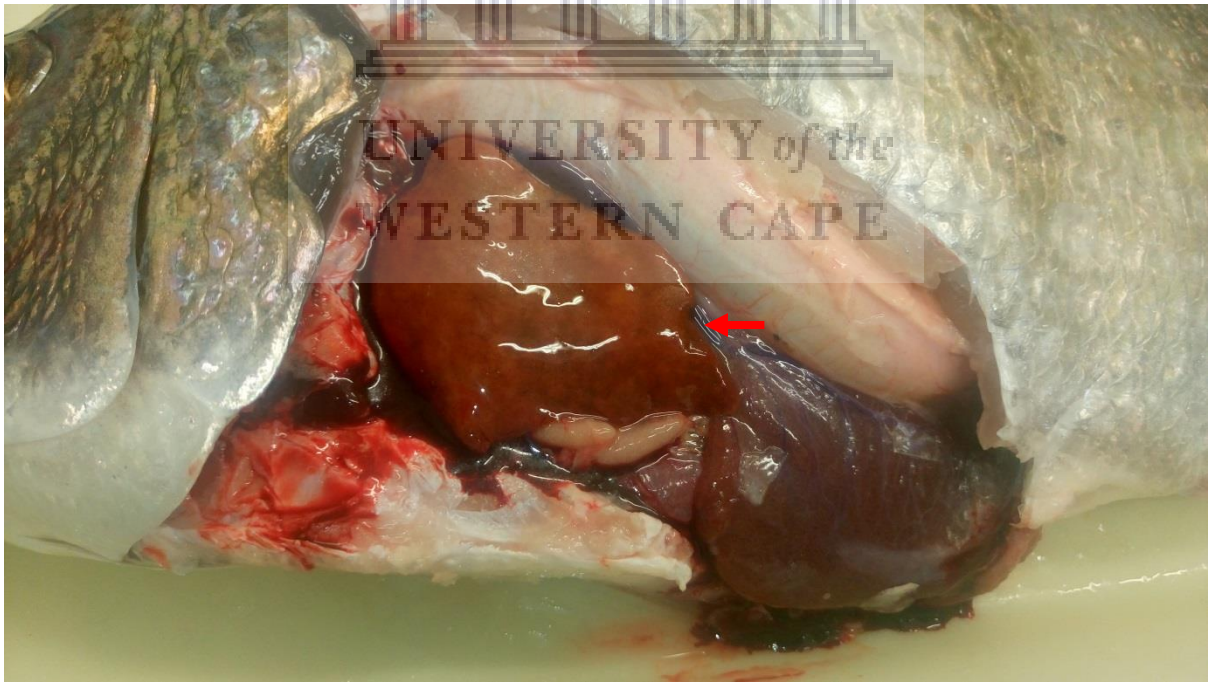

Figure 3.12: Red arrow indicating the biopsy site of the liver of fish 1573493 that underwent coelioscopy.

Fish were euthanised after the last blood sample was taken at week 6 and post mortem examinations were completed on all fish. There were a number of observations made at post mortem in the fish that underwent the two different surgeries. In the coeliotomy group all biopsy sites on all fish adhered to either the body wall, organs, coelomic fat or visceral tissue that was in close contact with the site once the biopsy was sampled. Some of the sites adhered to one or more of these tissues or organs. All biopsy sites had healed completely, some biopsy sites did show pigmentation which can be expected post-surgery. There were small adhesions that were present in the coelioscopy group but mainly to the mesentery, lower gonads and intestine. The body wall where adhesion occurred was discoloured with an orange/yellow colour and this could potentially be haemorrhaging that took place post-biopsy. This is a common post-surgical physiological response and there did not seem to be any complications internally from the incisions made with regard to this surgical procedure.

3.3 Fulton's condition factor

This was measured to determine the overall robustness or condition of the fish over time. The condition coefficient was calculated using

$$K = 100*W/L^3$$

Where W is the weight of the fish in grams and L is the length of the fish in centimetres. If a fish doubles its length it will potentially increase its' weight by almost eight times. This is taken from Galileo's law and was then subsequently referred to as the 'cube law'(Froese, 2006). The initial Fulton's condition factor was determined for the start of the experiment by using weights and lengths from the day of surgery and the final Fulton's condition factor was determined by weighing the fish on the day of post mortem at the end of the experiment. The same lengths were used for the final Fulton's condition factor to mitigate any sampling error and due to the experiment only running over 45 days. There was no difference in the average conditions in the coeliotomy group ($p=0.4$) and the coelioscopy group ($p=0.23$) over the course of the six weeks post-surgery (Fig. 3.13 and 3.14). The control group was not significantly different in condition at the start of the experiment when compared to the end ($p=0.082$) (Fig. 3.15). Paired t-tests for dependant samples were used for the analyses and the p-value was set at 0.05 for any statistical significances.

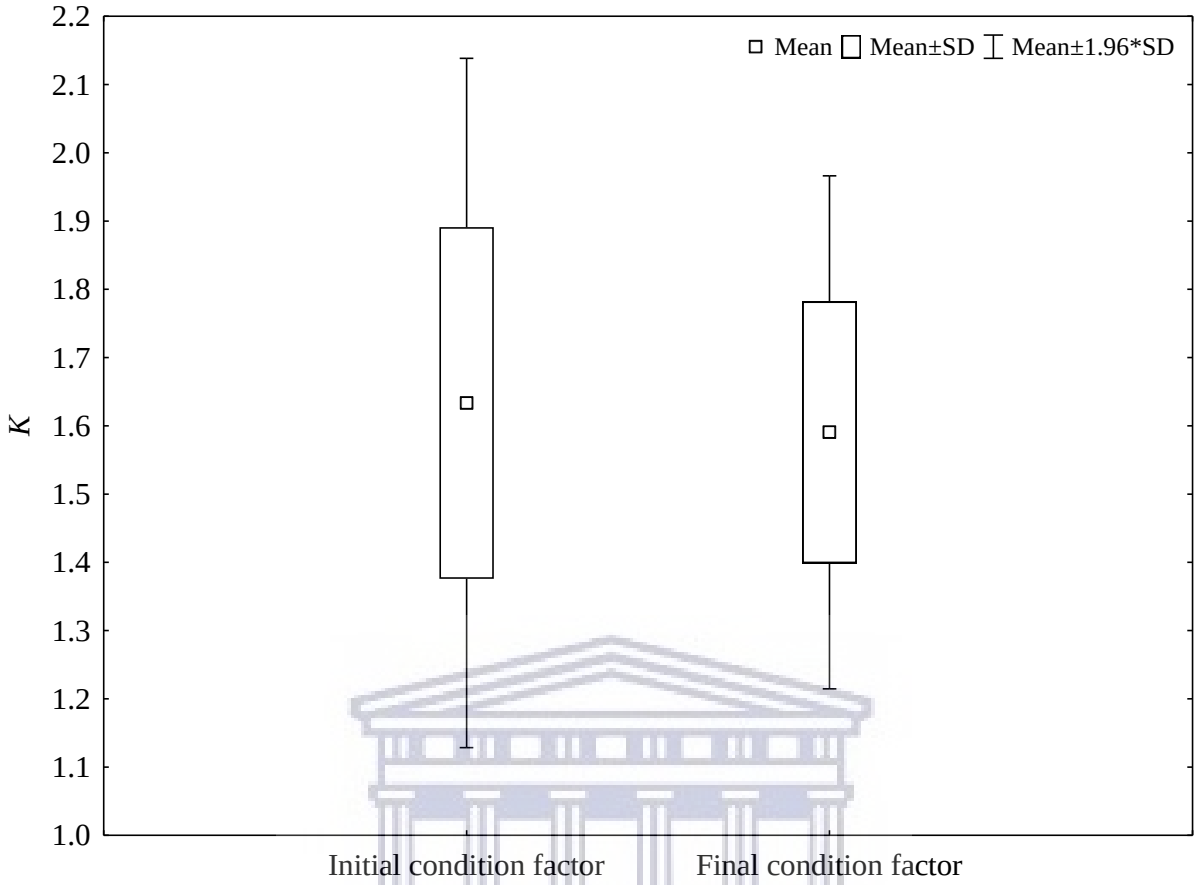

Figure 3.13: Fulton's condition factor (*K*) for the coeliotomy group fish at the start of the experiment (Initial condition factor) and the end of the experiment at post mortem (Final condition factor) where p=0.40.

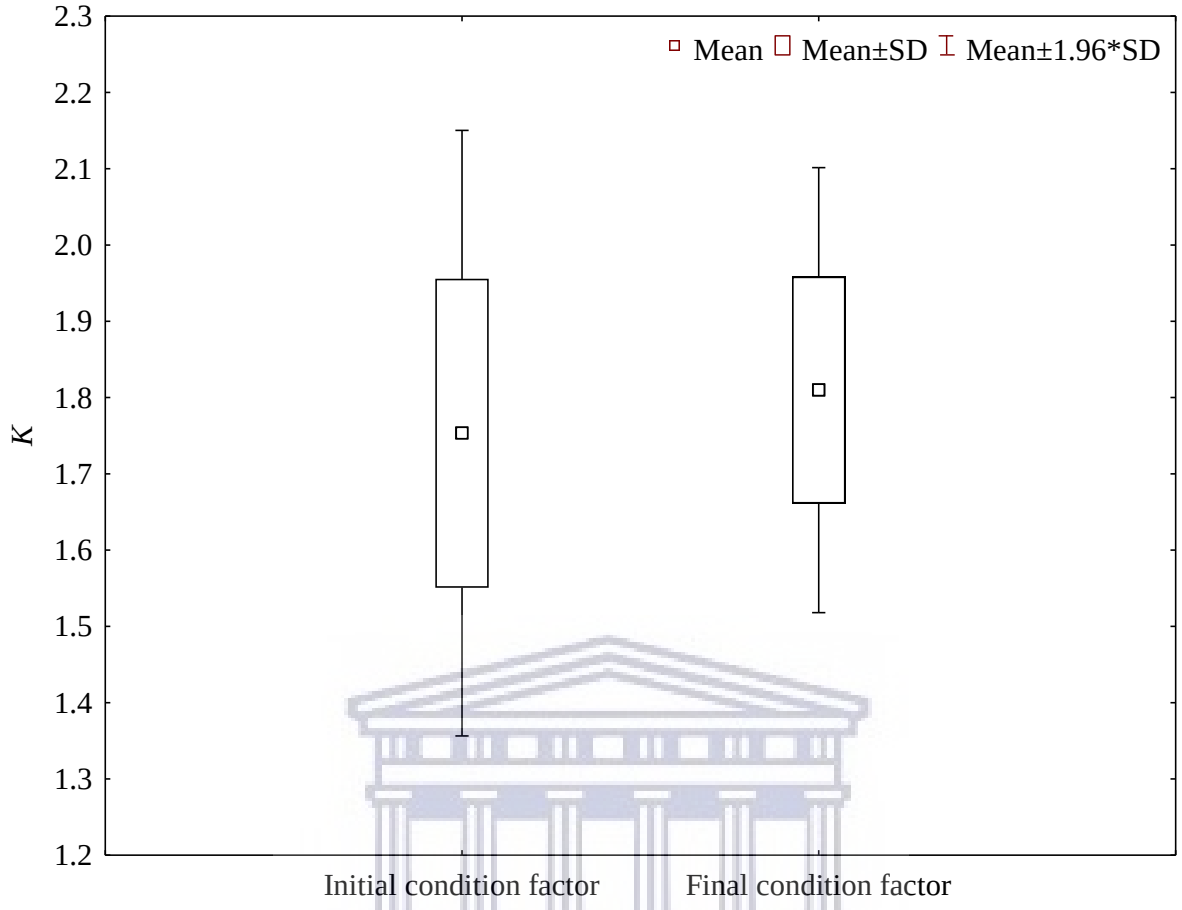

Figure 3.14: Fulton's condition factor (*K*) for the coelioscopy group fish at the start of the experiment (Initial condition factor) and the end of the experiment at post mortem (Final condition factor) where p=0.23.

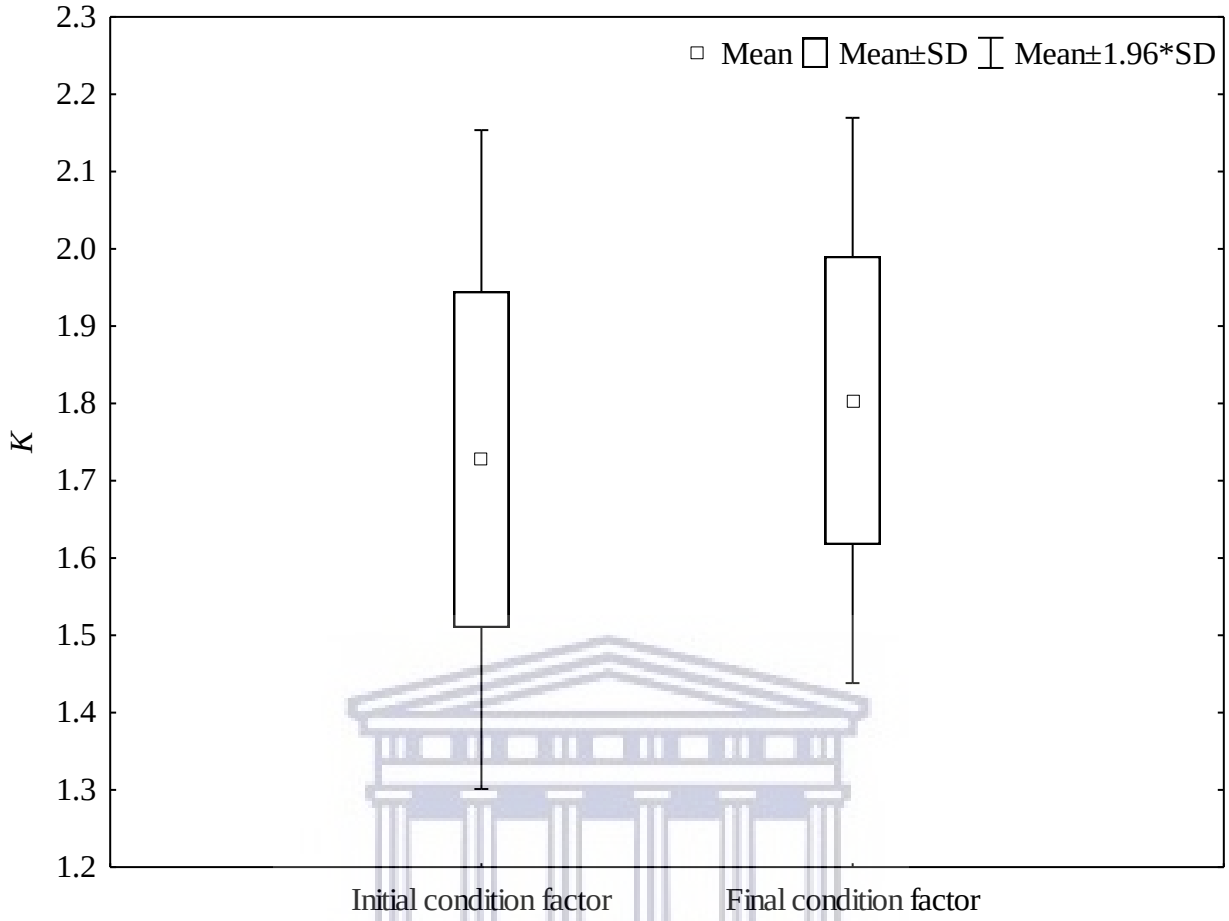

Figure 3.15: Fulton's condition factor (*K*) for the control group fish at the start of the experiment (Initial condition factor) and the end of the experiment (Final condition factor) where p=0.082.

3.4 Surgical times

Data was tested for normality and independent T-tests were used to determine the mean differences between the coeliotomy surgeries and the coelioscopy surgeries. A variable that was not accounted for was the experience gained by the surgeon through performing these surgeries, the surgeons experience increased in turn decreasing the total time of the surgeries for both surgical groups. These mean ± standard deviations for the coeliotomy surgeries were just over 28±10.71 minutes and the coelioscopy surgeries were completed in 23±6.23 minutes. It took an average of 5 minutes longer to complete a coeliotomy surgery than a coelioscopy surgery.

3.5 Recovery times

Recovery, referring to the period of time between termination of anaesthetic and subsequent return of the fish to a conscious state, times were longer in the coeliotomy group than in the coelioscopy group. The coeliotomy group took on average 21±21.86 minutes to recover with the coelioscopy group taking 9.66±5.02 minutes to recover.

Results: Post-surgical health

All fish from the three groups (coeliotomy, coelioscopy and control) underwent a post-monitoring phase which included blood sampling at week 0, 1, 2, 5 and 6. The blood parameters were tested and used as indicators to provide a measure of post-surgical health and healing (Campbell, 2015).

3.6 Post-surgical health

There were no mortalities over the duration of the entire experiment. All fish recovered from anaesthesia and survived six weeks post-surgery. Surgical sites on the fish were monitored over this period and photographs were taken, as previously described (Chapter 3.1). Sutures that had not been cut short enough caused dermal lesions in both surgical groups as previously described (Chapter 3.1). Once the surgical site had healed completely there were some fish where dark pigmentation occurred. In the fish where this occurred, the skin was scraped and examined microscopically for protozoan parasites such as scuticocilates or evidence of bacterial infection.

It was noticed that repeated blood sampling caused trauma to the caudal peduncle. In the study four fish presented with marked trauma related to blood sampling. It was found that repeated blood sampling from the same location caused erythema, trauma leading to scuticociliatosis in one case, and iatrogenic damage from capture (Table 3.3). 'Mild' trauma was classified where mild erythema and an accumulation of mucous was observed around the sample site. 'Marked' trauma was classified where diffuse erythema and substantial scale loss on the caudal peduncle was observed (Table 3.3). Scuticociliatosis was diagnosed by observing trauma consistent with excess mucus, scale and tissue loss in a localised area. Scuticociliatosis was confirmed

microscopically on a slide from a skin scrape of the area in question. One fish (1572245) presented with a severe case of scuticociliatosis on the caudal peduncle from repeated blood sampling and missed a blood sample while receiving treatment. The complete blood sampling schedule for all fish had to be altered to allow for the caudal peduncle to heal and recover between samples.

Table 3.3: Record of trauma to caudal peduncle from repeated blood sampling.

	Coeliotomy	Coelioscopy	Control	Total
No trauma	8	6	4	18
Mild trauma	1	7	4	12
Marked trauma	0	2	2	4
Scuticociliatosis	1	0	0	1
Total	10	15	10	35

3.7 Haematology

It should be noted that for the analysis of this blood parameter data, the coeliotomy group had a total n=10 and the coelioscopy n=15. The reason was that five fish underwent coeliotomy a few months prior but did not have all the blood parameters sampled at the same intervals as the other ten fish and thus the data could not be used. A control group of ten fish were also sampled and were used to provide a comparative analysis between the surgical group fish and fish that did not undergo surgery. There is not much research on blood reference ranges and blood parameter values for *R. globiceps* and this is problematic when interpreting the results. Results can be interpreted between surgical groups and the control group was used as a baseline within this study for comparison. A repeated measures ANOVA was used to analyse the means between each group from week 0 to 6. Differences within each group at differing weeks are

described using the outcome of Tukeys post-hoc test. Significant differences were determined at a $p<0.05$.

3.7.1 Packed cell volume

Packed cell volume is a way to measure the proportion of red blood cells within the blood and is an accepted test in fish to give a measure of overall condition (Weiss and Wardrop, 2011). A PCV can be used as an indicator of anaemia in fish and a high PCV can indicate dehydration or be related to other disease processes related to red blood cell production. Shen *et al.* (2011) showed PCV or haematocrit values in *R. sarba* to be approximately 30% providing a guide for the similar species *R. globiceps* (Shen *et al.*, 2011).

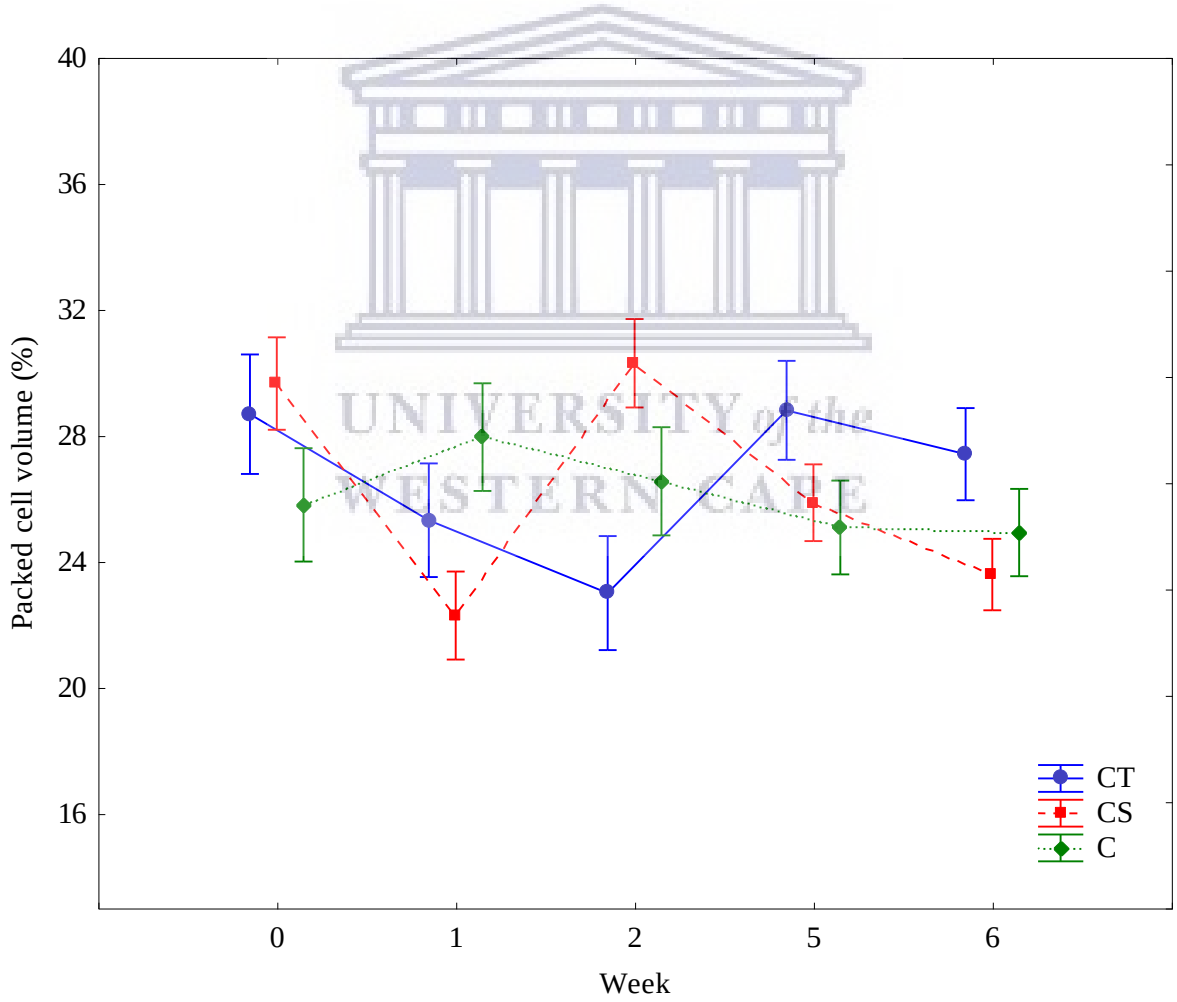

Figure 3.16: Mean packed cell volumes (%) from blood sampled in *Rhabdosargus globiceps* in three groups: coeliotomy (CT), coelioscopy (CS) and the control (C) group at week 0, 1, 2,

5 and 6 post-surgery. The points represent the means and the vertical bars denote the standard error providing a range of error within the data.

There were significant differences ($p<0.05$) between the coeliotomy group and the coelioscopy group at week 2 ($p=0.00047$) where the PCV means were 22% and 30%, respectively and week 6 ($p=0.031$) where PCV means were 27% and 24%, respectively (Fig. 3.16). The coelioscopy group showed a lower packed cell volume of 23% when compared to the control group of 28% PCV means at week 1 post-surgery ($p=0.016$). Coeliotomy shows a decrease of 6% in PCV from week 0 to 2 and then increases again by 6% from week 2 to 5 with a slight decrease again of 2% from week 5 to 6. There is a similar trend in the coelioscopy group with a decrease of 8% from week 0 to 1 and an increase of 8% from week 1 to 2 and then an average decrease of 6% from week 2 to 6. The control group shows a fairly stable trend with the PCV means increasing by 3% from week 0 to 1 and then gradual decrease of 4% from week 1 to 6.

3.7.2 Glucose

Blood glucose (mmol/l^{-1}) is used as a measure of stress in fish as it can be easily measured using machines developed for human medicine (Morgan and Iwama, 2011).

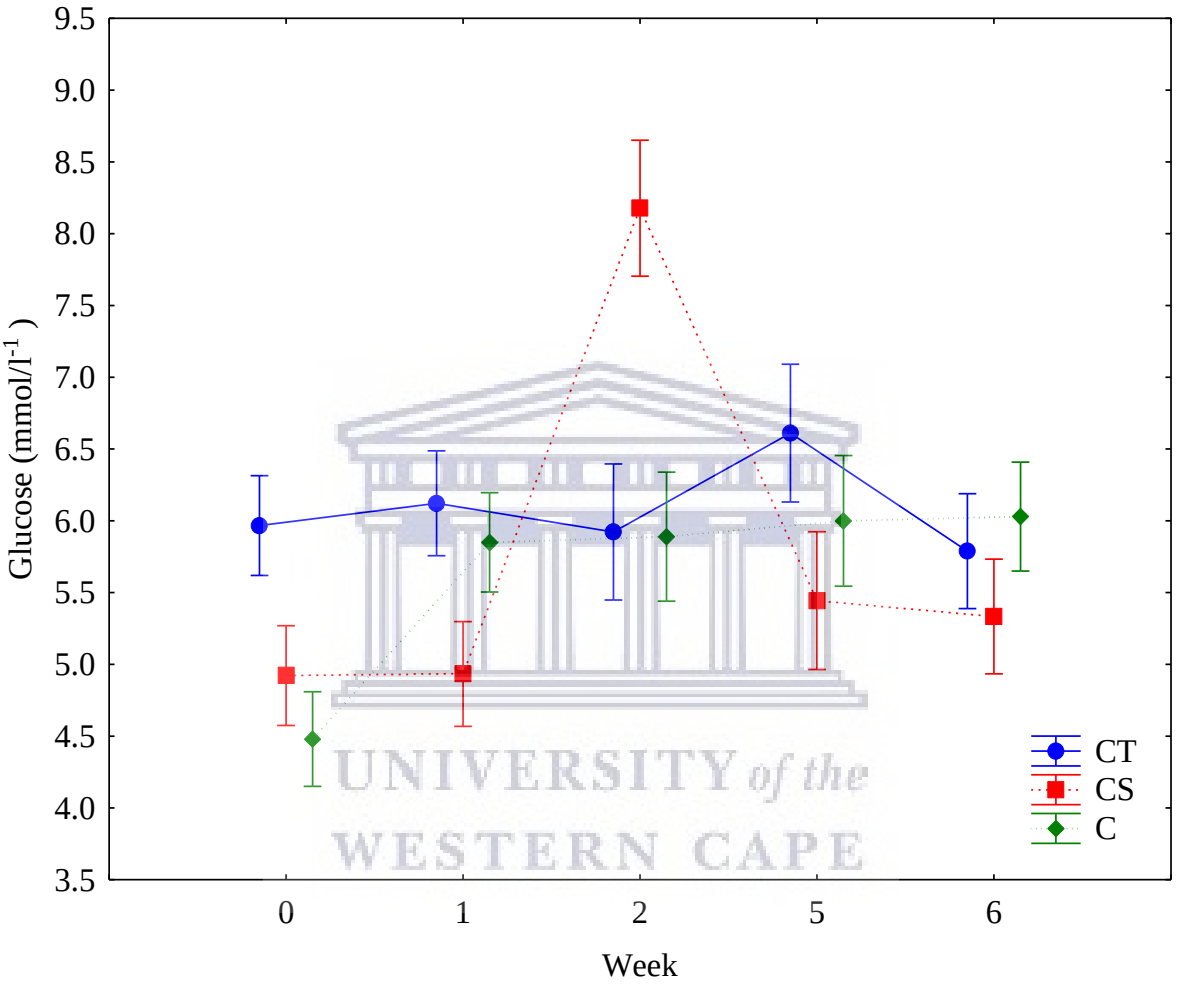

Figure 3.17: Mean glucose levels (mmol/l^{-1}) from blood sampled in *R. globiceps* in three groups: coeliotomy (CT), coelioscopy (CS) and the control group (C) at week 0, 1, 2, 5 and 6 post –surgery. The points represent the means and the vertical bars denote the standard error providing a range of error within the data.

The coelioscopy group showed a significantly raised glucose mean (8.5mmol/l^{-1}) at week 2 that was significantly higher than week 0, 1, 5 and 6 (Fig. 3.17). The coelioscopy glucose mean at week 2 was significantly different compared to the coeliotomy and control groups at week 2 ($p=0.0015$). The coeliotomy group was significantly higher than the coelioscopy group at week

5 (p=0.042). At week 0, or the day of surgery, the control group showed a lower mean glucose of 4mmol/l^{-1} when compared to the coeliotomy group with a mean glucose of 5.5mmol/l^{-1} (p=0.019). Coelioscopy average glucose levels are lower at week 1 when compared to the control group. The coelioscopy group showed significantly different glucose means when compared to the control group at week 1 (p=0.031) and 2 (p=0.0019), respectively. The general trend for the control group showed an increase of glucose mean values from week 0 to 6 with an average of under 4.5mmol/l^{-1} at week 0 and increasing to over 5.5mmol/l^{-1} at week 6 (Fig. 3.17).

3.7.3 Total Protein

Total protein concentrations were sampled directly after the blood was drawn and centrifuged, while the other serum chemistry was processed at a later stage. Total proteins provide supportive information and data with regards to the fish's health by aiding in determining the nutritional condition of the fish and providing an indication on liver function (Patriche *et al.*, 2009). A study done on hybrid bass used changes in haematology as a diagnostic tool in clinical pathology where total proteins were used as part of the blood analysis (Hrubec *et al.*, 2001). Hrubec *et al.* (2001) showed that in *Morone chrysops* x *Morone saxatilis* (Hybrid seabass) the total protein ranges between 4 to 6 g/dl. A study done on *Sparidentex hasta*, which like *Rhabdosargus globiceps* belong to the Sparidae family, demonstrated reference intervals between 3.2 g/dl and 3.9 g/dl (Mozanzadeh *et al.*, 2015). Since few studies exist on total proteins in fish and specifically *Rhabdosargus globiceps* it was necessary to have a control group to provide a comparison. Total proteins together with other blood parameters add to the overall result in determining the health of a fish.

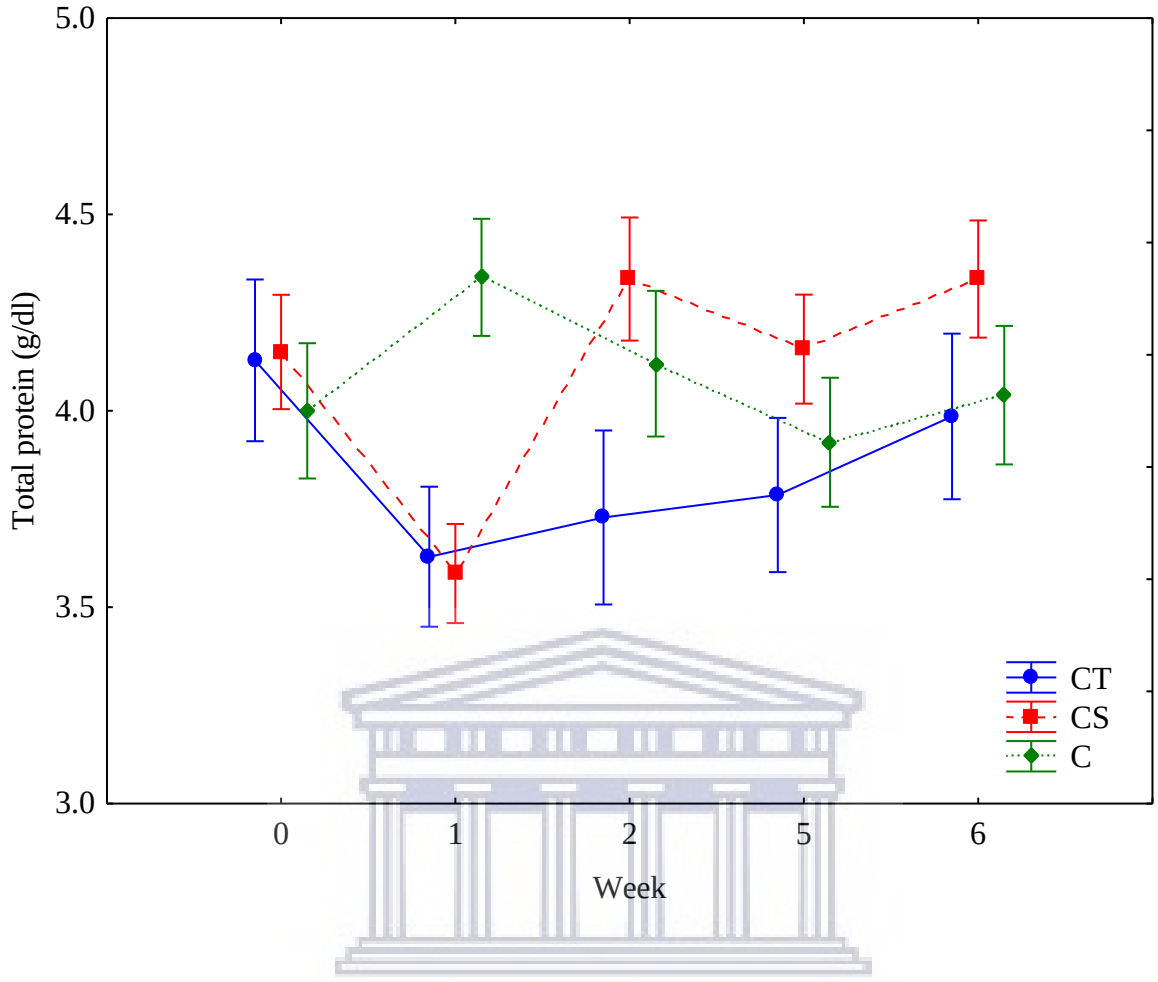

Figure 3.18: Mean total protein concentrations (g/dl) from blood sampled in *Rhabdosargus globiceps* in three groups: coeliotomy (CT), coelioscopy (CS) and the control group (C) at week 0, 1, 2, 5 and 6 post-surgery. The points represent the means and the vertical bars denote the standard error providing a range of error within the data.

Total protein concentrations were significantly higher in the coelioscopy group (4.3g/dl) at week 2 when compared to the coeliotomy (3.4g/dl) group (p=0.007). Both surgical groups showed a mean of 3.6g/dl at week 1 when compared to the control group of 4.3g/dl which was statistically significant (p=0.0014) and (p=0.00021). The control group shows an increase initially from week 0 to week 1. Both surgical groups showed a similar trend over time with a decrease in mean total protein concentrations from week 0 to 1 with an increase from week 1 to 6 (Fig. 3.18).

3.7.4 White Blood Cell Counts

White blood cell counts are indicative of potential infection, stress, inflammation, trauma, allergy, or certain diseases (Weiss and Wardrop, 2011). The change in white blood cell counts or the comparisons can be attributed to the potential stress from the surgical procedure, blood sampling and general handling of the fish over time (Fig. 3.19).

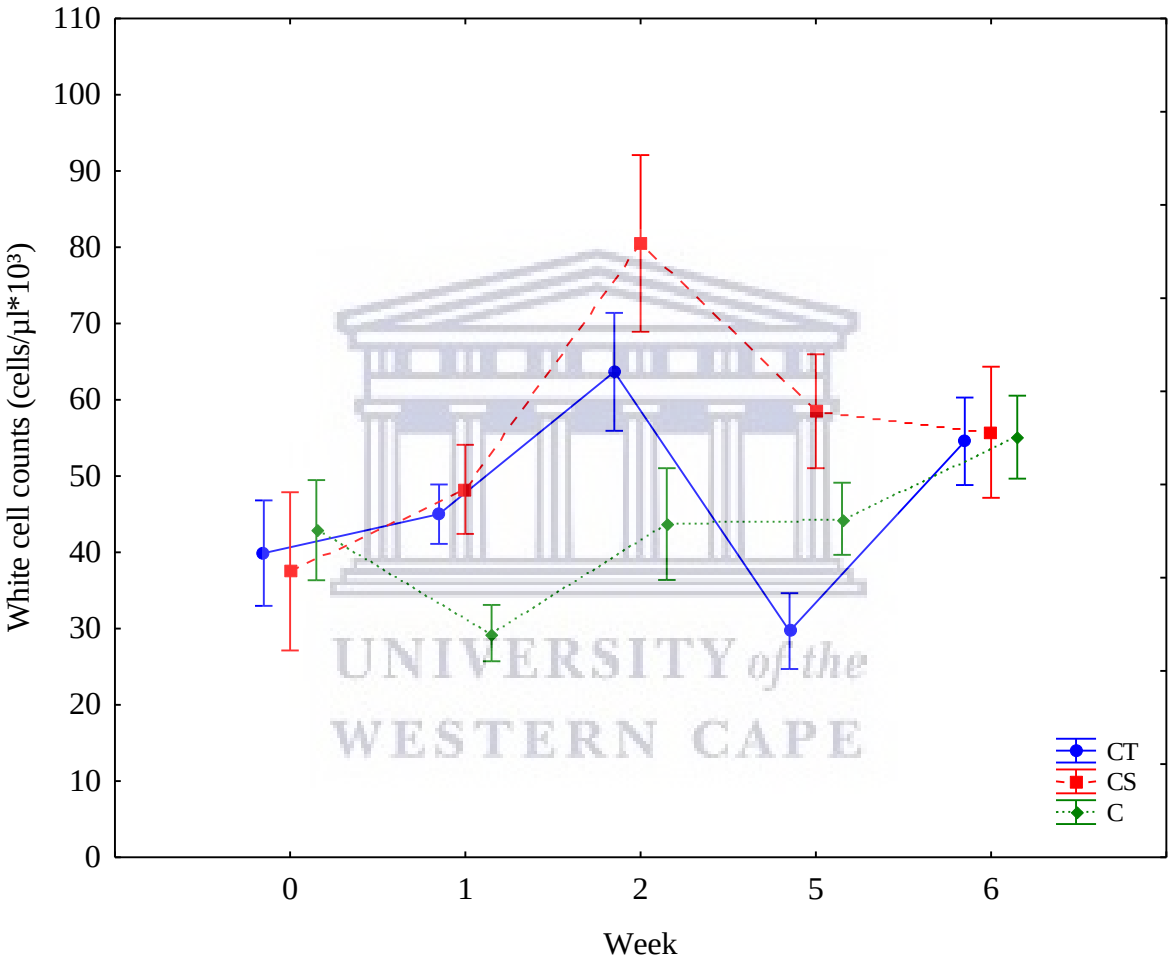

Figure 3.19: Mean white blood cell counts (cells/µl*10³) from blood sampled in *Rhabdosargus globiceps* in three groups: coeliotomy (CT), coelioscopy (CS) and the control (C) at week 0, 1, 2, 5 and 6 post-surgery. The points represent the means and the vertical bars denote the standard error providing a range of error within the data.

The mean white blood cell counts are higher in the coelioscopy group at 50 cells/µl*10³ compared to coeliotomy group with a mean count of 30 cells/µl*10³ at week 5 post-surgery

(p=0.0005). This is the only significant difference in white blood cell counts between the two surgical groups (Fig. 3.19). The following differences in white blood cell counts are between the surgical groups and the control group. At week 1 the coeliotomy group showed a significantly higher average white blood cell count than the control group post-surgery (p=0.006) (Fig. 3.19). At week 2 there was a higher average white blood cell count in the coeliotomy group when compared to the control group (p=0.012). At week 5 the control group had a statistically significantly higher average white blood cell count of 40 cells/µl*10^3 than the coeliotomy group with a count of 30 cells/µl*10^3 (p=0.041). The coelioscopy group average white blood cell counts was lower at 25 cells/µl*10^3 than the control group at 40 cells/µl*10^3 on the day of surgery at week 0 (p=0.034). The coelioscopy group had a higher average white blood cell count at week 1 post-surgery when compared to the control group that did not undergo any surgical procedure (p=0.0074). The coelioscopy group showed a significantly higher average white blood cell count at week 2 post-surgery compared to the control group (p=0.046). The surgical groups show a rise in white blood cell counts between weeks 0 to 2 whereas the control group shows a more gradual increase in the mean white blood cell count from week 0 to week 6. The coeliotomy group showed a decrease in the mean white blood cell counts between week 2 and week 5 with it increasing again between week 5 and 6. The coelioscopy group showed an increase in the mean white blood cell count between week 0 and 2 (Fig. 3.19). The overall trend is that all groups start out with a similar mean white blood cell counts and the mean counts increase to similar counts by week 6 at the end of the study (Fig. 3.19).

CHAPTER 4

The parasite *I. hoferi*, has been identified in numerous organ tissues of multiple species of fish (McVicar, 2011). Identification has been made through observation of tissue squash mounts, polymerase chain reaction (PCR), culture and histopathology. Identification of *I. hoferi* in the liver is well documented, however identification from a small section of liver taken from the biopsy of a live fish is novel (Kocan *et al.*, 2011). Biopsies from the liver of live fish have been obtained for histological examination as part of fish health evaluation (Tresise *et al.*, 2014). If *I. hoferi* could be identified from a biopsy, it may facilitate improved management of valuable fish housed in affected aquaria. In the initial experiments eight out of ten fish (*Rhabdosargus globiceps*) were diagnosed at post mortem examination to be infected with *I. hoferi*. Of the eight fish that were positive, seven were correctly diagnosed by microscopic examination using squash mounts of the liver biopsy. This produced a sensitivity of 87.5% and a specificity of 100% for detecting *I. hoferi* by liver biopsy. These preliminary results provided confidence in the potential for diagnosing *I. hoferi* from a biopsy and in selecting the liver as the organ to biopsy for the study.

Results: Diagnostics

In this study, 30 fish were anaesthetised using a fish anaesthetic rebreathing system (FARS), in order to obtain a liver biopsy from a live fish. Two surgical approaches were used: coeliotomy; where an incision was made allowing the surgeon to exteriorise and biopsy the liver, and coelioscopy; where two smaller incisions were made for an endoscope and biopsy forceps which were then triangulated to biopsy the liver *in situ*.

4.1 Biopsy analysis

The liver biopsy weights were recorded to the nearest 0.01g. Each biopsy was subsequently divided into three sections for the varying analyses.

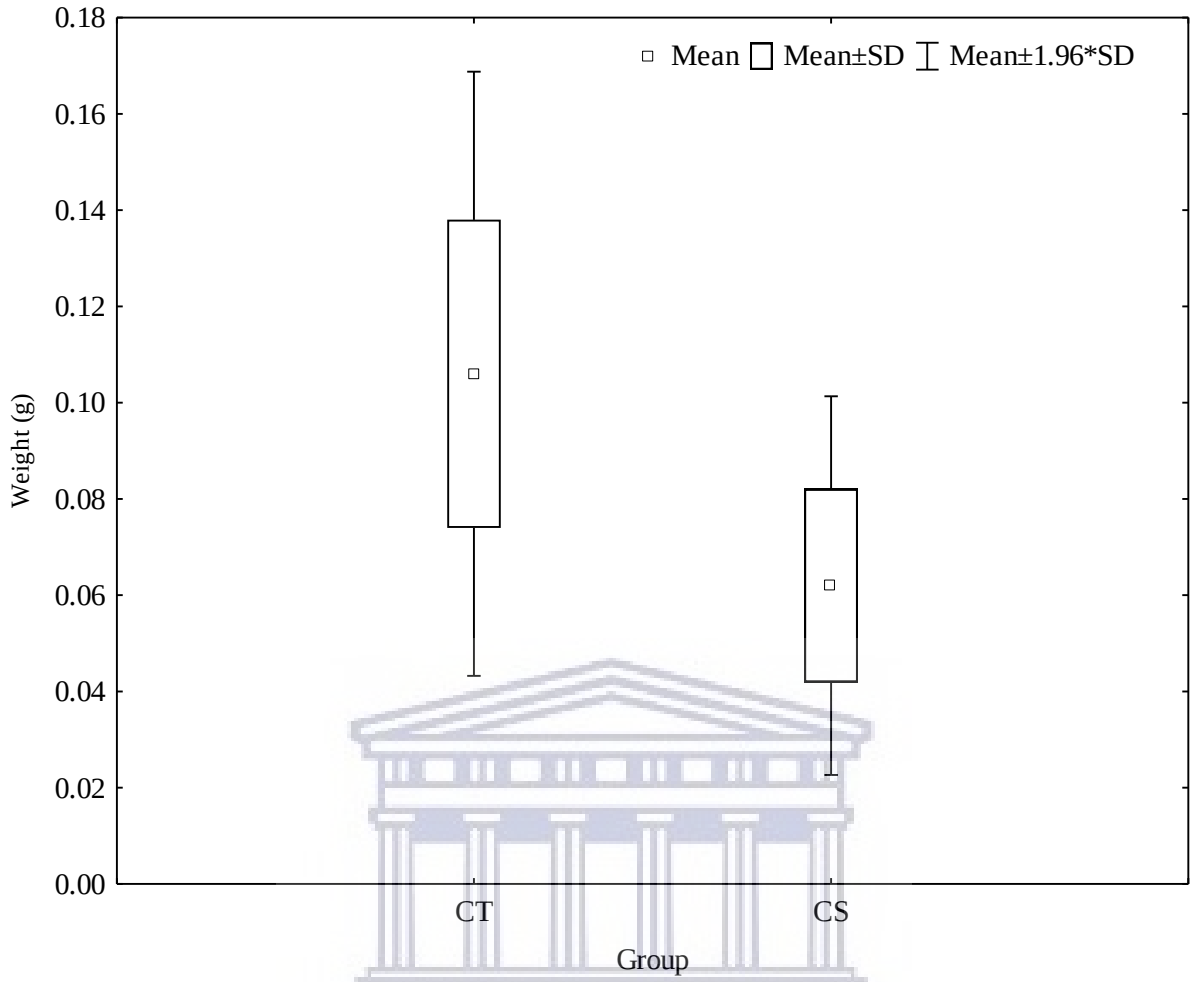

Figure 4.1: Size (weight in g) of liver biopsies obtained from *Rhabdosargus globiceps* through two surgical procedures, coeliotomy (CT) and coelioscopy (CS) where p=0.0001.

Data was tested for normality and an independent t-test was run on the biopsy weights sampled for both surgical procedures. The weights were significantly different ($p < 0.05$) where p= 0.0001 (Fig. 4.1). The coeliotomy surgical procedure allowed for a much larger sample of liver to be biopsied. The mean weight of the liver biopsy for coeliotomy was 0.11g and the coelioscopy group was 0.06g.

4.2 Diagnostic results

The biopsies of the live fish were divided into three sections for analysis:

- Squash mount preparation involved crushing the tissue between a microscope slide and cover slip prior to evaluation under a microscope. The squash mount preparation was independently evaluated by three personnel in house, a positive or negative decision was made unanimously.
- Culture involved incubation with an established culture medium for between 7-16 days, with analysis at two points in this time, only live *I. hoferi* were counted towards a diagnosis.
- Conventional and nested PCR, approaching diagnosis by DNA rather than morphology.

The fish were monitored for 43 days post-surgery to ensure the live biopsy methods were viable (see Chapter 3). Once healing of the surgical wounds were complete the fish were humanely euthanised in order to provide a final, gold standard diagnosis to which the biopsy samples could be compared. This post mortem diagnosis was reached through consensus of two of the established methods, including histology, using multiple organs.

Table 4.1: Test results for the diagnosis of *I. hoferi* by liver biopsy obtained by coeliotomy and coelioscopy, and at final post mortem in *R. globiceps*[5].

		BIOPSY					POST MORTEM						
	Fish tag number	Squash prep biopsy	Culture biopsy	Conventional PCR biopsy	Nested PCR biopsy	Final biopsy diagnosis (>2 tests)	Squash prep liver	Squash prep on all organs	Culture	Conventional PCR	Nested PCR	Histology (Liver, kidney, heart, spleen)	Gold standard post mortem diagnosis (>2 tests)
Coeliotomy	1573388	-	-	-	-	-	-	+	+	+	+	+	+
	1575929	+	+	-	-	+	+	+	+	+	+	+	+
	1573888	-	+	-	-	+	+	+	-	-	+	+	+
	1572733	+	+	-	-	+	-	+	+	+	+		+
	1573857	-	-	-	-	-	-	-	-	-	+	-	-
	1572245	-	-	-	+	-	-	-	-	-	+	+	+
	1573142	+	-	-	-	-	+	+	+	+	+		+
	1572616	+	-	-	-	-	+	+	-	+	+	+	+
	1574998	-	-	-	-	-	+	+	+	+	+	+	+
	1573280	+	-	-	-	-	-	+	-	-	+	+	+
Coelioscopy	1573720	-	-	-	-	-	-	-	-	-	-	-	-
	1573288	-	+	-	-	-	+	+	+	+	+	+	+
	1573226	+	-	-	-	-	+	+		+	+	+	+
	1572982	+	-	-	-	-	+	+	-	+	+		+
	1565431	+	-	-	-	-	+	+	-	+	+	+	+
	1573493	-	-	-	-	-	+	+	-	+	+	+	+
	1574552	-	+	-	-	-	+	+	-	+	+	+	+
	1565492	+	+	-	-	+	+	+	+	+	+	+	+
	1573519	+	-	-	-	-	+	+	-	+	+	+	+
	1574302	-	-	-	-	-	-	-	-	+	+	-	+
	1572522	+	-	-	-	-	+	+	+	+	+	+	+
	1572008	-	-	-	-	-	-	+	-	+	+	+	+
	1565700	+	+	-	-	+	+	+	+	+	+	+	+
	1575884	+	-	-	-	-	+	+	+	+	+	+	+
	1572075	+	+	-	-	+	+	+	-	+	+	+	+

Table 4.1: Test results for the diagnosis of *I. hoferi* by liver biopsy obtained by coeliotomy and coelioscopy, and at final post mortem in *R. globiceps* (Continued)[5].

		BIOPSY					POST MORTEM						
Group	Fish tag number	Squash prep biopsy	Culture biopsy	Conventional PCR biopsy	Nested PCR biopsy	Final biopsy diagnosis (>2 tests)	Squash prep liver	Squash prep on all organs	Culture	Conventional PCR	Nested PCR	Histology (Liver, kidney, heart, spleen)	Gold standard post mortem diagnosis (>2 tests)
Control	1574275						-	-	-	-	+		-
	1574479						+	+	+	+	+		+
	1564516						+	+	+	+	+		+
	1573398						+	+	-	+	+		+
	1565628						-	+	+	-	+		+
	1574923						-	-	-	-	+		-
	1575540						-	-	-	-	-		-
	1574499						+	+	-	-	+		+
	1572678						-	-	-	-	-		-
	1575038						+	+	+	-	+		+

[5] Tests performed on liver biopsies were squash prep biopsy, culture biopsy, conventional and nested PCR biopsies. For the final biopsy diagnosis a minimum of 2 tests had to be positive to classify that fish as positive for *I. hoferi*. The squash prep liver, squash prep on all organs, culture, conventional PCR, nested PCR and histology were all performed on tissues at post mortem. The last column is the gold standard post mortem diagnosis, here a fish was classified as positive if it tested positive in 2 or more of the 6 tests at post mortem.

Table 4.2: Sensitivity and apparent prevalence of diagnostic tests for *I. hoferi* performed on liver biopsies obtained by coeliotomy or coelioscopy, in *R. globiceps*[6].

		n-value	Sensitivity %	Apparent prevalence %
Coeliotomy	**Squash prep biopsy**	10	50	50
	Culture biopsy	10	30	30
	Conventional PCR biopsy	10	0	0
	Nested PCR biopsy	10	10	10
	Final biopsy diagnosis (>2 tests)	10	30	30
Coelioscopy	**Squash prep biopsy**	15	64	60
	Culture biopsy	15	21	33
	Conventional PCR biopsy	15	0	0
	Nested PCR biopsy	15	0	0
	Final biopsy diagnosis (>2 tests)	15	20	12
Totals	**Squash prep biopsy**	25	58	56
	Culture biopsy	25	25	32
	Conventional PCR biopsy	25	0	0
	Nested PCR biopsy	25	4	4
	Final biopsy diagnosis (>2 tests)	25	24	24

The following tests were performed on the biopsy samples: Squash preparation microscopic examination, culture, conventional and nested PCR. A post mortem examination was performed on all fish at the end of the experiment to determine the infection status of the fish. The following tests performed at post mortem on the liver and some organs were: Squash preparation microscopic examination of the liver; squash preparation microscopic examination on the spleen, kidney and heart; culture of the liver; conventional PCR, nested PCR and histology of the liver, spleen, kidney and heart. The results of these tests are displayed in Table 4.1. The final biopsy diagnosis was determined by classifying a fish as positive if two or more

[6] Squash preparation biopsy, culture biopsy, conventional PCR biopsy and nested PCR biopsy tests are listed for each surgical group and for the total group which is the combined results for all groups combined.

biopsy tests, tested positive. The gold standard at post mortem was determined by classifying a fish positive if two or more post mortem tests, tested positive. The gold standard post mortem was used to classify all the individual fish as either positive or negative for *I. hoferi* (Table 4.1). There were six fish that were negative out of the 40 fish used in the study (Table 4.1). All of the tests at biopsy show poor results with many of the liver samples testing negative. Contradictory to this, the post mortem tests all show a high number of positive tests with culture akin to the biopsy tests. Using the gold standard post mortem diagnosis to identify the true infection status of the fish, the ability of each test performed on the liver biopsy to correctly identify a fish as positive for *I. hoferi* was evaluated. This evaluation was done using the 2 x 2 tables to determine the sensitivity. Results are displayed in Table 4.2. Specificities were not shown here due to no false negatives in the coeliotomy resulting in all tests to be 0%. Biopsy squash preparation was the most sensitive single biopsy test in the coeliotomy, coelioscopy and combined groups with a sensitivity of 50%, 64% and 58%, respectively (Table 4.2). In both the coeliotomy and coelioscopy groups, the biopsy culture had a sensitivity of 30% and 21%, respectively. The sensitivity of conventional PCR performed on liver biopsies were 0% and 0% suggesting these tests to be undesirable. The nested PCR performed on the liver biopsy had a sensitivity of 10% in coeliotomy, and in coelioscopy a sensitivity of 0% suggesting a poor diagnostic test. The results of the final biopsy diagnosis test resulted in poor sensitivities when compared to the same post mortem diagnostic tests of the liver and other organs. The sensitivity of the final biopsy diagnosis resulted in a poor sensitivity of 24%. The liver samples tested at post mortem with both nested and conventional PCR tested a high number of fish as positive. Squash preparation and histology showed similar patterns in test detection. The nested PCR detected more positives than any of the other tests at post mortem but proved to be a poor diagnostic test for the biopsy samples. In the coeliotomy group histology showed seven out of eight fish to be positive and in the coelioscopy group there were 12 out of 14 fish that were positive. The results do not point to one clear answer as to which would be the ideal diagnostic test to test biopsy liver samples for *I. hoferi.*

4.3 Organ distribution of *I. hoferi* in *R. globiceps* at post mortem

Of the 40 fish used in this study 32 fish were diagnosed as positive for *I. hoferi* by squash mount examination of spleen, liver, kidney and heart at post mortem. *I. hoferi* schizonts or germination tubes were identified in the spleen in 94% of infected fish (30 out of 32), in the

liver in 78% of infected fish (25 out of 32), in the kidney in 72% of infected fish (23 out of 32) and in the heart in 69% of infected fish (22 out of 32 fish).

The larger mass (g) of the biopsies from the coeliotomy samples, identification of the parasite from the coelioscopy biopsy was not as accurate. The apparent prevalence for the gold standard for the biopsy of the coeliotomy group was 30% compared to the coelioscopy at 12% (Table 4.2). The PCR was considered to be of little value in diagnosis from biopsy, with squash preparation and culture being more sensitive. Although this studies' focus was on the liver biopsy, the results from the post mortem suggest that due to the predilection of *I. hoferi* in *R. globiceps,* the spleen may be the preferred site to sample.

CHAPTER 5

The aim of this study was to develop a non-lethal diagnostic tool for the diagnosis of the mesomycetozoan parasite, *I. hoferi*. Liver biopsies were successfully obtained surgically from 30 *Rhabdosargus globiceps* using either coeliotomy or coelioscopy. These fish were monitored post-surgery to determine the viability of a surgical approach as a diagnostic tool for live fish. Once the biopsy was collected, the presence or absence of *I. hoferi* was determined using microscopic tissue squash preparations, polymerase chain reaction (PCR) and culture. The diagnosis was then confirmed through rigorous testing of the fish at post mortem.

Discussion

5.1 Surgery

The use of 2-Phenoxyethanol (2-PE) proved to be an adequate anaesthetic agent in the fish anaesthetic rebreathing system (FARS) and was a practical means of maintaining anaesthesia during both methods of surgical procedures. 2-PE is an immersion anaesthetic that is added to the water and is moderately water soluble. The advantage of using 2-PE is that it is comparatively lower in cost in comparison to other fish anaesthetics and there is no need to buffer pH when added to seawater (Neiffer and Stamper, 2009). There have been reports of habituation from repeated anaesthetization (Weyl *et al.*, 1996), although it was not a concern of this study.

Surgery times were on average five minutes longer for coeliotomy when compared to the coelioscopy group. The recovery was on average 11 minutes longer in the coeliotomy group than in the coelioscopy group. A variable that was not taken into consideration was that the surgeons experience would increase with each procedure which in turn could potentially decrease the time of the surgery. It can be suggested that, with a shorter surgical time and recovery time, the fish was placed under less stress during coelioscopy (Murray, 2002). Coelioscopy is less invasive and the post-surgical recovery is quicker mitigating overall stress and trauma to the fish (Boone *et al.*, 2008). Coelioscopy can allow for a more complete visualization of the coelom which can be extremely useful and informative (Boone *et al.*, 2008). Due to increased time used in the coelioscopy group in identifying and visualising visceral

tissues in the coelom Boone *et al.* (2008) found no significant differences between the surgical times in both coelioscopy and coeliotomy. In this study the liver was searched for, identified and biopsied and then no further identification or visualization was carried out which may be why coelioscopy was faster than coeliotomy, unlike during the study of Boone *et al.* (2008). All surgeries were completed and a biopsy was obtained from each fish that underwent surgery. It is important to consider the topographical variation of the coelom between species. Ideally a member of the species should be sacrificed prior to surgical planning to determine coelomic topography. This may not be possible where there are few specimens of one species in an aquarium, in these cases a different species within the genus could be used, remembering the topography is unlikely to be identical. The anatomy of *R. globiceps* was studied prior to the surgical procedures for the current study using historical mortalities.

5.2 Post-surgical healing observations

All fish survived to week 6, the termination of the post-healing monitoring phase of the study. There were seven individuals across both surgical groups which displayed poor healing initially. By week 6, only one individual displayed fair healing, the healing of the rest of the fish were classified as good. As no fatalities occurred, and as by week 6 the majority of fish had healed to a good standard, this study was successful in obtaining a non-lethal biopsy through surgery.

Due to skin swelling not being a common response to injury in fish (as is found in mammals), it is suggested that the sutures are tied to form a secure knot with good skin apposition to prevent any water or contaminants entering the wound (Harms, 2005). Absorbable suture material was used, but in fish this suture material doesn't necessarily dissolve due to lower temperatures inhibiting hydrolysis (Harms, 2005). An additional aspect of this study looked at comparing two suture knots, either buried or exposed, within the coeliotomy group. Four of the ten individuals in the coeliotomy group were sutured with buried knots. Of these four individuals, two exhibited poor healing at week 1. There were also two (out of six) individuals in the coeliotomy group with exposed knots which healed poorly at week 1. Of the four fish with buried sutures there were only two fish with mild abrasions and one fish with no abrasions compared to the exposed suture method where three showed moderate abrasions and three showed mild abrasions. A small sample size makes it difficult to draw a decisive conclusion,

however, it could be suggested that the buried suture method is the preferred method of suturing. Within the coelioscopy group, once sutures were removed, fish healing improved. All fish that had their sutures removed due to signs of friction, healed well post-suture removal. This is in agreement with Harms (2005) suggestion, and could be a recommendation for future studies, to remove all visible or external sutures after two weeks to minimise any trauma and further complications.

Pigmentation change in and around the suture site areas of 12 fish was observed. This pigmentation in the fish was often associated with fair or poor healing around the sutures and once the sutures were removed the pigment faded. Pigment deposition is a common feature of the inflammatory response in fish, reported in Salmoniids by Roberts *et al.* (1971) as a response to ulcerative dermal necrosis. All fish produced an excess layer of mucous around the suture site which is a normal fish response to injury of the skin, and may have helped to reduce friction between the incision and pectoral fins. Scale loss and presence of sutures promoted mucous development around the area after surgery. Friction caused by rubbing of the pectoral fin on sutures (or scale disruption) would have likely loosened sutures at the incision site. Once loose, the sutures are free to move resulting in further inflammation characterised by erythema and excess mucous. This is seen in the coelioscopy group where 3/15 were classified poor at week one, increasing to 7/15 at week 2. Another issue which could have caused this inflammation is the repeated contact of the incision site with the bottom of the pool. It is possible that the fish made more contact with the pool due to an adverse reaction of the suture material. However friction (between pelvic fin and incision) was suspected to be the main concern here, as in one individual with no fin-incision contact there was good healing. Protruding suture material and scale disruptions caused damage in the form of abrasions on the pectoral fin in addition to a degree of erythema which was observed around the surgical site of some of the fish. The removal of scales at the surgical site may or may not be necessary depending on the individual, as was the case here. The loosening of scales at the incision site (so that the caudal edge of the scale was angled laterally away from the body wall) in some individuals caused marked abrasions on pectoral fins. If at all possible, this should be avoided. Regardless of the cause, the friction at the incision site caused abrasions to the pectoral fin. If feasible, contact between the pectoral fin and incision should be minimised.

Where other studies have experienced dehiscence post-surgery in fish (Boone *et al.*, 2008) in the current study dehiscence was not observed. This may be due to technique, suture material, water quality, use of antibiotics, or simply due to the variation of fish species used in studies. The skin and scales of *R. globiceps* are different than that of *I. punctutatus*. If a species has thin skin or muscle layer, the suture material may be more likely to tear than if the skin or muscle was thicker. Therefore species with a thicker or thinner epidermis than *R. globiceps* would potentially require different suture material/thickness and should be expected to heal differently. Comparing the two methods in week 1, healing was good or fair in 12/15 fish in the coelioscopy group when compared to only 6/10 in the coeliotomy group, coelioscopy yielded better, faster healing than coeliotomy. Coelioscopy allowed for good visualisation of the liver, without exteriorizing the organ, while demonstrating a reduced surgical time and a quicker recovery, subjectively the healing was better. The endoscopy equipment may be cost prohibitive for some, as well as logistically difficult for field work, but overall this study finds this to be the superior method.

5.3 Haematology

Obtaining a biopsy of the liver through either surgical method caused changes in haematology, packed cell volume, glucose, total protein, and white blood cell counts. This indicates that the fish were placed under physiological stress, as would be expected by surgery (Murray, 2002). Blood was sampled from the caudal peduncle in order to measure packed cell volume, total protein, glucose and white blood cell counts throughout the duration of the experiment. Repeated blood sampling did cause trauma on some of the fish's tails. There were no trauma related issues caused by blood sampling in the *I. punctatus* (channel catfish) study, likely due to the catfish being scaleless whilst *R. globiceps* possessing large ctenoid scales (Boone *et al.*, 2008). Blood sampling of fish was repeated more frequently in this study than for *I. punctatus*. With the fish on its side, when blood sampling, the needle needed to be placed on the ventral aspect of the caudal peduncle between the scales, pushed cranially then angled 90° (perpendicular to the ventral aspect of the caudal peduncle) and inserted towards the spine to find the blood vessel. This process caused some scales to be dislodged leaving exposed skin and muscle that could increase risk of infection. Blood sampling was then repeated in the same area of the caudal peduncle which compounded the risk of infection by repeating sampling.

Erythema of the skin and/or the remains of coagulated blood from the previous blood draw were observed in some fish on the caudal peduncle.

Scuticociliates (Ciliophora) are histophagous parasites that are problematic in a wide range of fish species at the Two Oceans Aquarium. They are generally associated with a high organic matter, bacterial and nutrient levels (Urrutxurtu *et al.*, 2003). Clinical signs include macroscopic skin lesions and often haemorrhagic ulcerations (Piazzon *et al.*, 2013). Metronidazole is used in veterinary medicine in the treatment of anaerobic infections, giardiasis and other protozoal infections (Ramsey, 2008). Scuticociliatosis is often systemic and metronidazole has good tissue penetration and mild side-effects (Samuelson, 1999). Fish 1572245 was positively diagnosed with scuticociliatosis (species unidentified) by taking a skin scrape of the infected area and observing the slide under a compound microscope. This fish received a 10 day course of 400mg Metronidazole at a concentration of 500mg/litre of water. Blood sampling of fish can cause scuticociliatosis due to the handler having to lift the scales and often accidentally removing a few scales to gain access to the skin. For fish 1572245, blood results were collected at weeks 0, 1, 2 and 6. The 4th sample was not performed due to the treatment and recovery of this fish. It is suggested to increase intervals between sampling and to rotate blood drawing sites to reduce the incidence of skin damage. These areas of rotation could include the ventral and lateral aspect of the caudal peduncle, if the anatomy of the species of fish allows for such blood sampling. Repeated blood sampling could have deleterious effects on the overall health of the fish affecting other outcomes that one may be looking to achieve. It should be considered that the blood parameters measured in this study may have been altered in fish which suffered more iatrogenic trauma due to blood sampling. Similarly the fish that suffered scuticociliatosis could have increased white blood cell counts, blood glucose and potentially lowered the total proteins and PCV thereby impacting on the results for that specific group. Irrespective of infection abnormal or raised white blood cell counts were not observed in fish 1572245 and this may be due to an effective treatment post diagnosis.

The mean PCV for 18 fish of a similar species *R. sarba* used as a control in a study was approximately 30% (Shen *et al.*, 2011). In the literature mean PCV values ranging from 28% to 34% were reported for four closely related species (Mozanzadeh *et al.*, 2015; Yildiz, 2009). The mean PCV decreased between week 0 and week 2 in both surgical groups whereas there was no drop in the mean PCV of the control group. It could be hypothesized that the decrease

in PCV seen in both surgical groups between week 0 and 2 is likely due to some blood loss at surgery. The lower PCV of 22% at week 1 compared to the control at 27% could be due to the surgical procedure. At week 2, there was a further decrease in the PCV in the coeliotomy group, however the PCV of the coelioscopy group increased and returned to baseline (day of surgery) levels. It is possible that the PCV showed a quicker recovery at week 2 in the coelioscopy group due to a significantly smaller biopsy sampled and the smaller size of the surgical site (and therefore less chance of haemorrhage from the biopsy site and also the surgical site). The smaller incision may have caused less discomfort or pain consequently leading to reduced stress and potentially a quicker recovery. In Boone *et al.* (2008), it was found that there was an initial decline in PCV of the coelioscopy group due to residual saline left in the coelom causing hemodilution. This is not considered to be disadvantageous to the fish but on the contrary provides fluid therapy post-surgery (Boone *et al.*, 2008). It was noted during the initial preliminary surgical testing that there was haemorrhaging at the surgical site in the coeliotomy fish. Conversely at week 6, the coeliotomy group had a higher PCV percentage than the coelioscopy group. The group PCV means at week 6 were 28% for coeliotomy and 24% for coelioscopy which is within the normal range and slightly lower than normal, respectively. This could suggest that some fish in the coelioscopy group were slightly anaemic. This anaemia could be due to the fish being infected with *I. hoferi*, as any infection or chronic disease may result in anaemia due to a reduction in the production of red blood cells in the body.

The varying changes in glucose level values at week 2 and week 5 in the coelioscopy group compared to the coeliotomy and control is an indication of potential stress during the capture process and the anaesthesia process. All fish were housed in the same pool so when a group (coelioscopy, coeliotomy or control) needed to be sampled, all fish had to be anaesthetised to a low level of anaesthesia in order to capture the required fish which would then be transferred to a separate pool with a higher level of anaesthesia. The fish would remain in this pool until all had been sampled. At week 0 the control group showed a significantly lower mean glucose level compared to the coeliotomy group. The control was also observed to have a lower mean glucose when compared to the coelioscopy group however this difference was not statistically significant. This does suggest that fish that did not undergo surgery would have less stress on them allowing for a lowered glucose mean level. This initial capture caused stress and these changes in glucose levels could be related to the time of sampling. At week 2 there is an outlier at 15.3 $mmol/l^{-1}$ which is indicative of a fish that was sampled last and had been under

anaesthesia for the longest period of time. This outlier is responsible for the raised group mean at week 2 in the coelioscopy group. By week 6 glucose levels for all three groups were all in the same range. Glucose is not a very reliable indicator of the post-surgical healing in this study and specific results of the blood glucose should be interpreted with caution. As glucose can also be elevated with infection or chronic disease, it may still be used to evaluate the health of fish but only if all fish were handled in exactly the same way. Even if fish are handled in the same way, fish may not perceive handling stress in the same way especially if there are differences between captive bred and non-captive bred individuals. Blood glucose would also likely be elevated by the anaesthetic procedure and any handling (even if handling was identical) so this will affect interpretation.

Total proteins provide supportive information and data with regards to the fish's health, by aiding in determining the nutritional condition of the fish (Patriche *et al.*, 2009). In conjunction with the PCV, the total protein can provide information on the hydration status of the fish. A study on hybrid bass used changes in haematology as a diagnostic tool in clinical pathology where total proteins were used as part of the blood analysis (Hrubec *et al.*, 2001). Structural liver alterations and altered fluid balance (dehydration) can result in an increase in total proteins (Mozanzadeh *et al.*, 2015). Starvation of an animal can cause a failure in protein synthesis causing a decrease in total proteins (Bernet *et al.*, 2001). Higher total protein in the coelioscopy group compared to coeliotomy at week 2 could suggest a quicker recovery and less trauma, stress and haemorrhage than in the coeliotomy group. Blood loss at surgery can result in lower blood protein levels due to direct loss of protein in the blood. The coeliotomy group had lower mean total protein concentrations at week 2 compared to the coelioscopy group and this could have been due to the increased total body demand for protein required for wound healing or increased loss of protein due to blood loss during surgery. At week 1 both of the surgical groups had lower total proteins than the control group which again may be due to increased demand due to wound healing and blood loss at surgery. Levels of total protein returned to baseline levels by week 6 in both surgical groups suggesting that there were no ongoing losses or increased demands caused by the surgery at this time and this is suggestive of recovery.

Increased blood white blood cell counts most frequently occur due to inflammation or infection. This has been observed in *Cyprinus carpio* (carp) infected with *Aeromonas hydrophilia* and *Onchorhnychus mykiss* (rainbow trout) suffering viral haemorrhagic

septicaemia (Harikrishnan *et al.*, 2003; Rehulka, 2003). There is a general trend in increasing white blood cell counts in both surgical groups from week 0 to 2 and this could be due to the surgery. The control group is observed as decreasing from week 0 to 1. The increase in white blood cell counts of both surgical groups at week one and two compared to the control group would have been due to inflammation as a part of the normal healing response to the surgery and is unlikely to be due to infection as no other signs of infection were seen in any of the fish. Surgery puts fish at risk of possible infection, as well as the anaesthetic itself causing a potential supressed immune system. At week 5 the coelioscopy group had a higher white blood cell count when compared to the coeliotomy group. The white blood cell count could be affected (elevated) as a result of trauma to the caudal peduncle from blood sampling, or due to the friction of the wounds when rubbed against the bottom of the tank (resulting in inflammation). There were nine fish that had mild trauma from blood sampling in the coeliotomy group which would have resulted in inflammation and an elevation in white blood cell counts as opposed to the coeliotomy group which only had one fish with mild trauma.

It is a complex analysis due to the fact that drawing blood repeatedly to determine/monitor the health status may in fact affect that result adversely and thus the interpretation of the blood parameters by group is challenging. Given the different healing responses that were seen in this experiment, and the individual variation seen with the trauma caused to the caudal peduncle, it might be much more valuable to evaluate the blood parameters on an individual fish basis rather than as a group. However this was beyond the scope of this project. Fish with poor healing or tail trauma are likely to have skewed the group results meaning that one must interpret with caution group comparisons and interpretation of group trends. A suggestion for future studies would be reducing the frequency of blood sampling and changing or rotating the site of venepuncture. The blood parameters have provided us with a comparison between the two surgical groups and the overall healing is very similar in the outcome by week 6 with no significant differences therefore with regards to the fish's health either surgical procedure would be adequate. Similar data is shown in the *I. punctatus* study (Boone *et al.*, 2008).

5.4 Diagnostics

In Pacific halibut the heart tissue was cultured to determine the concentration of *I. hoferi* within the heart tissue, in North Sea Herring the hearts were also sampled for the presence of *I. hoferi*

(Dykstra *et al.*, 2013; Mellergaard and Spanggaard, 1997). Hearts were also used to determine the cycling of *I. hoferi* within Chinook Salmon within their spawning migrations (Zuray *et al.*, 2012). It is well documented that in pelagic and migratory fish such as herring, salmon, and trout that *I. hoferi* is found in a greater concentration in the hearts compared to other organs or tissues of these species (Rahimian, 1998; Slocombe, 1980; Zuray *et al.*, 2012). In fish that are more resident slow swimming, benthic dwelling such as varying *Sebastes* sp., of the Sparidae family and flounders the heart is not necessarily the organ of interest when diagnosing *I. hoferi* (Gavryuseva, 2007; Halos *et al.*, 2005; Kocan and Hershberger, 2006; Mellergaard and Spanggaard, 1997; Sitja-Bobadilla and Alvarez-Pellitero, 1990). Researchers looking at two cultured mullet species in Spain found that the spleen had the highest mean infection infectivity when compared to the other organs (Franco-Sierra *et al.*, 1997). In the current study, 32 fish tested at post mortem were positive for *I. hoferi*. The heart was infected in 22 of the 32 fish, and in the kidney in 23 of the 32 fish. The heart being the least accessible organ and not being an organ that would be easily biopsied was ruled out first as an organ to use for diagnostic testing through non-lethal methods. The kidney is accessible and can be biopsied successfully using various non-lethal methods. Noga (2011) describes a fine needle aspirate (FNA) as a method of obtaining a biopsy from the kidney. This method would not be very useful in the diagnosis of *I. hoferi* as only a small quantity of tissue is obtained by this method. A FNA was successfully performed on rainbow trout in the attempt to diagnose *Yersinia ruckeri* (Noga *et al.*, 1988). It would be interesting and useful to obtain diagnostic results for *I. hoferi* using this method but this was not what was examined in this study. To obtain a larger biopsy of the kidney using endoscopy would be difficult because access is difficult due to the location of the swim bladder. *I. hoferi* was observed in the spleen of 30 of the 32 fish positive for *I. hoferi* and would have potentially provided a more sensitive diagnostic test, however, during preliminary studies it was found that accessing the spleen for biopsy was too difficult. The fish that were preliminary tested had large quantities of visceral fat around the spleen which would have made it more difficult to visualise and access easily. *I. hoferi* was observed in the liver of 25 of the 32 fish used in the study. The liver is also an organ that is able to manage a small loss in tissue from a biopsy without becoming dysfunctional. The spleen being a much smaller very vascular organ could result in complications from biopsy and the danger of severe haemorrhaging would be far greater than that of the liver. The consideration of organ distribution of *I. hoferi* should however be considered on a species by species basis as the predilection of *I. hoferi* for the spleen may only be true for *R. globiceps* and may not be true

for other fish species. The liver was the practical option for biopsy as it could be easily identified, easily visualised on endoscopic video and exteriorised easily through a small surgical opening. It has been previously demonstrated that liver samples could be non-lethally sampled with minimal difficulty from fish with no or minimal adverse effects (Boone *et al.*, 2008; Divers *et al.*, 2013).

The final gold standard results identified that there were only two negative fish in the surgical groups combined. This made it very difficult to accurately assess the specificity of the biopsy tests and to draw correct conclusions from the results. Two fish is not enough to accurately assess specificity in any test. This may or may not be the true infection status of the fish. The gold standard is not perfect and the accuracy of the final diagnosis is entirely dependent on the outcome (and therefore accuracy) of the tests used to make the diagnosis. There were more positive results using PCR at post mortem compared to culture, squash preparation and histology. The reason there were true false positives was potentially due to contamination in the PCR testing methodology, or the test was too sensitive (not inaccurate), or the PCR test was identifying DNA from non-viable *I. hoferi* material. This DNA material could have been from fish that had been exposed to other fish infected with *I. hoferi* and had then recovered from the infection. The biopsy sample was divided into three sections for squash preparation, culture and PCR. The PCR at biopsy did not yield sensitive results and this was likely due to the small size of tissue that was prescribed to run the PCR testing and because *I. hoferi* is not evenly distributed in the liver.

According to the recommendations of the World Organization for Animal Health a minimum of two diagnostic tests should be applied to any surveillance or sampling for a disease (OIE, 2019). The suggested gold standard was determined by classifying a fish positive only if it presented a positive test in two or more diagnostic tests performed at post mortem. The selection of an appropriate diagnostic method will vary among studies and is dependent on the goals or outcomes required for that specific study. Non-lethal sampling is also limiting as the organ that is most heavily infected may not be the most practical option for sampling. Tissue squash preparation showed the highest sensitivity for both surgical groups. The tissue squash preparation at post mortem showed similar results to the biopsy tests with a high number of positives in both groups and the majority of the same fish being classified as positive suggesting good reproducibility in the test. A study done on *I. hoferi* in *Clupea harengus* showed that

microscopic evaluation of tissue squash preparation found more than four times more infected herring than macroscopic evaluation (Rahimian and Thulin, 1996). Kocan *et al.* (2011) suggests that tissue squash preparations are valuable for cursory evaluation during Ichthiophoniasis epizootics. The PCR diagnostics on the biopsy samples proved to be the least sensitive, and with the surgical groups combined the sensitivities for conventional and nested PCR were 0% and 4%, respectively. This could have been due to the relatively small tissue sample used for the test. As mentioned in Chapter 2, the biopsy was divided into three sections for each test. However, conversely the nested PCR at post mortem was the most sensitive for both surgical groups. If one could obtain a larger biopsy from the fish, this could allow for a more sensitive test. Histology could also be added as a fourth diagnostic method due to the similar detection patterns seen in this study with tissue squash preparation, if a larger biopsy could be obtained. Histology showed a large number of positive fish at final post mortem which suggests it could add value to the biopsy diagnostic testing in future studies. When only a small tissue sample is available it is easier and quicker for the sampler to observe and diagnose using a tissue squash preparation under light microscopy with the option of using the sample afterwards to run PCR, place in culture media and send samples for histology.

Depending on the time and resources available, researchers should apply as many diagnostic tests to determine the status of a fish when using non-lethal sampling techniques (Kocan *et al.*, 2011). This is shown in the current study and it will provide a more reliable result for an institution or facility to make confident research based decisions. There were challenges that were experienced in working through all the diagnostic tests. Tissue squash preparation of an organ allows for a quick diagnosis and can be done directly after sampling but does require the examiner to be familiar with the organism in question and it does take time to methodically examine the slide so areas are not missed. Identifying the organism can be challenging and examiner error may occur with this method of diagnosis and it was decided that more than one examiner would be used to confirm diagnosis for squash preparation. This method in this study required more than one examiner to confirm a diagnosis, however this test performed the best on the liver biopsy in this study. Culturing *I. hoferi* takes 7-14 days to confirm a diagnosis and does require the researcher to wait for a result. This diagnostic method is most commonly used to determine if the disease was viable within the host. A culture will only grow if viable *I. hoferi* tissue was placed into the culture vial. Sensitivities for the culture samples were low which could have been due to not enough viable *I. hoferi* growing in the tissue sample. Squash

preparation tissue could be added to the culture vials after microscopic examination and increase the chances of more sensitive test as long as this is done in the most sterile way possible. If *I. hoferi* is present in a tissue sample either living or non-living, PCR will detect it with great accuracy when tested on dead samples (Kocan, 2019). This was not true for the current study and the PCR presented a poor diagnostic sensitivity. PCR is not considered the gold standard for *I. hoferi* due to its ability to detect living and non-living *I. hoferi* DNA making it too ambiguous when trying to diagnose an animal (Kocan, 2019). Conventional PCR and nested PCR were not sensitive enough in any of the biopsy tests and this, like culture, could have been due to the size of tissue sample used. When larger sizes of tissue were used for the liver sampling at post mortem all of the tests showed significantly higher sensitivities. Conventional PCR and nested PCR both showed higher numbers of positives when larger tissue samples were used. One challenge in diagnosing *I. hoferi* may be obtaining a large enough tissue sample without compromising the fish. Researchers recommend validating microscopic techniques using culture and PCR when lethal sampling is done (Whipps *et al.*, 2005). PCR does require expensive equipment and also skilled personnel to process the samples which can be challenging. If all the samples tested negative using PCR, optimization would be required as negative test results don't necessarily mean the samples are negative for *I. hoferi.*

Histology had a high rate of detection and a similar pattern of detection to the squash prep in the post mortem samples from the heart, liver, kidney and spleen, and one should consider this in the biopsy diagnosis if there is enough tissue available. Histology testing was performed by an independent fish pathologist at the Fish vet group and thus removing any bias from the testing. Detections patterns were similar to the squash preparations and the histology results also act a potential validation for the squash preparation results. Histology is performed on a very thin section of tissue (5µm) and thus the potential of not seeing *I. hoferi* might be increased and this would need to be considered when choosing diagnostic tests. Kocan *et al.* (2011) found that histological examination in *O. tshawytscha* showed a sensitivity in detecting low level infections or early infections at 55% and when infections had been left to progress for several weeks the sensitivity was 87%. A higher sensitivity in histology at post mortem in this study could be indicative of a population of fish that had been infected with *I. hoferi* for an extended period of time and possessed a high level of infection. This is however speculative as this was not tested. Unless the researcher can process and cut histology sections for analysis they need to be sent to a laboratory to be prepared. This processing and analysis can be expensive and

not all diagnostic laboratories have pathologists that have specific fish disease prowess. The time factor of sending samples to an external laboratory should also be considered.

These diagnostic tests presented a number of challenges but all provided good insight into what tests should be considered in the future and what provided adequate diagnostics when testing for *I. hoferi*. Due to all the fish being housed in the same pool for the entirety of the study there is verification bias associated with this as fish could have potentially entered the pool as being non-infected but over the course of the study become infected. It should be mentioned that a variable in this study that could not be accounted for was that some of the fish could have been negative for *I. hoferi* at the start of the study and may have been infected while in holding for the post-monitoring phase. The fish could have then showed different diagnoses at post mortem compared to biopsy diagnoses. This was not possible to test as the fish needed to be kept alive to monitor post-surgery. There is ambiguity in the literature with regards to the length of time required for the parasite to transmit between hosts as well as the rate at which the parasite moves through the host itself. This is problematic due to the nature of the parasite and the differences it presents within a host and between species.

5.5 Future studies and concluding remarks

A diagnostic tool has been developed to diagnose *I. hoferi*. If lethal sampling is not an option and a diagnosis is required then this method can work to aid in obtaining a diagnosis. The need to apply more than one diagnostic tool would be advisable to obtain the most optimal diagnostic sensitivity and specificity. Specific recommendations will be discussed here.

The FARS, designed for this study, was an efficient way to maintain an anaesthetic in these fish. The anaesthetic depth was sufficient throughout all surgeries. This system could be used without the need for alteration in further studies, although it may prove cumbersome if used for field work. Either surgical approach allowed for visualisation of the liver and successful biopsy. Coeliotomy produced a larger (g) biopsy. A larger biopsy in theory could have yielded better diagnostic power by virtue of sample size. When combining any two of the three methods (tissue squash preparation, culture or PCR) to diagnose *I. hoferi* a larger sample provided an increased sensitivity (30%). If the gold standard allowed for only one positive test to confirm *I. hoferi* diagnosis, using squash preparation, the sensitivity of coelioscopy (64%) is greater

than that of coeliotomy (50%). Coelioscopy as a surgical approach resulted in quicker surgical and recovery times, as well as fewer adhesions post mortem. With valuable, potentially delicate, aquarium species the potential negative impact of the surgical approach, the invasiveness, increased stress, larger incision, increased physical trauma, as in coeliotomy, is important and so coelioscopy is recommended. There were individual differences in healing at the incision site. It is a recommendation for future studies that any sutures should be removed after two weeks, in line with Harms (2005), to remove any irritation that can delay wound healing. It is also suggested suture ends should be cut as short as possible to prevent any unnecessary abrasions on the fins close to the sutures. If trauma on the fins can be avoided it will benefit any fish recovering from a surgical procedure (Harms and Lewbart, 2000).

The monitoring of all the experimental fish using blood parameters and wound healing observations provided a practical way in which the researcher could monitor each fish at various post-surgical intervals. Important decisions could be taken at the time of observation such as removing sutures, examining caudal peduncles for trauma, examining the fish for any other damage or lesions and also taking photographs to be able to compare them to observe the healing over time. The blood analysis also provided insight into overall health over time, with differences seen in PCV, glucose, total protein and white blood cell counts between the control and the surgical groups, however examination of trends on an individual level may be more appropriate to enable monitoring of health in individual fish. There were detrimental effects of repeated blood sampling and so although monitoring itself is recommended - the frequency of monitoring should be minimal.

Due to the nature of *I. hoferi*, how it presents itself in various species of fish and the nebulous nature of its life cycle, it is not easy to diagnose using one diagnostic test. If a liver biopsy is to be examined using only one test, squash mount (58%, sensitive across both surgical groups) is the singular diagnostic test of choice. However, this should be confirmed in a study with a larger sample size. More information on the life cycle of the parasite in different species would be valuable. From a practical perspective, ideally future research of *I. hoferi* should focus on improving the diagnostic sensitivity. Potentially another organ, such as the spleen, could be used for biopsy. After diagnosis more research into management or treatment within the aquarium is vital.

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