THE EVALUATION AND DEVELOPMENT OF DIAGNOSTIC TOOLS FOR THE DETECTION OF *ICHTHYOPHONUS HOFERI* IN FISH HOST TISSUE SAMPLES

Bret Mark Wurdeman

A dissertation submitted in fulfilment of the requirements for the degree of *Magister Scientiae* in the Department of Biodiversity and Conservation Biology, Faculty of Natural Science, University of the Western Cape.

Supervisors:

Dr. Kevin W. Christison (Department of Biodiversity and Conservation Biology, University of the Western Cape and the Department of Agriculture, Forestry and Fisheries, Aquaculture Research)

Co-supervisors

Dr. Georgina Cole (BVetMed MSc MRCVS)

Prof. Mark Gibbons (Department of Biodiversity and Conservation Biology, University of the Western Cape)

2019

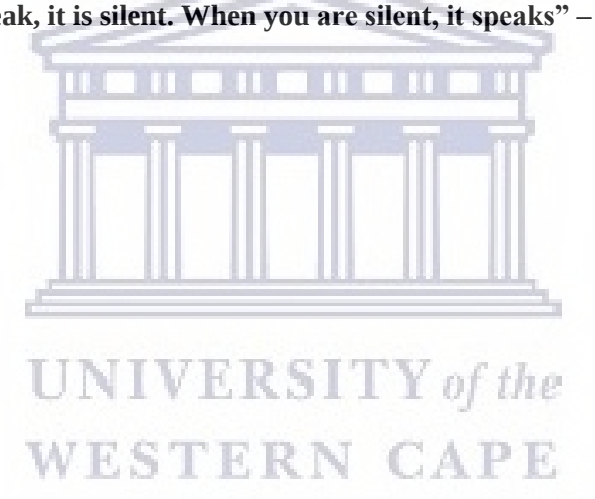
DECLARATION

I declare that this is my own work, that '**The evaluation and development of diagnostic tools for the detection of *Ichthyophonus hoferi* in fish host tissue samples'** 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.

Mr. Bret Mark Wurdeman **Date 15/11/2019**

Signed .....

"You cannot catch hold of it, nor can you get rid of it. In not being able to get it, you get it. When you speak, it is silent. When you are silent, it speaks" – Zen Poem



KEYWORDS

Ichthyophonus hoferi

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Validation

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ABSTRACT

The evaluation and development of diagnostic tools for the detection of *Ichthyophonus hoferi* in fish host tissue samples

Ichthyophonus hoferi is a highly pathogenic histozoic parasite that has low host specificity capable of producing mass mortalities of epizootic proportions in marine commercial fish populations. Currently in Southern Africa, *I. hoferi* has been reported from flathead mullet (*Mugil cephalus*) from the Kowie lagoon and from multiple species on exhibit at the Two Oceans Aquarium. Since epizootiologists rely on accurate assessments of prevalence to establish patterns of morbidity and mortality within populations, using the most accurate diagnostic techniques for accurate assessments of infection is imperative. Currently, several diagnostic techniques have been employed to detect *I. hoferi* in infected fish hosts. These include macroscopic examination of tissues, microscopic examinations of wet-mount squash preparations of tissue, histological examination of tissue sections, *in vitro* culture of tissue explants, the polymerase chain reaction (PCR) using *I. hoferi*-specific primers and real-time quantitative PCR (qPCR) using *I. hoferi*-specific primers and a hydrolysis probe. When evaluating infection prevalence of the same tissues using different diagnostic techniques these produce different results. The disparities observed between these evaluations of the same tissues can result from an uneven distribution of the parasite within the host tissues, the size of the tissue sample relative to the infected organ and the analytical sensitivity of the diagnostic technique.

In this study a SYBR green real-time quantitative PCR assay for species-specific detection and quantification of *I. hoferi* was developed and validated based on strict criteria according to the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines. The techniques lowest detectable limit to detect *I. hoferi* was determined by the production of a standard curve based on known schizont concentrations using a simple direct counting method to accurately estimate *I. hoferi* concentrations *in vitro.* To evaluate the comparability of this qPCR technique with microscopic examinations of wet-mount squash preparations of tissue, histological examination of tissue sections and *in vitro* culture of tissue explants for detecting *I. hoferi* in *Rhabdosargus globiceps* fish samples, thirty five fish samples were collected from the Two Oceans Aquarium and examined for *I. hoferi*-infection. The diagnostic sensitivity, the diagnostic specificity, the positive predictive value (PPV), the negative predictive value

(NPV) and the apparent prevalence of each diagnostic method was tested. The results from the test were used to determine the screening and diagnostic test to determine whether *I. hoferi* is present in local sardine, anchovy, mullet and hake subpopulations of South Africa.

The qPCR assay developed in this study was optimised to accurately detect a 299 bp fragment of the small subunit ribosomal DNA gene from *I. hoferi*. The qPCR assay was optimised to accurately detect as little as 0.005 schizonts in a 25 µL reaction volume. Among the diagnostic techniques, the test with the highest diagnostic sensitivity and NPV was the wet-mount squash preparations of all organ tissues (95.24 % and 88.89 % respectively) followed closely by qPCR of homogenised liver tissue (90.48 % and 87.50 % respectively). Quantitative real-time PCR, *in vitro* culture and histology of liver had diagnostic specificities of 100 % with positive predictive values of 100 % while the wet-mount squash preparations of all organ tissues conversely had the lowest diagnostic specificity (57.14 %) and PPV (76.92 %). We observed a 14.28 % (71.43 % to 85.71 %) diagnostic sensitivity increase of *in vitro* culture and a 9.53 % (80.95 % to 90.48 %) diagnostic sensitivity increase from qPCR after the liver tissues were homogenized in buffer. The data further showed that examination of all tissues of *Rhabdosargus globiceps* is imperative to accurately determine the true infection prevalence. The wet-mount squash examination technique was selected as a viable screening test to detect *I. hoferi*-infection, and used in conjunction with qPCR for diagnosis and to prevent misidentification. None of the fish collected from bycatch locations around South Africa during this study tested positive for *I. hoferi*-infection. An expanded surveillance assessment is recommended due to the small sample sizes within the subpopulations of each species examined.

In selecting appropriate diagnostic techniques considerations such as the objective of the study, the sampling costs, logistics, test performance, speed required from diagnosis and the technology available should be acknowledged and that a combination of diagnostic methods is often required for a definitive diagnosis. In this study the qPCR protocol developed has the potential for utilisation as a routine diagnostic tool for *I. hoferi* surveillance and management.

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CHAPTER 1

LITERATURE REVIEW

1.1) Introduction

Ichthyophoniasis is a systemic granulomatous disease caused by infection of the mesomycetozoan parasite, *Ichthyophonus hoferi* Plehn and Mulsow (1911) (Mendoza *et al.*, 2002). Ichthyophoniasis has been implicated in epizootics resulting in high mortality and significant economic losses among many freshwater and marine fish populations (Rahimian and Thulin, 1996; Mellergaard and Spanggaard, 1997; Mcvicar, 1999; Óskarsson and Palsson, 2011). *Ichthyophonus hoferi*, which was first identified in cultured brown and brook trout in Germany by von Hofer in 1893, is now considered to be globally distributed with a wide host range (Mcvicar, 1999; Gregg *et al.*, 2014). The low host specificity of *I. hoferi* and its natural adaptability to different environments has created an economic and political concern due to its capability of producing mass mortalities of epizootic proportions in commercial fish populations (Rahimian and Thulin, 1996; Mcvicar, 1999; Óskarsson and Palsson, 2011). The most common form of *I. hoferi* seen in infected hosts are the spherical multinucleate schizonts (Paperna, 1986; Franco-Sierra and Alvarez-Pellitero, 1999a; Mcvicar, 1999; Hershberger, 2012). In infected host tissue, schizonts are usually encapsulated with inflammatory host cells, visible as granulomas containing host lymphocytes, macrophages, neutrophils and fibrous connective tissue (Spanggaard and Huss, 1996; Mcvicar, 1999). *Ichthyophonus hoferi*-infections can be found in the spleen, liver, kidney, heart, intestine, stomach, brain, skeletal muscle, gills and blood of infected hosts which can vary greatly among individuals and host species (Eaton *et al.*, 1991; Rahimian, 1998; Franco-Sierra and Alvarez-Pellitero, 1999b; Mcvicar, 1999; Kocan *et al.*, 2009, 2013; Huntsberger *et al.*, 2017).

Infections with *I. hoferi* can be detrimental to the host (Kocan *et al.*, 1999, 2006; Mcvicar, 1999; Kramer-Schadt *et al.*, 2010; Vollenweider *et al.*, 2011) and can reduce the quality of the flesh of the fish (Kocan *et al.*, 1999, 2004a; Mcvicar, 1999). This can reduce the economic value of the fish, negatively affecting commercial fisheries and aquaculture industries (Bush *et al.*, 1997). The transmission of *I. hoferi* occurs through the ingestion of schizonts in *I. hoferi*-infected flesh. In cultured fish transmission has been reported through the feeding of un-processed tissues from infected marine fishes or infected fish harbouring

I. hoferi introduced into the systems (Slocombe, 1980; Miyazaki and Jo, 1985; Sitja-Bobadilla and Alvarez-Pellitero, 1990; Mcvicar, 1999; Kocan and Hershberger, 2006). Research into the epidemiology of infected wild fish populations is relevant to identify areas of disease and the potential pathways for *I. hoferi* introduction into aquaculture farms and aquaria. With the improvement of diagnostic tests, epidemiological studies can make a significant contribution to disease prevention through improved disease surveillance, improving biosecurity management and assessing the risk of disease introduction. For epidemiological studies to accurately estimate parasite prevalence and to identify potential high risk areas to captive fish populations, diagnostic assays are required to be sensitive, specific and reproducible (Thrusfield, 1986; Hunt, 2011; Peeler and Taylor, 2011).

1.2) Aquaculture overview

Aquaculture has been identified as one of the fastest growing sustainable animal production sectors in the world and is considered globally to be a sector which has emerged as a major contributor to the growth in fishery production (FAO, 2018). A combination of increased demand for fishery products and a levelling-off in the world's capture fisheries has provided the stimulus for aquaculture industries across the world to expand (DAFF, 2018; FAO, 2018). Global production of aquaculture has grown from 16.8 million tons in 1990 to producing 110.2 million tonnes in 2016, providing half of all fish for human consumption (FAO, 2018). Intensive aquaculture, with a recognized high production capacity, is becoming an important food producing industry. Aquaculture makes a significant contribution to the ever increasing demand for aquatic food in most world regions and also plays an important economic role in many countries offering opportunities in development to improve incomes and providing employment opportunities (DAFF, 2018; FAO, 2018). In 2016 employment in the aquaculture sector supported the livelihoods of 19.3 million people of the world's population (FAO 2018).

Within South Africa the aquaculture industry has also experienced growth. The total aquaculture production in 2015 was 5 418 tons, increasing by 2 775 tons (105 %) since 2006 (DAFF, 2018), with total marine aquaculture producing 3591.86 tons and total freshwater aquaculture producing 1826.29 tons. The total number of aquaculture farms in South Africa reported in 2015 was 189 with 37 being marine aquaculture farms and 152 freshwater aquaculture farms. The Western Cape Province leads the marine aquaculture with 23 farms (encompassing 62 % of total South African marine farms) and the

Mpumalanga province leads the freshwater aquaculture with 33 farms (encompassing 21 % of total South African freshwater farms). The sector employed 3826 staff directly onto farms in 2015, with the marine aquaculture industry being the largest employer (DAFF, 2018). The increase in employment can be attributed to the increase in aquaculture production, investments and increased support from the government. In 2014 an aquaculture initiative, Operation Phakisa, to promote the sector was released and achieved 261 additional jobs in 2015. This operation involves a detailed plan of how policies and projects are going to be combined to grow the sector, increasing revenue and employment. Currently 24 projects spread across the country have been identified by this operation with a revenue stream of about half a billion rand to R1.4 billion in 2019. There are further initiatives aimed at skills development, improving access to markets and improving awareness of aquaculture (Operation Phakisa, 2014; DAFF, 2018).

The successful development of aquaculture projects has been flawed by the dissemination of diseases. Diseases have caused significant economic losses in both freshwater and marine fish species worldwide (Bondad-Reantaso *et al.*, 2005; Timi and Mackenzie, 2014; Lafferty *et al.*, 2015). Parasites are ubiquitous and pose significant threats to successful aquaculture by reducing species growth and survivorship, and decreasing seafood quality (Windsor, 1998; Lafferty *et al.*, 2015). Fish farming practices, by maintaining large numbers of fish confined in a small area, create conditions that can increase the risk of outbreaks of diseases in fish populations by providing increased density of fish (Reno, 1998; Timi and Mackenzie, 2014). At present, the rapid development of aquaculture has been associated with the emergence of diseases, some new and some previously known (Lafferty *et al.*, 2015; FAO, 2018). Awareness of the effects of these emerging diseases on fish populations has sought devotion into parasite control and management (Timi and Mackenzie, 2014). Understanding the impact opportunistic diseases have on populations stocks and investing in good management practices becomes imperative for farms to stay profitable.

1.3) Taxonomy of *Ichthyophonus hoferi*

The taxonomic position of *I. hoferi* has been controversial since its first description by von Hofer (1893) from *Salmo trutta* and *Salvelinus fontinalis* in Germany (Table.1.1). The causative organism was first considered to be a member of the Protozoa, because it was thought to have some similarities to the Protozoa described by Caullery and Mesnil (1905)

as *Ichthyosporidium gastrophilum.* Several years later Plehn and Mulsow (1911) regarded the parasite as a fungus and described the organism causing the disease as *Ichthyophonus hoferi*, classifying it with the *Phycomycetes* close to the *Chytridinae* (Mcvicar, 1999). The organism regularly appeared in the subsequent literature under the generic names *Ichthyosporidium* and *Ichthyophonus* which created confusion. Sprague (1965) reviewed the taxonomy of *Ichthyosporidium* and concluded that the generic name, *Ichthyosporidium*, is reserved for the protozoans with *I. giganteum* selected as the type-species of the genus and therefore reverted the name to *Ichthyophonus hoferi* Plehn and Mulsow (1911).

The original descriptions of *I. hoferi* by Plehn and Mulsow (1911) however were incomplete due to limited resources and techniques. This caused many researchers in classifying the causative agent of ichthyophoniasis to either follow the most recent trends or to group the organism on the basis of the most obvious morphological features (Mcvicar, 1999). These morphological studies of *I. hoferi*, based on the histopathological examination of infected tissues, failed to clarify the systematic classification of this organism. According to Paperna (1986) all the organelles traced in the endoplasm (inner fluid region of the cytoplasm) of *Ichthyophonus hoferi* could be identified with, or at least were structurally similar to organelles to the P*hycomycetes* fungi. Paperna (1986) however concluded that the fine structural data presented did not provide sufficient supportive evidence for affinities with any particular taxa of fungi. Alderman (1982) suggested placing *Ichthyophonus hoferi* in the fungi *incertae sedis* pending further evidence. Although most authors considered the organism a fungus on the basis of its life history stages that superficially resemble fungal spores and hyphae, *Ichthyophonus hoferi* are internal parasites that exhibit highly plastic morphology in host tissues (Sproston, 1944) and *in vitro* (Okamoto *et al*., 1985; Spanggaard *et al*., 1994). This made them very difficult to classify by histology or microscopic examination of live cells in culture. The application of molecular biological techniques questioned such classification (Ragan *et al.*, 1996, 2003; Spanggaard *et al.*, 1996; Mendoza *et al.*, 2002).

Studies conducted by two groups of researchers, Spanggaard *et al.* (1996) and Ragan *et al.* (1996), used sequencing of small subunit ribosomal ribonucleic acid (SSU rRNA) sequences in these and other related microbes (eg: rosette agent) to demonstrate that *I. hoferi* was not a fungus, but rather a member of a unique and ancient clade of protistan parasites with origins near the animal-fungal divergence. These two groups of researchers independently reached the conclusion that *I. hoferi* was a member of a lower protistan

group, which has been referred to as the 'DRIP' clade (an acronym for the incorporation of the *Dermocystidium*, rosette agent, *Ichthyophonus hoferi* and *Psorospermium* groups of organisms) and shared ancestry with the *choanoflagellates* (Ragan *et al.*, 1996, 2003; Spanggaard *et al.*, 1996; Mendoza *et al.*, 2002). The DRIP's clade introduced by Ragan *et al.* (1996) was later extended to a new class *Ichthyosporea* in the subphylum *Choanozoa* (Cavalier-Smith, 1998). The name *Ichthyosporea* for the class was chosen because these organisms produce unicellular walled spores and infect mostly fish. Franco-Sierra and Alvarez-Pellitero (1999) demonstrated in their study that although *Ichthyophonus hoferi* shares some morphological and biological features with fungi, other characteristics indicate affinities to pseudofungi or to other protist organisms. More recently Mendoza *et al.* (2002) established the class mesomycetozoea to accommodate the *Dermocystidium*, rosette agent, *Ichthyophonus* and *Psorospermium* groups of organisms. Two orders have been described in the mesomycetozoeans: the dermocystida, which have flat mitochondrial cristae and the ichthyophonida, which have tubulovesicular cristae (Mendoza *et al.*, 2002). This ended most of the controversy over the phylogenetic placement of *I. hoferi*. *Ichthyophonus* is now generally considered to be the valid generic name of the organism.

Ichthyophonus hoferi for some time had been the only species of the genus *Ichthyophonus* recognized to cause disease in fish. However, based on morphological criteria and phylogenetic DNA sequences, Rand *et al.* (2000) found that an unusual species, morphologically different from *I. hoferi*, had been recovered from yellowtail flounder (*Limanda feruginea*) in Nova Scotia. When the 18S SSU rDNA from this particular isolate was sequenced, these investigators proposed that their isolates were a new species, which they named *Ichthyophonus irregularis*. This revealed that the genus *Ichthyophonus* comprises of more than one species and that all may be fish pathogens (Rand *et al.*, 2000; Mendoza *et al.*, 2002). Currently *I. hoferi* Plehn and Mulsow (1911) (Mcvicar, 1999) and *I. irregularis* (Rand *et al.*, 2000) are the only 2 recognized species in the genus.

1.4) Morphology and life cycle of *Ichthyophonus hoferi*

Data on the morphological characteristics and life cycle of *I. hoferi* have illustrated the presence of parasitic stages in fish hosts and free non-parasitic forms (Mcvicar, 1999; Mendoza *et al.*, 2002). The morphology and life cycle of *I. hoferi* has been investigated by studying host species with natural and experimental infections and through *in vitro* culture.

Table 1.1: Systematic classification of *Ichthyophonus hoferi* Plehn and Mulsow (1911) since its first identification.

Year	Taxonomic position	Genus Species	Reference
1893	Recorded as 'dizziness disease'		(Von Hofer, 1893)
1905 - 1965	Protozoa	*Ichthyosporidium gastrophilum*	(Caullery and Mesnil, 1905)
1911	Fungi (*Phycomycetes*)	*Ichthyophonus hoferi*	(Plehn and Mulsow, 1911)
1982	Fungi (*incertae sedis*)	*Ichthyophonus hoferi*	(Alderman, 1982)
1986	Fungi (*Phycomycetes*)	*Ichthyophonus hoferi*	(Paperna, 1986)
1996	Animal-fungal divergence (DRIP clade)	*Ichthyophonus hoferi*	(Ragan *et al.*, 1996) and Spanggaard *et al.*, 1996)
1998	Protozoa (*Ichthyosporea*)	*Ichthyophonus hoferi*	(Cavalier-Smith, 1998)
2002 - present	Mesomycetazoan, Ichthyophonida	*Ichthyophonus hoferi*	(Mendoza *et al.*, 2002)

As a whole, studies have shown *I. hoferi* to have a limited range of morphological structures (Fig. 1.1.A - 1.1.D, 1.2.A - 1.2.F & 2.6.A - 2.6.F), and have empirically demonstrated a direct transmission life cycle for *I. hoferi* in piscivores hosts (Athanassopoulou, 1992; FrancoSierra *et al.*, 1997; Jones and Dawe, 2002; Kramer-Schadt *et al.*, 2010; Kocan *et al.*, 2013; Sindermann and Chenoweth, 1993). The most frequent morphological forms of *I. hoferi* observed in living hosts are the spherical cells variously referred to as resting spores, multinucleate spores, the multinucleated spherical body, the M-spore or schizont (Fig. 1.1.A, 1.2.A, D & 2.6.A) (Okamoto *et al.*, 1985; Paperna, 1986; Mcvicar, 1999; Kocan, 2013). Kocan (2013) deemed the term schizont to most closely describe the multinucleate *I. hoferi* cell and will therefore be referenced as schizont throughout the rest of the dissertation.

These schizonts vary in size (10 - 250 µm in diameter) and occur either singly or in small groups and are surrounded by granulomatous tissue in infected hosts (Fig. 1.2.A, B, C) (Paperna, 1986; Mcvicar, 1999). These spherical schizonts are characterized by a fibrillar thick strongly periodic acid-Schiff (PAS) positive wall (Fig. 1.1.A, 1.2.A & 2.6.A), an undulating cell membrane, a fine granulated cytoplasm packed with ribosomes, scattered vesicles (spherical dense body vacuoles and globular electron-lucent bodies), mitochondria with tubulovesicular cristae (composed of small tubes and sacs) and a varying number of nuclei (Paperna, 1986; Spanggaard *et al.*, 1995; Rahimian, 1998; Franco-Sierra and Alvarez-Pellitero, 1999b; Mcvicar, 1999). The cell wall of *I. hoferi* consists of parallel arrangements of micro-fibrils and the wall thickness varies with schizont age and size. In young schizonts the walls are usually thin and display no fibrillary organization. In large schizonts fibrillar organization is more evident in the inner layers with the central layer usually being more compressed and the border of the outer layer usually being less organized with variable thickness, density and consistency (Paperna, 1986; Franco-Sierra and Alvarez-Pellitero, 1999). An increase in wall thickness is seen with an apparent reduction of the endoplasm volume and an apparent loss of the fibrillary configuration of the wall (Paperna, 1986). Studies done by Spanggaard *et al.* (1996) found chitin in the wall of *I. hoferi*, in contrast to the results reported by Rand (1994), who could not demonstrate the presence of chitin in the different forms of the parasite. The cytoplasm of *I. hoferi* schizonts are structurally uniform in appearance and are normally lightly staining with haematoxylin and eosin (H & E) (Fig. 1.2.D) (Mcvicar, 1999).

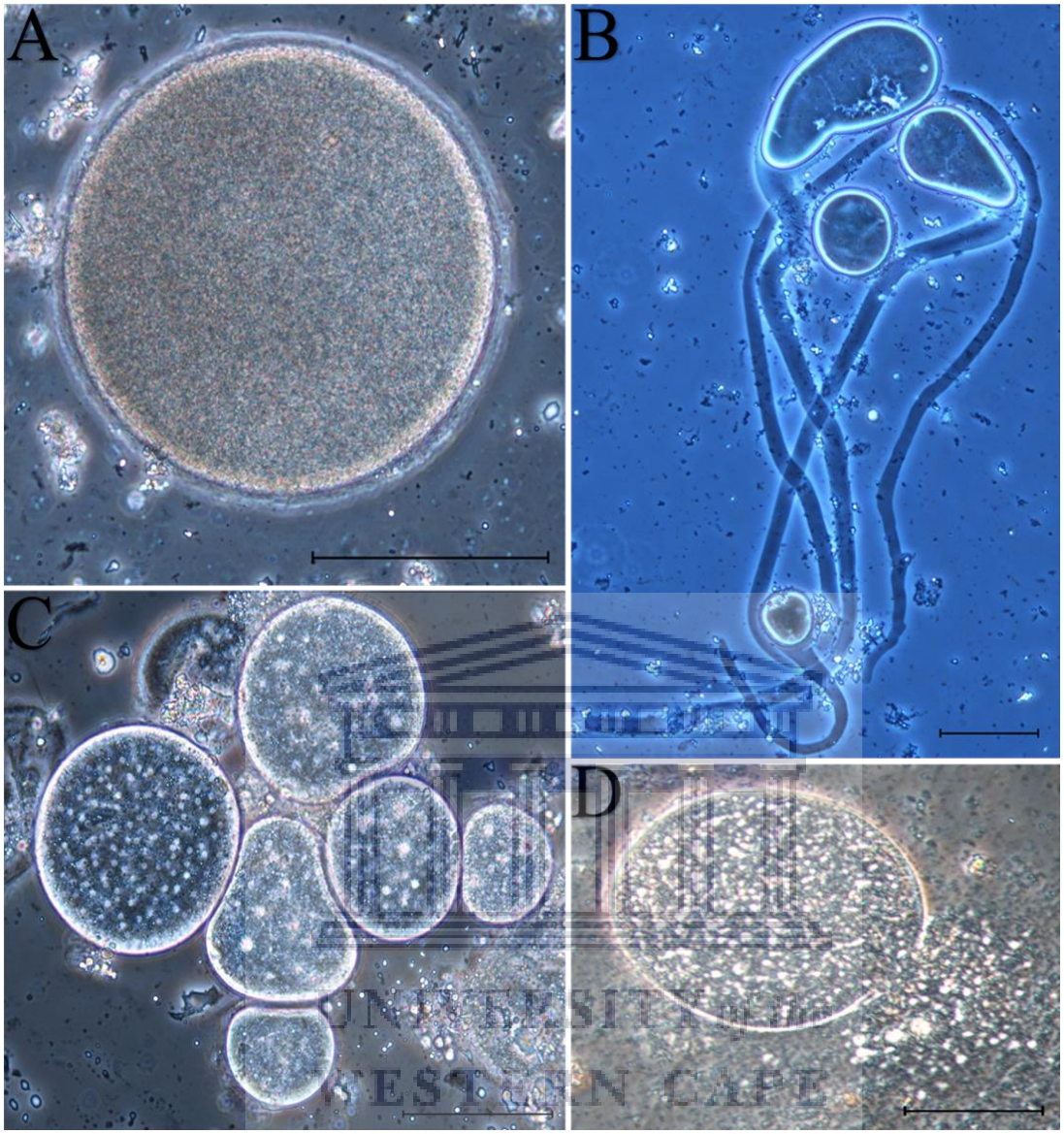

Fig. 1.1.A - 1.1.D: *Ichthyophonus hoferi* stages cultured *in vitro* in Eagles minimum essential medium (EMEM). A: The multinucleated spherical body or schizont of *I. hoferi.* Note the thick non-cellular multilaminate membrane. B: Hyphal development of *I. hoferi.* C: The aggregation and formation of numerous *I. hoferi* schizonts. D: Further proliferation of *I. hoferi* through the release of merozoites. Scale bar: 100 µm.

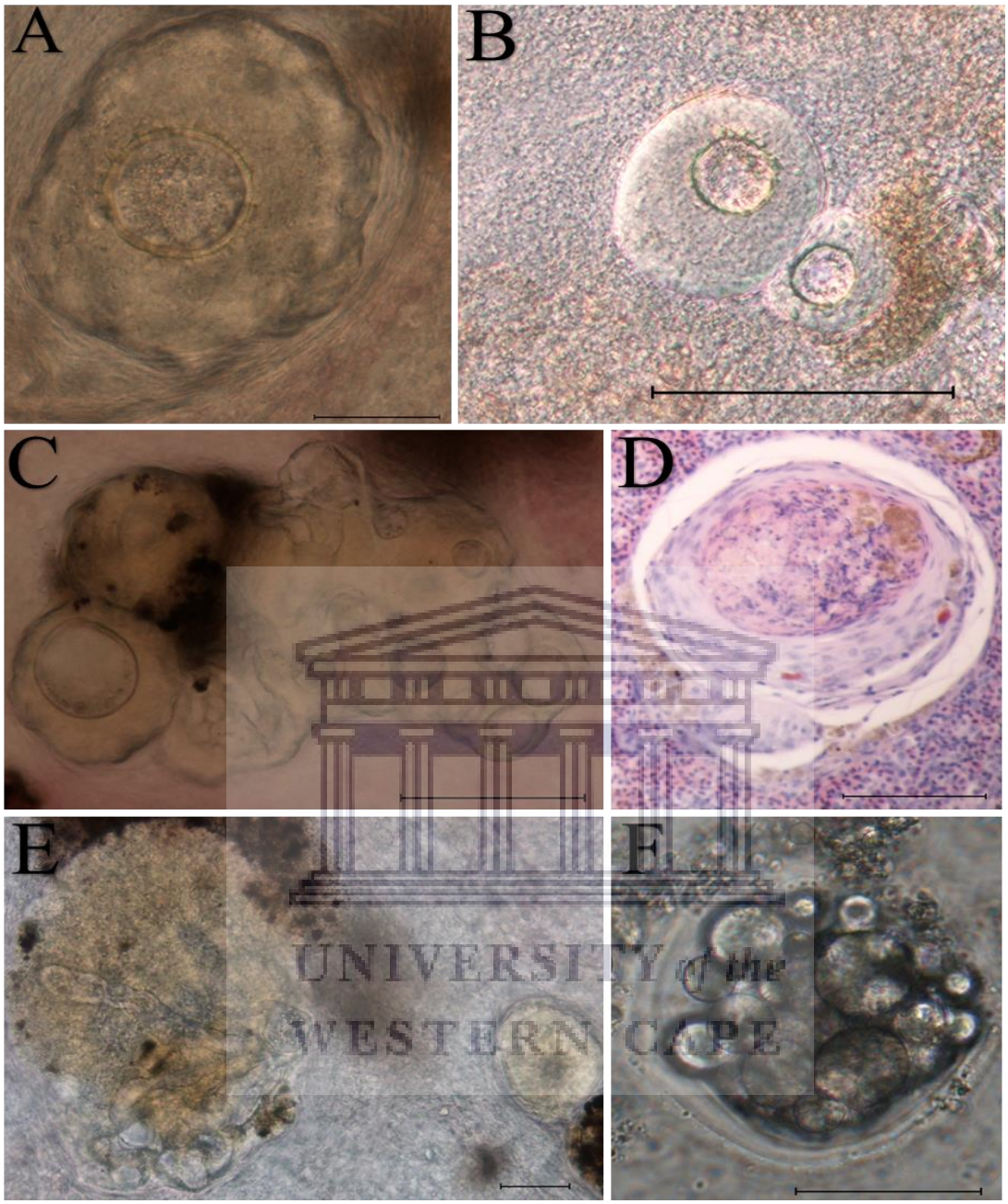

Fig. 1.2.A - 1.2.F: *Ichthyophonus hoferi* stages from *Rhabdosargus globiceps* (light microscope images). A & B: Thick walled multinucleated schizont surrounded by host granulomatous liver tissue reaction. C: Aggregation of *I. hoferi* in spleen tissue granuloma. D: Histological staining of *I. hoferi* cytoplasm with Hematoxylin-Eosin. E: Characteristic germination seen after the death of the infected host. F: Merozoites embedded within the schizont awaiting proliferation. Scale bar: 100 µm.

In live fish, small mono-nucleate schizonts develop into multinucleate schizonts, however following the death of the host and *in vitro*, other developmental stages of *I. hoferi* can be observed. *Ichthyophonus hoferi* schizonts have been observed to either produce germination tubes which bud to produce schizonts (Fig. 1.1.B, 1.2.E & 2.1.C - F) (Spanggaard *et al.*, 1994; Franco-Sierra and Alvarez-Pellitero, 1999; Mcvicar, 1999), or fragment into multinucleate motile merozoites that migrate away from the schizont (Fig. 1.1.D) (Mcvicar, 1999; Mendoza *et al.*, 2002; Kocan *et al.*, 2013). The stimulation of the schizont to develop germination tubes or hyphae (non-septate hyaline tubules) has been related to low pH (Spanggaard *et al.*, 1995; Franco-Sierra and Alvarez-Pellitero, 1999b). Germination occurs very rapidly and can occur within four to five hours at a low pH (Spanggaard *et al.*, 1995). The rise in the carbon dioxide levels as the host dies is said to stimulate the *I. hoferi* schizonts to germinate (Mcvicar, 1999). The outcome of this proliferation results in a large increase in the number of potentially infective units, which is advantageous at the point of transmission to infect new hosts. *In vitro* studies by Kocan *et al.* (1999) showed that hyphal-like structures were evident growing from the schizonts by 4 days in culture, and by 11 days in culture spherical structures developed at the hyphal tips and were released into the medium. Rapid growth of merozoites (Fig. 1.1.D) (Okamoto *et al.*, 1985; Paperna, 1986; Spanggaard, 1995; Kocan *et al.*, 1999; Mcvicar, 1999) ultimately lead to further subdivision of the schizonts and the formation of new infective stages (Spanggaard *et al.*, 1994; Mcvicar, 1999; Mendoza *et al.*, 2002; Kocan *et al.*, 2013).

Ichthyophonus hoferi growth and the different development stages seen in naturally infected fish can be achieved in cultured media, according to the medium used (Okamoto *et al.*, 1985; Spanggaard *et al.*, 1994; Franco-Sierra and Alvarez-Pellitero, 1999b; Mcvicar, 1999). Spanggaard *et al.* (1994), Spanggaard (1995) and Franco-Sierra and Alvarez-Pellitero (1999) showed that pH seems to be one of the factors crucial for the growth and development of *I. hoferi.* They showed that in media with an acidic pH (pH of 3 and 5), germination of multinucleate schizonts and the formation of terminal cells in the hyphal tips can be observed. In media at a pH of 7 optimal growth of schizonts occurs as well as the internal cleavage and release of the terminal cells through the germination tubes can be observed. Spanggaard and Huss (1996) carried out studies of the growth of *I. hoferi* in North Sea herring products and concluded that pH 3 and 7 did not affect the growth ability of the parasite. Mcvicar (1999) noted that variable carbon dioxide, glucose availability and salinity can also affect the growth of *I. hoferi.*

In the infected host, following the ingestion of resting schizonts, the schizonts of *I. hoferi* can be observed to undergo a series of morphologic changes in the host. These changes have been observed to consist either of a transition from the large thick walled multinucleate schizont to smaller infective stages that enter the blood from the stomach after several days (Kocan *et al.*, 2013), or the germination of the schizont in the low pH of the stomach to produce hyphae that penetrate the digestive tract and produce schizonts to be transported in the blood to the viscera (Spanggaard *et al.*, 1994; Mcvicar, 1999). Surrounding the tips of the germinating tubes, a strong reaction occurs which has said to be an enzymatic system associated with the ability of the hyphae to penetrate through host tissue (Mcvicar, 1999). Once within the host body *I. hoferi* shows all of the characteristics of a typical system invader. The infective units spread passively throughout the body through the blood (Mcvicar, 1999; Kocan *et al.*, 2013) or lymphatic systems (Fish, 1934) and pass through the capillary beds into the blood rich organs where they grow to maturity (Kocan *et al.*, 2013). Once the circulating cells become too large to pass through the capillary beds, they become lodged in the various organs. Their growth then often leads to the destruction initially of the host cell and then of adjacent cells (Paperna, 1986). In these areas proliferation of the parasite also occurs, leading to a secondary invasive spread in the immediate vicinity and to a release of further infective stages into the circulatory system (Mcvicar, 1999; Kocan *et al.*, 2006, 2013). Most or all of the pathogenesis of *I. hoferi* can be linked directly to the replacement, disruption and atrophy of infected tissues due to the proliferation of the parasite. Sometimes replacing the organ tissue completely, leading to their malfunction and to organ failure (Mcvicar, 1999; Kocan *et al.*, 2006).

Infection elicits a chronic inflammatory response, which depending on the duration of infection and host species, consists of variable amounts of macrophages, epitheliod cells, giant cells, fibrocytes and eosinophilic granular cells (Mcvicar, 1999). Phagocytosis is the primary defence mechanism of the host to invading schizonts (Paperna, 1986). The result is often white or cream-colored nodular lesions appearing throughout the vascularized organ due to the separation of the muscle cells by the large spherical multinucleate schizonts, infiltration by inflammatory cells early in the infection and accumulation of fibrous tissue around the parasite (Paperna, 1986; Rahimian, 1998; Mcvicar, 1999; Kocan *et al.*, 2004b, 2013). Post mortem germination tubes occur in the infected fish, due to the lowering of the host pH, and has been believed that these may be a mechanism for dispersal of infectious cells into the environment following the death of the host, or possibly a means of infecting an intermediate host (Spanggaard *et al.*, 1994; Mcvicar, 1999). The outcome

of this proliferation in the tissues of a dead host results in a large increase in the number of potentially infective units which ultimately leads to an increase in the confidence of transmission to a new host which has eaten live infected prey (Mcvicar, 1999).

1.5) Epizootiology of *Ichthyophonus hoferi* in captive and wild populations

Ichthyophonus hoferi has resulted in mass mortalities in many fish populations from a variety of localities due to its adaptability to a range of environments. *Ichthyophonus hoferi* was first identified in cultured brown trout, *Salmo trutta*, and brook trout, *Salvelinus fontinalis*, in Germany by von Hofer in 1893. Since then, *I. hoferi* has been reported in many species of fish from freshwater, brackish water and the marine environment. *Ichthyophonus hoferi* has been recorded from many temperate and tropical waters both North and South of the Equator and currently has a global occurrence in sea water, making ichthyophoniasis one of the most widespread diseases of fish (Fig. 1.3) (Mcvicar, 1999).

Fig. 1.3: Records of *Ichthyophonus hoferi* infections of fish.

Historical records of *I. hoferi* infecting wild populations have come from the North Atlantic region (Fish, 1934). Epizootics of the disease have occurred among commercial fish species in the North Atlantic and Eastern North Atlantic, particularly in Atlantic herring (*Clupea harengus*). In Europe in the early 1990's an *I. hoferi* related epizootic killed an estimated 300 million Atlantic herring in marine waters around Sweden and Denmark, resulting in masses of dead and dying herring floating on the surface and on the sea bottom which caused clogging of trawls and masses washing up on the shores (Rahimian and Thulin, 1996). This was the first record of a mass mortality of herring due to *I. hoferi* infection along the Eastern coast of the North Atlantic. The factors which led to the development of the epizootic are unknown. In 2008 in Icelandic waters a major epizootic of *I. hoferi* in herring was reported (Óskarsson and Palsson, 2011). Epizootics among salmonids thought to be caused by *I. hoferi* have also been reported from trout farms in Europe and North America (Mcvicar, 1999). In the Mediterranean region, the parasite has been reported in Greece (Athanassopoulou, 1992) and Spain (Sitja-Bobadilla and Alvarez-Pellitero, 1990). The first description of *I. hoferi* in the North East Pacific occurred in 1986 (Olson, 1986) and subsequently has expanded dramatically possibly as a result of increased testing. In wild populations of salmonids a high prevalence of *I. hoferi* was reported for Chinook salmon (*Oncorhynchus tshawytscha*) from the Yukon River (Zubchenko and Karaseva, 2002). During the 1990's when this disease emerged in the Yukon River Chinook salmon it caused pre-spawn mortality in returning adults and affected the fillets of the fish creating serious product quality issues for subsistence fishers, commercial fishers, and processors. The Yukon River is one of the three major rivers flowing into the Pacific Ocean, others being the Fraser and Columbia. In the Eastern North Pacific up to Alaska many species are now affected (Kocan *et al.*, 1999, 2010; Mcvicar, 1999; Criscione *et al.*, 2002; Marty *et al.*, 2010; Gregg *et al.*, 2014). In Japan *I. hoferi* broke out among cultured ayu (*Plecoglossus altivelis*) in 1979 (Miyazaki and Jo, 1985). In Australian waters marine mullet (*Mugil cephalus*) and farmed trout (*Salmo trutta*) have been infected (Slocombe, 1980; Mcvicar, 1999). In Africa, *I. hoferi* were identified in marine mullet (*Mugil cephalus*) from the Kowie lagoon (brackish water), located on the shore of the South-Eastern Cape, South Africa (Paperna, 1986) and in aquarium held Cape white stumpnose (*Rhabdosargus globiceps*) at the Two Oceans Aquarium in Cape Town (this dissertation) (Fig. 1.3) (Mcvicar, 1999; Kocan *et al.*, 2004a; Kocan and Hershberger, 2006). In response to the discovery of these infected areas, an expanded study including expanded sites and a larger size and age range of fish should be conducted to better understand infection prevalence and intensity globally. The difference in distribution of *I. hoferi* from

tropical and temperate waters is probably due to research efforts in those areas as a failure to report epizootics may be attributed to the lack of epizootiological research and the difficulty in establishing the effect of the disease in the affected populations.

Since the discovery of *I. hoferi*, the known host range has expanded rapidly. This has created considerable economic concern because of its capability of producing mortalities of epizootic proportions in wild marine commercial fish populations (Rahimian and Thulin, 1996) and in fresh and seawater aquaculture (Athanassopoulou, 1992; FrancoSierra *et al.*, 1997). Commercially important *I. hoferi* hosts include: Atlantic herring (*Clupea harengus),* Atlantic cod (*Gadus morhua*), Atlantic mackerel (*S. scombrus*), turbot (*Scophthalmus maximus),* European plaice (*Pleuronectes platessa),* Pacific herring (*C. pallasi),* sea trout (*Salmo trutta*), haddock (*Melanogrammus aeglefinus),* European flounder *(Platichthys flesus*) and salmonids (McVicar, 1984; Mcvicar, 1999; Gregg *et al.*, 2014). Salmonids, include farmed rainbow trout (*Oncorhynchus mykiss*) probably due to the feeding of unprocessed infected marine fish, migrating Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*) and Chinook salmon (*Oncorhynchus tshawytscha*) (Mcvicar, 1999; Zubchenko and Karaseva, 2002; Kocan *et al.*, 2004a; Prabhuji and Sinha, 2009; Rasmussen *et al.*, 2010). *Ichthyophonus hoferi* has been reported in two cultured marine fish; thinlip grey mullet (*Mugil capito*) and leaping grey mullet (*Liza saliens*) (FrancoSierra *et al.*, 1997). In the case of aquarium ornamental fish, *I. hoferi* has been reported in sumatra barb (*Systomus tetrazona*) and black tetra (*Gymnocorymbus ternetzi*) (Mcvicar, 1999). Rahim Peyghan (2014) also reported this parasite from black tetra and also from tiger barb *Pentius tetrazona* in Ahvaz-Iran (Fig. 1.3). Although epizootics of fish diseases are fairly common in fish farms, the most commonly reported ecological and economic impact of ichthyophoniasis have been in wild marine fish. Most of the records of *I. hoferi* from the available literature have been recorded from the open sea, estuaries or from fish migrating between marine and freshwater. This has suggested that *I. hoferi* is primarily of a marine origin. Kocan *et al.* (2004a) showed that adult Chinook salmon entering into the Yukon River were already infected at a prevalence level of 25 % - 35 %, proposing *I. hoferi* to be of marine origin. This conclusion was supported in experimental studies done by Jones and Dawe (2002), which showed that *I. hoferi*-infected Chinook salmon only developed signs of the disease 25 - 35 days following exposure, which meant that the Chinook salmon that had entered into the Yukon River had already been infected at least 25 – 35 days before entering the river.

Results have shown that *I. hoferi* can grow in a wide range of temperatures which contributes to the appearance of the disease in different climatic conditions. Sitja-Bobadilla & Alvarez-Pellitero (1990) demonstrated that *I. hoferi* could grow between 3 °C and 20 °C with its optimum temperature for growth being 10 °C. Spanggaard and Huss (1996) however detected no significant differences in the growth ability of the parasite in North Sea herring at temperatures between 0 °C and 25 °C, but revealed that the schizont is non-viable *in vitro* at temperatures above 25 °C and is killed when heating at 40 °C and blast freezing at -20 °C. There have been reports on correlations between water temperature and *I. hoferi* infections in fish species (Okamoto *et al.*, 1987; Kramer-Schadt *et al.*, 2010). Studies done by Okamoto *et al*. (1987) reported that 100 % mortality occurred among groups of infected rainbow trout (*Oncorhynchus mykiss*) maintained for 35 days at temperatures of 15 °C and 20 °C but only 10 % mortality at 10 °C and no mortality at 5 °C. Spanggaard and Huss (1996) reported that infection trials with mice showed no indication of toxicity or pathogenicity and found survival of the parasite to be less than 3 min at 40 °C. There have also been no cases of human infections reported; concluding that humans or other mammals are not at risk from infection. The wide ranges of geographic and host distributions suggest that *I. hoferi* has very low host specificity and that fish predominate as hosts. Pathogenicity of *I. hoferi* varies significantly between species. Species such as plaice (*Pleuronectes platessa*) and herring (*Clupea pallasii*) are highly susceptible, haddock (*Melanogrammus aeglefinus*) and rainbow trout (*Oncorhynchus mykiss*) show an intermediate susceptibility while other species such as rock cod (*Gadus morhua*), goldfish (*Carassius auratus*), guppy (*Lebistes reticulatus*), squawfish (*Ptychocheilus oregonensi*) and catfish (*Ameiurus nebulosus*) show a high level of resistance (Mcvicar, 1999). These differences may be due to variability in host species, immune response, the stage of the parasite delivered, the route of exposure or the infectious dose and age of the fish (Rahimian and Thulin, 1996; Rahimian, 1998; Mcvicar, 1999; Kocan and Hershberger, 2006). In haddock (*Melanogrammus aeglefinus*) the most obvious lesions occur in the white muscle, in herring (*Clupea pallasii*) in the heart and in plaice (*Pleuronectes platessa*) in the liver and kidney (Mcvicar, 1999). It too is therefore necessary to take such differences into account when determining the prevalence of infection.

Transmission of *I. hoferi* is thought to occur through the ingestion of the multinucleate schizonts with infected food particles (Kocan *et al.*, 1999; Mcvicar, 1999). Mcvicar (1999) suggested that instances of infection in cultured freshwater trout and aquarium fish could be the result of feeding contaminated flesh of marine fish to the cultured fish. This has led

to the conclusion that the movement of infected fish or feeding infected unprocessed marine fish to susceptible cultured fish populations in both marine and freshwater farms (knowingly or unknowingly) by humans has been a reason for *I. hoferi* occurring in cultured fish populations (Mcvicar, 1999). Hansen & Pethon (1985) also supported this view on horizontal trophic transmission when they stated salmon have a dietary preference for other fish species, such as herring. This concluded it is quite likely that salmon may contract *I. hoferi* through feeding on these infected species and that transmission in piscivorous and scavenger hosts likely occurs through the consumption of infected prey. Alternatively it seems probable that hosts can also become infected directly by ingestion of free multinucleate schizonts in the water. Kocan *et al.* (1999) suggested that *I. hoferi* first targets the internal organs and then migrates to the skin to exit as small schizonts to infect or to be ingested by an invertebrate or larval fish. Rahimian and Thulin (1996) proposed that since small herring do not usually school with adult herring, it is unlikely that infection is transferred directly from adults and therefore the small herring found infected with *I. hoferi* in the western North Atlantic might have been infected by swallowing schizonts that were visible and of a suitable size to serve as food for larval herring. Other routes such as through the gills or skin lesions, as suggested by Sproston, (1944) require further investigation. Further research is needed to understand the possible involvement of intermediate hosts and other natural routes of infections.

Although records of infection with *I. hoferi* have suggested a high level of transmission and infection success with a high pathogenicity, it cannot be expected that infection with *I. hoferi* will certainly lead to a systemic infection or the beginning of an epizootic (Mcvicar, 1999). The successful establishment of a parasite within the host's body is dependent on the ability of the infective stage to penetrate the host's first line of defences and invade the target tissues before being neutralized by the host's humoral and/or cellular immune defences (Mcvicar, 1999; Kocan *et al.*, 2006, 2013). Fish host records and reports of natural hosts are likely a reflection of whether a particular fish species has been thoroughly and appropriately examined. With new diagnostic techniques now available, the records of *I. hoferi* from other groups of hosts need to be reconsidered carefully as it is not possible to determine the extent to which misidentifications have appeared in the literature without a thorough reassessment of the original material. However as the distribution of *I. hoferi* is not uniform between areas and within fish populations determining the true prevalence of infection in natural populations may be challenging.

1.6) Diagnosis of ichthyophoniasis

The diagnosis of *I. hoferi* infection is currently performed by several methods of pathogen identification. The ability for observers to detect *I. hoferi* from gross signs is linked closely to the level of infection in the host, the pathogenicity and the tissue being sampled. When pathogenicity is high, several clinical signs associated with organ failure can accompany the advanced stages of infection; such as loss of appetite, decreased swimming performance, lethargy, emaciation, colour abnormalities and nervous disorders (Mcvicar, 1999). These abnormalities are readily observed in captive fish but are often missed in wild populations. When infected fish are examined macroscopically, externally they may exhibit a rough sandpaper skin texture with occasional skin ulcerations surrounded by tissue granuloma (Rahimian and Thulin, 1996). The sandpaper skin is caused by large numbers of raised papules under the skin surface. The parasite is eventually released from these papules, ultimately leaving ulcers that resemble flakes of pepper on the skin surface (Mcvicar, 1999; Hershberger, 2012). The condition is often most pronounced on the caudal third of the body surface (Kocan *et al*., 2011). When infected fish are examined internally, it is common for gross nodular pigmented lesions (1 - 5 mm in diameter) to be visible throughout most tissues, usually surrounded by granulomatous tissue reaction (Mcvicar, 1999). The lesions that occur in the skeletal muscle of heavily infected fish result in the inability of fisheries and aquaculture industries to market the affected fillets. This technique is useful for field evaluations when rapidly screening large numbers of fish in infected populations. However, without doubt some infected fish will be missed if only macroscopic evaluations are relied on and should therefore be verified by the use of other more specific techniques (Rahimian and Thulin, 1996; Mcvicar, 1999).

Microscopic examination of fresh squash preparations of infected soft tissues usually reveal the presence of the thick-walled spherical schizonts (10 – 250 µm in diameter) usually surrounded by host granulomatous tissue reaction (Fig. 1.2.A) (Paperna, 1986). Germination tubes (hyphae) can be typically observed after the infected host has been dead for a period of time (Fig. 1.2.E) (Mcvicar, 1999). Microscopic examination of tissue squash preparations often detects higher infection prevalence than macroscopic examination of the same tissues. Rahimian and Thulin (1996) revealed that only 1.1 % of herring (*Clupea harengus*) showed macroscopically visible nodules of *I. hoferi* on their hearts, whereas microscopic examination revealed 3.8 % to have signs of the infection in the heart. The authors also showed that when the results between the micro- and macroscopic prevalences

were compared, the ratio is not the same in different size groups with the ratio decreasing as the size of the fish increases. This technique is most useful for providing partial confirmation of moderate to heavy infection intensities, where clinical signs are present (Hershberger, 2012). However, because of the lack of distinguishing features in the schizonts of *I. hoferi*, Mcvicar (1999) stated that caution should be exercised in diagnosing the disease solely from the observation of wet-mount squash preparations.

Ichthyophonus hoferi schizonts can be easily observed by histological evaluation of stained tissue sections from moderate and heavy intensity infections. The cytoplasm is normally lightly staining with Heamtoxylin-eosin (Fig. 1.2.D) (Hershberger, 2012). This method however uses only a section of tissue approximately 5 µm thick, which is not always suitable for accurately screening populations for *I. hoferi*, due to the distribution variations of the parasite among organs (Mcvicar, 1999; Kocan *et al.*, 2011). Periodic acid-Schiff (PAS) is another commonly used special stain in the histology lab and is used to identify polysaccharides in the outer walls of the *I. hoferi* schizonts (Schmidt-posthaus and Wahli, 2002; Kocan *et al.*, 2011). The disadvantage with using PAS staining is that other spherical organisms in the 50 - 250 µm size range also stain PAS-positive and superficially resemble *I. hoferi* schizonts. This can lead to false positives and reduce diagnostic sensitivity. Histological evaluation is beneficial when confirming the identity of the parasite and determining the cellular damage and host cell response, but is not always suitable for accurately screening fish populations for *I. hoferi*-infection prevalence.

A diagnostic technique that is more sensitive than histological examination at detecting *I. hoferi* infections involves the *in vitro* culture of tissue explants. *In vitro* culture has the advantages of allowing a live *I. hoferi* cell to reproduce during incubation thereby multiplying the amount of analyte in the sample and also uses a larger amount of tissue than histological evaluation, thus significantly increasing the analytical sensitivity (probability of detecting low-level subclinical infections) of the assay (Mcvicar, 1999; Kocan *et al.*, 2011; Hershberger, 2012). Kocan *et al.* (1999) showed that *in vitro* culture of herring (*Clupea harengus*) tissue was more sensitive than histologic examination for detecting subclinical infected fish; because of the 30 adult wild Pacific herring (*Clupea harengus*) simultaneously evaluated, 70 % (21/30) were positive by culture, while only 7 % (2/30) of the same fish were positive by histologic examination. In a different study using a different species done by Halos *et al*. (2005), 11 % (33/302) of Puget Sound rockfish (*Sebastes emphaeus*) were positive by the *in vitro* culture method, while less than

1 % (1/302) of the same fish were positive by histology. Cultures are initiated by placing tissues in Tris or Hepes-buffered Eagles minimum essential medium (EMEM) supplemented with foetal bovine serum and incubated between 5 °C and 25 °C for 10 - 21 days (Spanggaard and Huss, 1996; Mcvicar, 1999; Kocan *et al.*, 2011, 2013). *Ichthyophonus hoferi* schizonts and hyphae grow readily in culture media at < 1 part tissue: 5 parts media (w/v) (Hershberger, 2012). The addition of antibiotics in the medium such as gentamicin, penicillin and streptomycin are recommended to decrease the possibility of bacterial and yeast contamination. The culture is then screened microscopically for signs of *I. hoferi* growth. The pathogen is confirmed by the presence of *I. hoferi* schizonts and germinating stages (Spanggaard *et al.*, 1994; Spanggaard and Huss, 1996; Mcvicar, 1999). It is important to note that tissue samples from recently-dead specimens should be used as there is an increased risk of potential yeast and mould contamination from autolyzing (disintegrating) tissues of fish that have been dead for some time (Mcvicar, 1999). The *in vitro* explant culture method is recommended as the diagnostic 'gold standard' (Kocan *et al.*, 2011), however, this culture method is not practical when collecting samples at remote field sites when a short presumptive diagnosis is required, or when frozen fish are sampled, and under these constraints PCR is a viable alternative (Hamazaki *et al.*, 2013a; b)

The polymerase chain reaction (PCR) which targets a specific DNA sequence is a method which has become a routine diagnostic tool for many fish pathogens and is potentially more sensitive at detecting *I. hoferi*-infection than explant culture (Whipps *et al.*, 2006; Bott *et al.*, 2010; Adams and Thompson, 2011; Hamazaki *et al.*, 2013a). Whipps *et al.* (2006) developed and evaluated a polymerase chain reaction (PCR) assay as a tool to detect the presence of *I. hoferi* DNA from preserved host tissues. After the genomic DNA from the *I. hoferi*-infected fish tissues were isolated using standard methods (QIAGEN protocol for the DNeasy Tissue kit), PCR amplification of a 371 bp segment of the small subunit (SSU) rDNA was achieved using specific primers and PCR conditions described by Whipps *et al.* (2006). The products were visualized by conventional agarose gel electrophoresis (containing 0.1 $\mu g.mL^{-1}$ ethidium bromide) and the presence of the 371 base pair band indicated a positive reaction. Whipps *et al.* (2006) demonstrated that the diagnostic test is a specific (94.0 - 100 %) and moderately sensitive (47.8 - 100 %) tool for detecting *I. hoferi* DNA, with diagnostic sensitivity of the test being higher in fish with heavy infections than those with light infections. This is expected as testing a single sample from a tissue of a fish may not reliably detect all infections, especially due to the uneven distribution of the parasite within individual tissues (Kocan *et al.*, 2011). Whipps *et al.* (2006) also evaluated

the detection of the parasite in the blood using PCR in order to non-lethally identify infected fish. The parasite however was rarely detected in the blood, with sensitivity results of the PCR test on the blood being very poor (8.7 %). Criscione *et al.* (2002) also used PCR as a confirmatory diagnostic test for their phylogeographic study however amplified fragments consisting of approximately 640 base pairs (bp) (region A) and 673 bp (region B) using specific primers and PCR conditions described by Criscione *et al.* (2002).

There are certain advantages of PCR over culture and other diagnostic techniques. The samples collected for PCR can be archived and stored indefinitely in 95 % ethanol thus making this method ideal for field collections where storage, controlled environment, and timely sample treatments can be problematic; a large number of samples can be tested relatively easily using PCR; less time is required for diagnosis; morphologically similar strains can be separated and samples can be tested repeatedly (Walker and Subasinghe, 2000; Whipps *et al.*, 2006; Bott *et al.*, 2010). Whipps *et al.* (2006) suggested that because individual cells contain multiple copies of the small subunit rDNA the test is likely to detect the presence of "prespore" stages and lighter infection when the appropriate tissue is sampled. Another advantage of PCR over culture is when aseptic techniques are not practiced during the culture method, the cultures can periodically become over-run with yeast and mould and the contaminants can out-compete *I. hoferi* growth, causing the cultures to become unreadable. The polymerase chain reaction is effective in confirming *I. hoferi*-positive cultures that become overrun with yeast (Hershberger, 2012). The disadvantage in using PCR is that since PCR detects fragments of DNA, it will detect both living and dead parasites, and cannot determine whether live parasites are present. This potentially can produce false positives, which occurs when a host carrying a non-viable pathogen is diagnosed as 'infected' (Whipps *et al.*, 2006; Kocan *et al.*, 2011). Despite this drawback the advantage of PCR makes it an excellent tool for the diagnosis of fish diseases and is a highly specific tool to detect *I. hoferi* infections (Hamazaki *et al.*, 2013a). With advances in molecular biology, real-time quantitative PCR (qPCR) has become a routine technique for pathogen detection and surveillance and when compared to conventional PCR, qPCR possesses added benefits of increased diagnostic and analytical sensitivity and allows for parasite DNA quantification (White *et al.*, 2013).

When comparing the accuracy of one diagnostic test to other tests using field samples, it is important to acknowledge that a diagnostic test is rarely 100 % accurate and that false positive and false negative results do occur. It should also be acknowledged that the

prevalence levels recorded can only be related to the detection method used and should only be used as an estimate of infection prevalence in the subsample of fish surveyed (Rahimian and Thulin, 1996; Kocan *et al.*, 1999b, 2011; Mcvicar, 1999; Halos *et al.*, 2005; Hamazaki *et al.*, 2013). Kocan *et al.* (2011) confirmed that the differences observed between the evaluations of the same tissues may result from an uneven distribution of the parasite within the host tissues when they observed the schizonts in herring (*Clupea harengus*) skeletal muscle. The schizonts were either randomly distributed throughout the white muscle, occurred in clumps or were concentrated in the dark muscle under the skin. This concluded that the wide variability of parasite distribution within the same tissue of a single host contributes to the variable differences in sensitivities observed between the different diagnostic techniques. Because of the discrepancies in parasite detection sensitivity between different diagnostic techniques and the importance in obtaining accurate prevalence for epizootiologic studies, it would be beneficial to evaluate the comparability of the several diagnostic techniques for detecting *I. hoferi* infection in fish populations before and after homogenization of the same tissue. Homogenization is a process whereby a sample is brought to a state such that all fractions of the sample are equal in composition, meaning that when removing some of the sample it does not alter the overall molecular make-up and is identical to the fraction being removed. In practice the choice of method is based on considerations such as logistics of sampling, specimen handling, cost and speed of diagnosis, and the objectives of the assessment (Mcvicar, 1999).

1.7) Validation of diagnostic tools

Validation is a concept that focuses on the evaluation of a diagnostic tool to ensure that the technique and its application is practical and suitable when applied for its specific purpose (Hiney and Smith, 1998; Walker and Subasinghe, 2000; OIE, 2016). The development and validation of an assay is an incremental process (Hiney and Smith, 1998; OIE, 2016). The World Organisation for Animal Health (OIE) manual of diagnostic tests for aquatic animals highlights specific criteria for assay development and validation and describes a 4-stage validation pathway to assess a tests "fitness" for its intended purpose. These stages define the analytical characteristics, the diagnostic characteristics, the reproducibility and implementation of the technique under validation. When evaluating the validity of a technique, examining its qualitative and quantitative ability and reliability is the ultimate consideration of assay validation to accurately predict the infection or exposure status of

the animal or population of animals (Hiney and Smith, 1998; OIE, 2016). Qualitative tests look at the specificity of the technique. Analytical specificity refers to the ability of the assay to correctly identify the right target organism, rather than other non-target organisms in a sample. Diagnostic specificity can be defined as the ability of a technique to detect only the organism of interest in a complex mixture of non-target organisms. Quantitative criteria look at the analytical sensitivity of the assay. Analytical sensitivity represents the ability of an assay to accurately measure the smallest amounts of substance present in a sample often referred to as the lowest detection level. Diagnostic sensitivity is the percentage of samples that are identified by the assay as positive for a specific disease (Saah and Hoover, 1997). Validation of a technique for specificity and sensitivity is important to assist with the interpretation of the data where false positive and negative results have been obtained. Reliability and whether a test can produce similar results when repeated is also an important characteristic of any detection technique, to ensure that the researcher can be confident that the value of the measure in the given case is accurate.

The framework proposed in Hiney and Smith (1998) shows that for validation of PCR-based techniques three major criteria at four levels of experimental complexity should be evaluated. The three criteria cover the qualitative, the quantitative, and reliability of the technique for the different levels of experimental complexity, namely: *in vitro* studies; studies of seeded matrices; studies of incurred matrices and analysis of naturally occurring environmental samples which dictate field studies. As one progresses from one experimental complexity to the next, there is a loss in the level of control of some parameters because at each level of complexity, the matrix type is different. *In vitro* studies are those performed in the traditional 'test-tube' matrix. In these studies all parameters are under the researchers control and the basic properties of a technique can be established. Seeded studies are those performed in a matrix designed to be a sterile simulation of environmental conditions. In seeded studies known concentrations of laboratory grown cultures of the target organism are introduced into selected sterile matrices. Studies in seeded matrices provide information concerning the impact of physical and chemical properties of the matrices themselves on the basic properties of a technique. Incurred studies are those performed in a matrix composed of non-sterilised samples of naturally occurring environments. In incurred studies, laboratory grown cultures of the target organism are introduced to the matrix and allowed to establish themselves within the matrix before any analyses are attempted. These studies provide information concerning the possible impact of physiological changes in the target cell on the performance of a

technique. The final level of the framework is the non-sterile field study which evaluates the performance of the technique in a naturally infected environment.

Transparent reporting of key elements of diagnostic accuracy studies for infectious diseases in cultured and wild aquatic animals prevents the introduction of biases and inappropriate interpretation or generalization (Gardner *et al.*, 2016; Laurin *et al.*, 2018). Gardner *et al.* (2016) provided a checklist for reporting of animal diagnostic accuracy studies (STRADAS-aquatic) for finfish, crustaceans, and molluscs. The STRADAS-aquatic checklist, was developed and refined by an expert group of 14 transdisciplinary scientists with experience in test evaluation studies using field and experimental samples, in operation of reference laboratories for aquatic animal pathogens, and in development of international aquatic animal health policy. Laurin *et al.*, 2018 also identified and addressed important metrics for consideration at the design phase of validation that should be taken into consideration. With the advancement in molecular diagnostic techniques, in particular PCR-based techniques, several other factors need to be considered during validation (Hiney and Smith, 1998; Bott *et al*., 2010; OIE, 2016). These factors include an effective and repeatable extraction method of genomic DNA from environmental samples, the identification of an appropriate DNA marker that is specific to the DNA of the target organism, and avoiding PCR inhibition and false positives caused by contaminants or non-optimal molecular methods (Walker and Subasinghe, 2000; Bott *et al.*, 2010; OIE, 2016). The minimum information for the publication of real-time quantitative PCR experiments (MIQE) guidelines by Bustin *et al.* (2009) reports on a reference framework to promote transparency of experiments and a blueprint for good PCR assay design to follow to ensure unambiguous reporting of real-time quantitative PCR experiments. For appropriate interpretation of test results for presumptive diagnosis, confirmation of clinical disease, targeted surveillance for risk assessment and epidemiology studies, these validation and standardisation criteria should be considered. A test that is carefully optimized and standardized through the accrual of validation data provides confidence in the assay's results and ability to perform during the intended purpose. The World Organisation for Animal Health (OIE) manual of diagnostic tests for aquatic animals also makes mention of the continuous monitoring of assay performance and the maintenance of validation criteria during routine use of the assay to ensure that assay results are valid.

1.8) Study objectives

The primary aim of this study is to evaluate the current diagnostic techniques to detect *I. hoferi* in fish tissue samples and to provide preliminary epizootiologic information of *I. hoferi* in South Africa. The following objectives were investigated to achieve this:

1. Development of a physical enumeration technique to be able to enumerate *I. hoferi in vitro* to allow for standardization of real-time qPCR.
2. Developing and optimizing a real-time qPCR assay based on the criteria proposed by Hiney and Smith (1998) and Bott *et al.* (2010) and which followed MIQE guidelines.
3. Evaluating the various diagnostic techniques in detecting *I. hoferi* in fish tissue samples and evaluating whether *in vitro* culture and qPCR assays ability to detect low concentrations of *I. hoferi* in the biological samples (test sensitivity) is improved after the homogenization of tissue samples.
4. To preliminary assess the natural prevalence and geographical distribution of *I. hoferi* in infected hosts along South Africa to identify risk assessment for commercially important fisheries through the evaluation of certain pelagic species that are used as aquaculture feed.

CHAPTER 2

Direct counting method to quantify laboratory grown *Ichthyophonus hoferi*

Abstract

Enumeration of cells is important for standardizing cellular concentrations to minimize error and variation in downstream results and to ensure experimental reproducibility and accuracy. In this study we report a simple direct counting method to accurately estimate *Ichthyophonus hoferi* concentrations *in vitro.* The method involved the microscopic analysis of 100 µL of cell suspension from a homogenous sample pipetted onto a glass slide and covered with a 50 x 22 mm coverslip with the entire area under the cover slip being analysed and the enumeration of *I. hoferi* recorded as schizonts.mL^{-1}. The validation protocol applied was designed to evaluate accuracy, recovery and repeatability of the method. The growth of laboratory grown cultures of *I. hoferi* at 15 ˚C pH 7 was then assessed using the method as described. We recorded the lowest detectable limit as 8.6292 schizonts.mL^{-1}, however the lowest detectable limit can be further decreased through further replication. The test was able to accurately determine the range of dilution on the study and fitted with a linear model within the studied ranges (150, 135, 120, 105, 90, 75, 60, 45, 30 and 15 schizonts.mL^{-1}) with an R^2 value of 0.9918 and met the assumptions for parametric testing. When counts of cultures prepared in media were compared to cultures in homogenized tissue at low, medium and high concentration standards (100, 500 and 1000 schizonts.mL^{-1}), the results demonstrated that schizont concentrations in homogenized tissue measured by the enumeration method closely matched those concentrations in media. Repeatable measurements were achieved with an average percent coefficient of variation (CV) increasing at lower schizont concentrations. Growth of *I. hoferi* showed a linear growth curve from day 0 to day 12 with a predictable sequence of development. The method described in this paper is repeatable and is intended that this assay could be utilised for determining concentration of cultures, and therefore standardizing sample preparations for downstream applications such as qPCR to allow the analytical sensitivity of qPCR to be determined by the production of a standard curve based on known schizont concentrations.

2.1) Introduction

Studies that require accurate and consistent numbers of input microorganisms such as those analysing growth and longevity (Meyrath, 1963; Meletiadis *et al.*, 2001; Melgar *et al.*, 2013) or those requiring the seeding of microbes for subsequent experiments (Phister and Mills, 2003; Mendonca and Arkush, 2004; Phister *et al.*, 2007), require enumeration prior to beginning the experiment to allow standardization of microbial concentrations between samples. Standardizing and obtaining consistent concentrations ensures experimental reproducibility and accuracy and aids in minimizing variation between samples and in downstream results (Doyle and Griffiths, 1998; Petrikkou *et al.*, 2001; Adams and Thompson, 2011; Sandle, 2015).

While methods exist to enumerate a range of microorganisms (Bowden, 1977; Marie *et al.*, 1999; Maruyama and Sunamura, 2000; Yokomaku *et al.*, 2000; Kaminskyj, 2008; Steinberg *et al.*, 2012; Cadena-Herrera *et al.*, 2015) and of the several diagnostic techniques that have been employed to detect *Ichthyophonus hoferi* (Paperna, 1986; Rahimian and Thulin, 1996; Whipps *et al.*, 2006; Kocan *et al.*, 2011; Hershberger, 2012; White *et al.*, 2013), some attempts have been made to quantify *I. hoferi* (Franco-Sierra and Alvarez-Pellitero, 1999; Whipps *et al.*, 2006; Kocan *et al.*, 2011; Friedman *et al.*, 2014). To evaluate parasite density in skeletal muscle, Kocan *et al.* (2011) quantified *I. hoferi in situ* by counting the total number of PAS-positive *I. hoferi* schizonts, as well as their distribution in Pacific herring (*Clupea harengus*). Whipps *et al.* (2006) estimated the number of parasite spores in infected heart tissue histologically to determine the lowest limit of PCR as 10^{-5} spores. White *et al.* (2013) provided a qPCR assay to quantify *I. hoferi* and identified the lowest limit of detection (analytical sensitivity) using plasmid vectors as 1 copy per reaction. Although these methods attempted to quantify *I. hoferi*, no physical enumeration method was used to quantify *I. hoferi in vitro.* Franco-Sierra & Alvarez-Pellitero (1999) however evaluated the growth of *I. hoferi* at various pH conditions (3, 5, 7 and 9) as colony forming units per milliliter ($CFU.mL^{-1}$). Although the growth in $schizont.mL^{-1}$ was assessed by inoculating the cultures grown in MEM-10 to solid media (Earle's fish saline agar), no definitive description on quantification was explained.

Of the current diagnostic methods, Kocan *et al.* (2011) describes that pathogen culture from infected tissues is the diagnostic standard for assessment of *I. hoferi* infection prevalence. Employing pathogen culture however is not feasible when samples are collected at remote

field sites and short time of diagnosis is required. In light of this Whipps *et al*. (2006) and Hamazaki *et al*. (2013a; b) have discussed the the equivalency of PCR to culture. Whipps *et al*. (2006) reported that sensitivity and specificity of PCR when evaluating heart muscle on infected Chinook salmon (*Oncorhynchus tshawytscha*) both exceeded 90 % (range 90.9 – 100 % and 91.2 – 98.9 % respectively) but that sensitivity of PCR decreased (range between 50 and 100 %) in samples with low pathogen loads or low infection intensity, suggesting that PCR can underestimate the prevalence of *I. hoferi* infections (Kocan *et al*., 2011). Hamazaki *et al*. (2013a) however clarified the results of Whipps *et al*. (2006) by re-examining their data and assessed the accuracy of PCR in diagnosing *I. hoferi* infection with a larger sample size. Hamazaki *et al*. (2013a) reported that PCR is equivalent to or could be more accurate than pathogen culture for diagnosing *I. hoferi* in infected tissue.

Although Hamazaki *et al*. (2013a) have suggested that PCR is comparable to culture in detecting *I. hoferi*, only the diagnostic sensitivities of the assays have been compared and not their analytical sensitivities. Diagnostic sensitivity is the likelihood (expressed as percentage) of a positive result occurring from those known to have the disease (Saah and Hoover, 1997). Analytical sensitivity is the detection limit of the assay, that is the smallest amount of the analyte that an assay can accurately measure (Adams & Thompson, 2011; Saah and Hoover, 1997). Standardizing concentrations will allow the analytical sensitivity of PCR to be determined by running the PCR on extracted DNA from cultures with known concentrations. The objective of this study was to identify a method to enumerate *I. hoferi in vitro* to be able to standardize cellular concentrations for downstream applications applied further in this dissertation. In this study we report on a simple reliable counting method to enumerate *I. hoferi in vitro* based on direct counting of the organism under a microscope slide. This method was validated based on linearity, accuracy and repeatability and the repeatability of the test was further assessed against enumeration using a hemocytometer, Mc Masters counting chamber and image analysis to highlight the suitability of the method to accurately quantify *I. hoferi in vitro* for further application in this dissertation.

2.2) Materials and Methods

2.2.1) Microorganisms and culture media

Ichthyophonus hoferi was cultured from the infected liver of euthanized Cape White Stumpnose, *Rhabdosargus globiceps* at the Two Oceans Aquarium in Cape Town South Africa. The fish were captured, euthanized with an overdose of 2-phenoxyethanol and laid on ice before being necropsied. The euthanasia of these fish was part of the aquarium's disease control strategy which was conducted by their animal health team under the supervision of their veterinarian. Liver tissue (≈ 0.5 g) was aseptically excised from the infected fish and cultured according to Franco-Sierra & Alvarez-Pellitero (1999), Hershberger (2012) and Okamoto *et al*. (1985). *I. hoferi* infected tissue (≈ 0.5 g) was inoculated into tubes containing 5 mL HEPES-buffered (pH 7) Eagles minimum essential medium (Sigma Aldrich, Cat# M0268) supplemented with 10 % foetal bovine serum, 100 IU.mL^{-1} penicillin (Sigma Aldrich, Cat# P3032), 100 µg.mL^{-1} streptomycin sulphate (Sigma Aldrich, Cat# S9137), and 100 µg.mL^{-1} gentamicin sulphate (Sigma Aldrich, Cat# G1264). Cultures were maintained at 15 ˚C for 14 days and subsequently examined microscopically for the presence of *I. hoferi* schizonts and hyphae using 200 X magnification (20 X objective with 10 X ocular) with an Olympus BX51 microscope with phase contrast. Cultures were maintained by subculturing 1 mL of the culture into new media every 14 days as described above.

2.2.2) Enumeration of I. hoferi *cultures*

Cultures (obtained from 2.2.1) were vortexed briefly, homogenized using a hand held pellet pestle (Sigma Aldrich, Cat# Z359971) for 1 min 30 seconds and vortexed again to distribute *I. hoferi* evenly. Before the cells had a chance to settle, 100 µL of cell suspension from each sample was pipetted onto a glass slide and covered using a 50 x 22 mm coverslip. Care was taken to ensure that the culture filled the entire surface under the coverslip resulting in a uniform film of ~10 µm thick between the slide and the coverslip. Slides were examined using an Olympus BX 51 compound microscope and assessed at 200 X magnification (20 X objective with 10 X ocular) using phase contrast. Assessment of the slides involved first placing the objective of the microscope directly over the top left corner of the cover slip before moving down the cover slip to complete its first pass. The slide was then moved slightly over to the right and counts again were recorded as movement up the

cover slip followed. This process was repeated until the entire coverslip had been examined. Spherical schizonts (Fig. 2.6.A), spherical schizonts with hyphae (Fig. 2.6.B - 2.8.F) and spherical schizonts containing hyphae displaying the rounding up of the tips (Fig. 2.6.E - 2.6.F) were counted. Schizonts with budding hyphae were enumerated as two or separate schizonts as they could give rise to separate colonies (Okamoto *et al.*, 1985; Franco-Sierra and Alvarez-Pellitero, 1999). The overall concentration was determined by averaging the total number of schizonts counted (schizonts per 100 µL) multiplied by 10 to obtain a concentration of schizonts.mL^{-1}.

2.2.3) Validation of I. hoferi *culture enumeration method*

The validation protocol was designed to evaluate accuracy, recovery and repeatability of the *I. hoferi* culture enumeration method described above (see 2.2.2).

2.2.3.1) Enumeration accuracy

The accuracy of an analytical method is the degree of agreement of test results generated by the method to the true value and is measured by spiking the sample matrix of interest with a known concentration of analyte standard and analysing the sample using the method being validated (Rao, 2018). Enumeration efficiency was evaluated using concentration standards prepared from four independent stock cultures (see 2.2.1). Stock cultures were enumerated and concentration standards of ≈ 150, 135, 120, 105, 90, 75, 60, 45, 30 and 15 schizonts.mL^{-1} were prepared by diluting the stock cultures in growing media. Ten samples from each concentration standard (10 cell concentrations from each of the four independent stock cultures) were prepared and analysed. The results for the enumeration of the ten replicates were averaged for each independent stock culture at each respective cell concentration. The average schizont concentration for each independent culture were plotted by linear fit against the expected concentrations in the range mentioned above. Intercept, slope and correlation coefficient values were reported.

2.2.3.2) Recovery

The recovery of an analyte in an assay is determined when a known amount of analyte is spiked into a natural biological matrix and its response is measured (recovered) in the assay by comparison to an identical spike of the analyte in in the standard diluent (Andreasson *et*

al., 2015). The recovery of the *I. hoferi* culture enumeration method was assessed at low, medium and high concentration standards prepared in media as described above and in media containing homogenized uninfected *Rhabdosargus globiceps* liver tissue. For the latter, homogenized liver tissue of an uninfected *R. globiceps* was used at concentration of 0.15 g.mL^{-1}. Schizonts were enumerated from four independent *I. hoferi* cultures and concentration standards of 100, 500 and 2000 schizonts.mL^{-1} were prepared in triplicate by diluting the cultures in their corresponding media. Five samples from each concentration standard (3 schizont concentration standards from four independent *I. hoferi* cultures) were prepared and analysed. Mean recovery at each concentration was determined by relating the average schizont concentration values (schizonts.mL^{-1}) between samples achieved in media prepared in 2.2.1 to average schizont.mL^{-1} values achieved in homogenized tissue. Mean recovery was expressed as (%) ± S.D.

2.2.3.3) Repeatability

Repeatability was evaluated as the average percent coefficient of variation (CV) among sample counts. CV was determined for 2.2.3.1 and 2.2.3.2 using the following calculation where 'mean sample count' was the average schizonts enumerated per sample and SD was the standard deviation between the sample counts: $\% CV = \frac{SD}{mean\ sample\ count}\ x\ 100$. Repeatability of the method mentioned in 2.2.2 was also compared to other methods of quantification, namely using the hemocytometer, the Mc Masters counting chamber and Image analysis. Six independent samples of one another were enumerated for 5 counts each. Shown are the average % CVs per method. Below explains each of the methods in detail.

2.2.3.3.1) Hemocytometer

Twenty microliters of homogenized sample as described in 2.2.2 were loaded into each chamber of the hemocytometer. Counts were performed by triplicate by one analyst under 200 X magnification using an Olympus BX 51 compound microscope according to the standard methodology (Laing, 1991).

2.2.3.3.2) Mc Masters counting chamber

One millilitre of homogenized sample as described in 2.2.2 was used to fill the Mc Masters chamber by pipetting the suspension using capillary action (Boyne *et al.*, 1957). The suspension in the counting chamber was then assessed at 200 X magnification using an Olympus BX 51 compound microscope.

2.2.3.3.3) Image analysis

One hundred microliters of homogenized sample as described in 2.2.2 was pipetted onto a 50 x 22 mm glass slide. Two drops of 5 % Giemsa (Sigma Aldrich, Cat# GS500) were added to the slide and left for 2 min before being covered with a coverslip (Fig. 2.6.H). Slides were examined using a Nikon eclipse N1 compound microscope fitted with a Nikon Digital sight DS-U3 camera system and interfacing with NIS-Elements BR software. Each slide was assessed using five random fields of view at 100 X magnification. The image analysis method was adopted from Meijering and Cappellen (2006), Ollivro *et al.* (2012) and Papagianni (2014). The digital images of each field of view were enhanced by adjusting the exposure settings on the NIS-Elements software to generate a good contrast of the sample against the background. Clean-up functions on the NIS-Elements software were also utilized to remove background noise. Using the RGB colour space, NIS-Elements segmented the digital image and created a digital binary image. The digital binary image was then used to manually measure the area *I. hoferi* occupies within each field of view by utilizing the various manual measuring tools. The results were recorded simultaneously to a simple statistics table which were later exported to a Microsoft Excel file. The area calculated represented the total volume the parasite occupied in 100 µL of media. Schizont diameters were also measured with the use of image analysis.

2.2.4) Growth of laboratory grown cultures of I. hoferi *at 15 ˚C pH 7*

Growth was assessed using the method described in 2.2.2 on day 1, 3, 9, 15, 21, 27 and their duplicates on days 6, 12, 18, 24, 30. These duplicates were assessed on respective days to avert any inaccurate readings due to sample volume declines caused from counting. Each culture was inoculated into media prepared as described in 2.2.1 to obtain a final concentration of 10, 100, 500 and 1000 cells.mL^{-1}. Cultures were maintained at 15 °C and growth was assessed on days as above post inoculation. Five replicates were carried out

per sample and four replicates were carried out per concentration. Growth was evaluated as the increase in the number of schizonts.mL^{-1}. Cultures were quantified until a plateau of growth was reached (i.e., until no new schizonts become available within the tubes). Mean and standard errors were recorded.

2.2.5) Statistical analysis

To ascertain whether the data conform to the assumptions of linear regression in 2.2.3.1; linearity, homoscedasticity, normality and independency were tested. Linearity of the data was confirmed from a scatter plot of the data; homoscedasticity and independency was confirmed using a residual plot to ensure the random scattering of points and normality was confirmed using a QQ-Plot showing data fit to line.

2.3) Results

2.3.1) Enumeration efficiency

The schizont concentration curve obtained from enumerating the dilution series prepared from stock cultures (as described in 2.2.2), fitted with a linear model within the studied ranges (concentrations of 150, 135, 120, 105, 90, 75, 60, 45, 30 and 15 schizonts.mL^{-1}) (Fig. 2.1). The R^2 value for the concentration curve was 0.9918 and the slope 0.8849. Using the linear regression model equation, the lowest detectable limit for the method is 8.6292 schizonts.mL^{-1}.

2.3.2) Recovery

The results shown in Table 2.1 and Fig. 2.2 demonstrate that schizont concentrations in homogenized tissue measured by the enumeration method closely matched those concentrations in media. The mean recovery percentage for samples over the low, medium and high dilutions for all 5 samples are displayed in Table 2.1. According to the results the recovery of *I. hoferi* was lower at lower schizont concentrations.

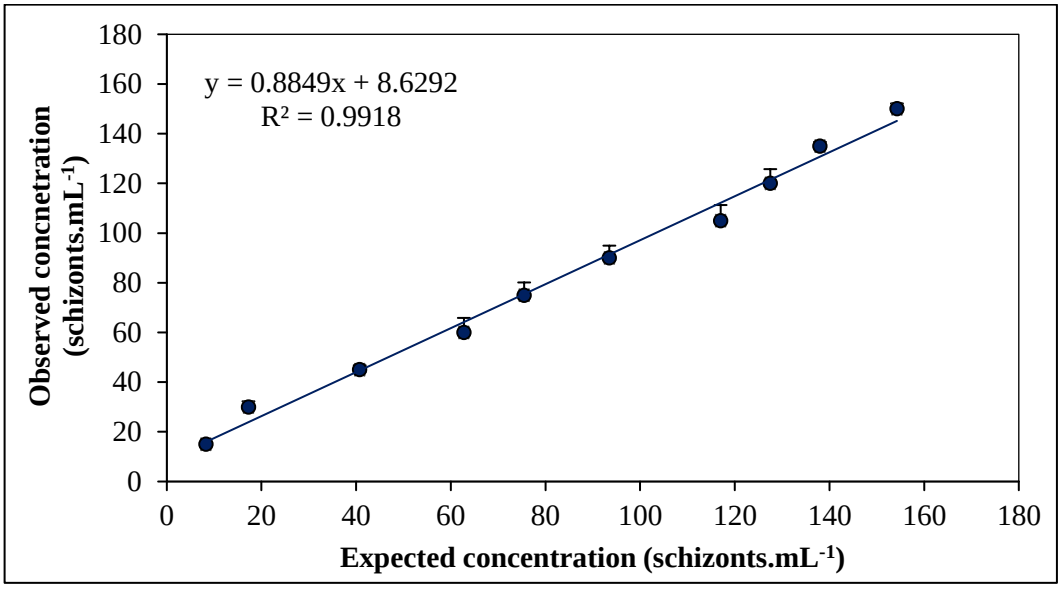

Fig. 2.1: Concentration linearity obtained for *I. hoferi* schizonts.mL^{-1} by the direct counting method to quantify laboratory grown *I. hoferi*. Each point indicates the mean ± SD of 10 counts from 4 independent samples.

Table 2.1: Recovery values at concentration standards 100, 500 and 2000 obtained by the direct counting method to quantify laboratory grown *Ichthyophonus hoferi*. Mean recovery at each concentration was determined by relating the average schizonts.mL^{-1} values between samples enumerated in media to average schizonts.mL^{-1} values enumerated in uninfected homogenized tissue. Mean recovery is expressed as (%) ± S.D. Values represent the average of four sample replicates with 5 counts per sample. CV between replicates is indicated in brackets and expressed as percentage.

Concentration standards (schizonts.mL^{-1})	**Quantified in standard media**	**Quantified in homogenized tissue**	**% Count recovery**
100	100.8 ± 21.9 (21.7)	98.0 ± 21.5 (21.9)	97.2 ± 1.0
500	494.9 ± 42.2(8.5)	486.0 ± 45.0 (9.3)	98.2 ± 1.1
2000	2026.4 ± 101.7 (5.0)	2030.1 ± 143.6 (7.1)	100.2 ± 1.4

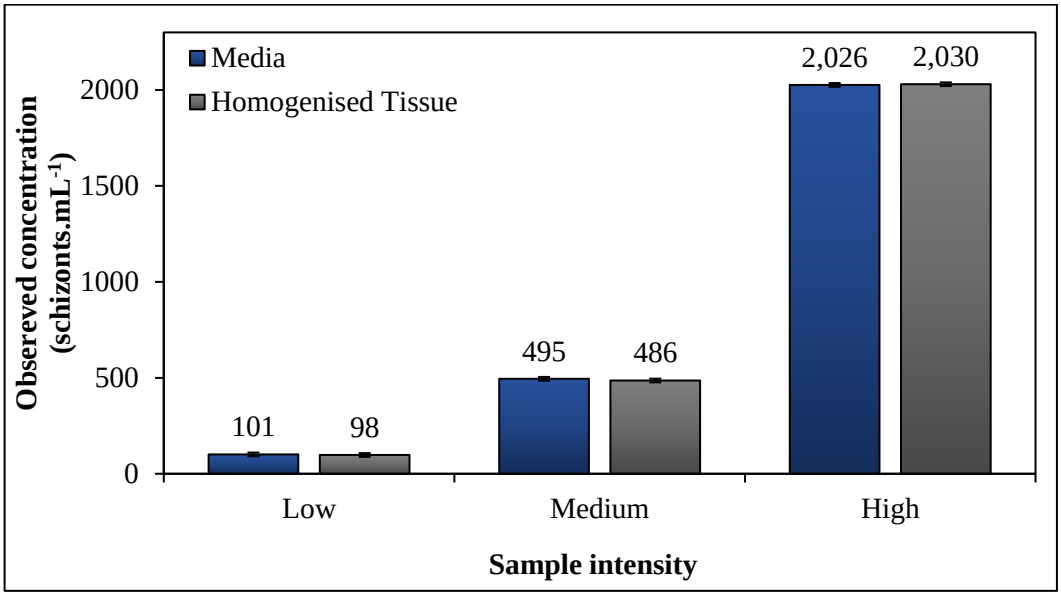

Fig. 2.2: Recovery of *I. hoferi* schizonts.mL^{-1} counted by the direct counting method to quantify laboratory grown *I. hoferi*.in media and homogenized tissue.

2.3.2) Repeatability

The average CV among sample counts for linearity was observed to increase as dilution increases (Table 2.2). Among sample counts in 2.2.2.2 the CV for samples quantified in media were 21.7 %, 8.5 % and 5.0 % for concentration standards 100, 500 and 2000 schizonts.mL^{-1} respectively. For samples quantified in homogenized tissue the average CV values were 21.9 %, 9.3 % and 7.1 % for concentration standards 100, 500 and 2000 schizonts.mL^{-1} respectively. Quantifying *I. hoferi* using the enumeration method had the highest repeatability between sample counts with an average CV value of 22.5 %, when compared to quantifying using the hemocytometer, Mc Masters counting chamber and the image analysis method described in 2.2.3.3 (Fig. 2.3).

2.3.3) Growth of laboratory grown cultures of I. hoferi *at 15^{o}C at pH 7 for 30 days*

I. hoferi grown at conditions described as above at different concentrations showed a typical sigmoid population growth curve for medium and high starting schizont concentrations (Fig. 2.4). After an initial lag phase (Day 0 – 3), the *I. hoferi* cultures grew exponentially until around day 9 where after population growth started decelerating and reached a stationary phase after 12 days at both medium and high starting concentrations.

Table 2.2: The percentage coefficient of variation (CV) for 4 homogenous *I. hoferi* culture samples at reducing schizont concentrations. Ten counts were done per sample (n=400). Recorded is average concentration ± S.D.

Dilution %	Observed schizonts.mL^{-1}	% C.V
Neat	154.3 ± 4.4	2.8
10	138.0 ± 3.4	2.4
20	127.5 ± 11.5	9.0
30	117.0 ± 12.56	10.7
40	93.5 ± 9.9	10.6
50	75.5 ± 10.3	13.6
60	62.8 ± 11.7	18.6
70	40.8 ± 3.6	8.8
80	17.3 ± 4.6	26.5
90	8.3 ± 1.7	20.7

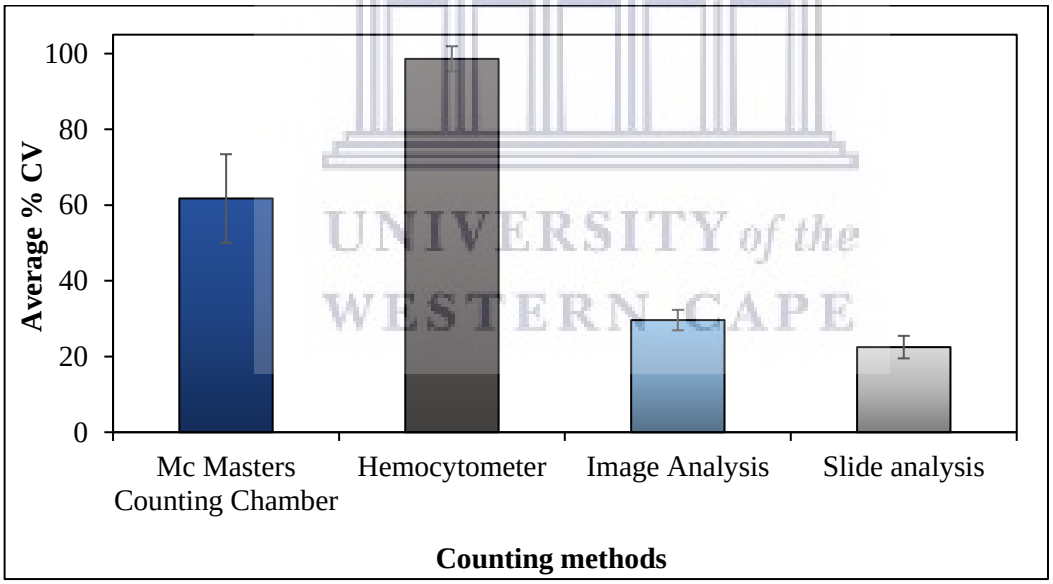

Fig. 2.3: The percentage coefficient of variation (CV) observed for each counting method. All the samples are independent of one another. Shown are the average % CVs.

Growth was mainly observed as spherical schizonts (Fig. 2.6.A), varying in size. Limited hyphenation was observed with hyphae usually branching out twice developing from spherical multinucleate schizonts (Fig. 2.6.B – 2.6.F). The development of hyphal tubes occurred from distension of the thick walled spherical multinucleate bodies throughout day 3 – day 15 (Fig. 2.6.B – 2.6.D). Development typically proceeded by the cytoplasmic contents of the spherical body flowing up the hyphae to accumulate in the tips, with subsequent rounding up varying in size (Fig. 2.6.E). Around day 21 dissolution of hyphae were observed (Fig. 2.6.F). The size of the multinucleated schizonts measured (n= 319) varied and had a mean diameter of 80.7 ± 41.5 µm (range of 14.5 – 270.4 µm) (Fig. 2.5).

2.4) Discussion

In the present study we report on a simple enumeration technique to quantify *I. hoferi in vitro.* The method involved microscopic analysis of 100 µL of cell suspension from a homogenous sample pipetted onto a glass slide and covered with a 50 x 22 mm coverslip with the entire area under the cover slip being analysed and the enumeration of *I. hoferi* recorded as schizont.mL^{-1}. The results shown in Fig. 2.1 demonstrate that concentrations recorded closely match theoretical cell concentrations (R^2 = 0.9918). The degree of linearity demonstrates that the method is a reliable method to quantify *I. hoferi*, across a wide linear operating range (Fig. 2.1 and Table 2.2). The lower recovery data and higher CV recorded in in low schizont concentration samples in Table 2.1 and Table 2.2 respectively display a loss in recovery and accuracy of the method when compared to higher concentrations. The linear regression model determines the minimum detectable concentration of the method as 8.6292 schizonts.mL^{-1}. This suggests that there is error in the method and that the enumeration method slightly overestimates the actual concentration. The method however is used to determine stock concentrations and so in effect measurements are not independent from stock concentrations and variations between samples can be carried through. It is noted that the lowest detectable limit can be further decreased through further replication. Since every schizont in the 100 µL volume on the slide is counted, minimal mathematical adjustments are required to estimate the cell concentration. Furthermore testing the assay when samples were spiked in non-sterile conditions demonstrated that there was minimal interference between and within sample counts when compared to samples enumerated in media (Table 2.1 and Fig. 2.2), confirming that the physical and chemical properties of homogenized tissue has minimal interference on the performance on the direct counting method to quantify laboratory

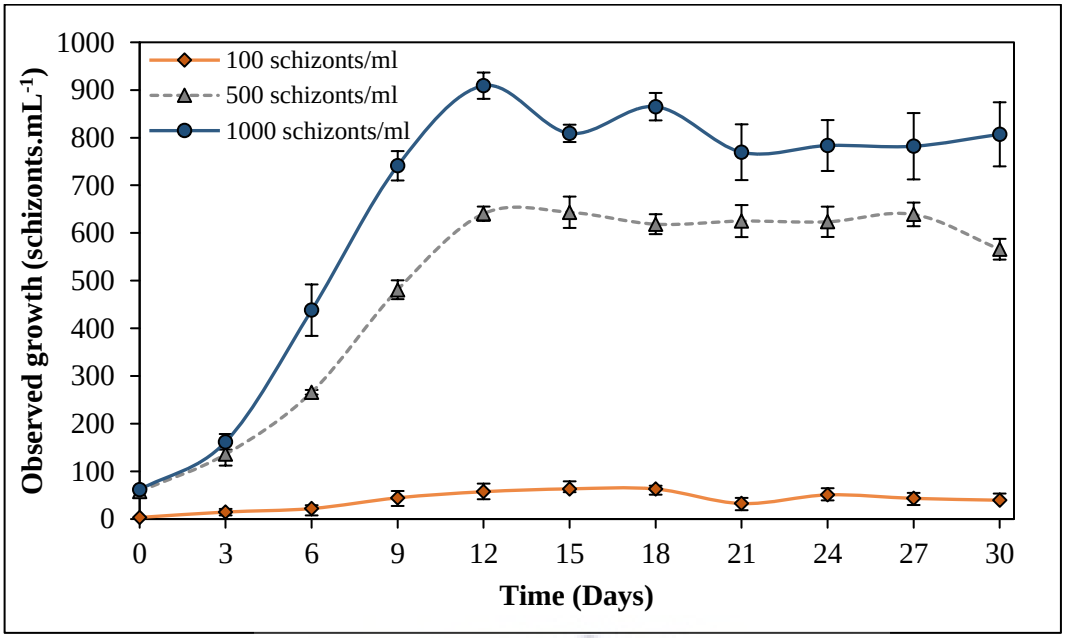

Fig. 2.4: Sigmoid population growth curve for *I. hoferi* cultures at 15 °C at pH 7. Points indicate the mean ± SD from 4 replicates.

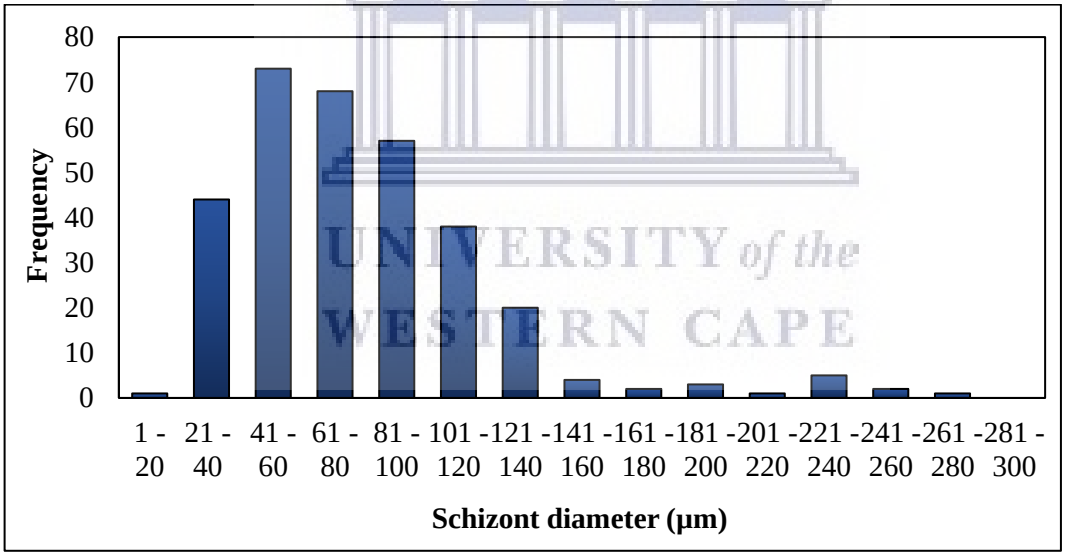

Fig. 2.5: Frequency occurrence of schizont diameter *in vitro* based on the measurement of 319 schizonts. Mean diameter of 80.7 µm with standard deviation of 41.5 µm. These data represent measurements from numerous independent cultures.

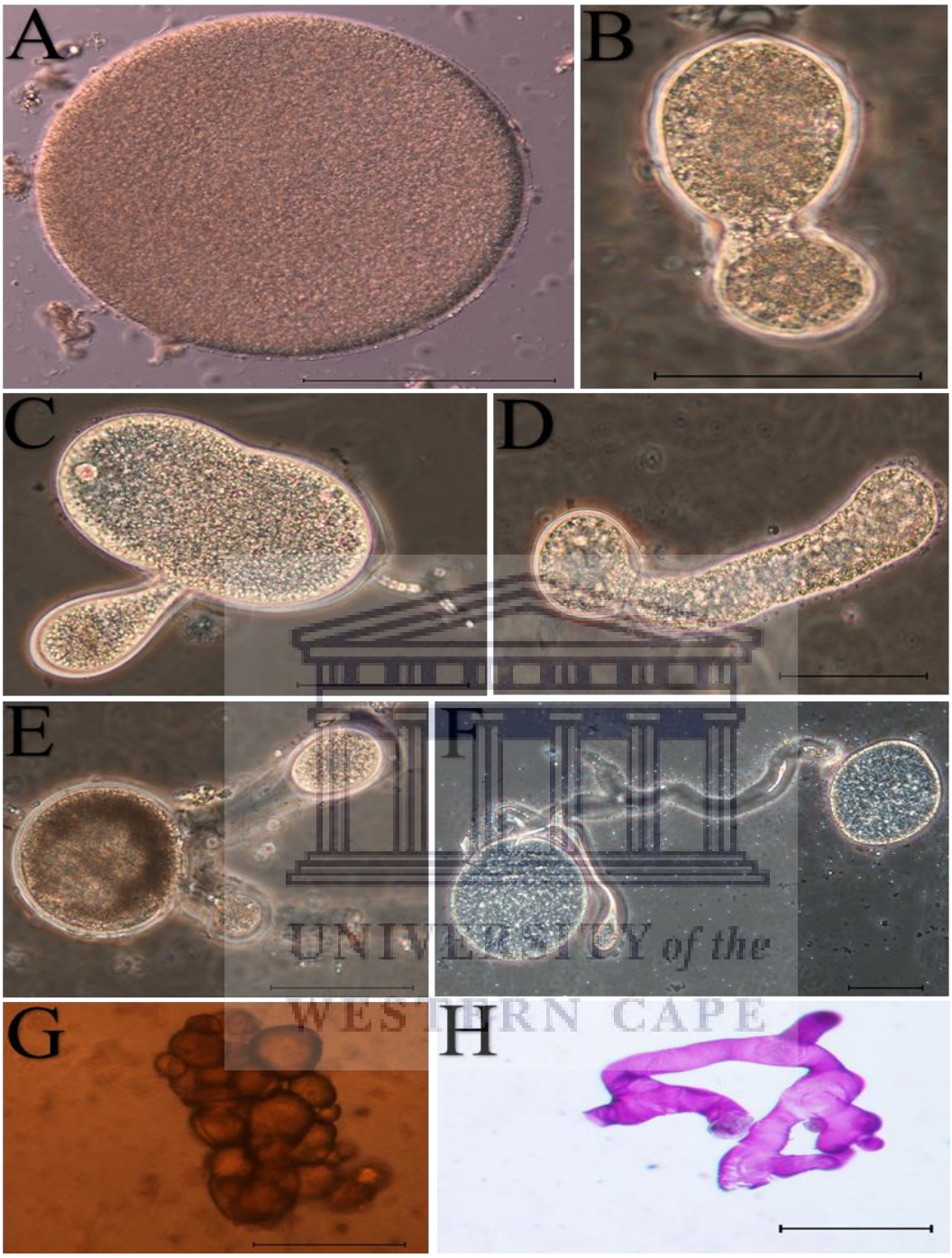

Fig. 2.6.A - 2.6.F: *Ichthyophonus hoferi* stages cultured *in vitro* in Eagle's minimum essential medium (EMEM) at 15 °C at pH 7. A: The multinucleated spherical body or schizont of *I. hoferi*. Note the thick non-cellular multilaminate membrane. B, C & D: Cultures after 6 days. The initiation of hyphal growth of *I. hoferi* from a multinucleate spherical body. E: Cultures after 15 days. The cytoplasmic contents protruding through wall migrating towards the apex. F: Cultures after day 21, dissolution of hyphae. G: Aggregated *I. hoferi* cells viewed using a Mc Masters counting chamber. H: Giemsa stained hyphae. Scale bar: 100 µm

grown *I. hoferi*. The enumeration method was repeatable with CV increasing as concentration decreases. The precision of counting using this method therefore increases as the cell concentration increases (Table 2.1 and Table 2.2). The observed differences within sample counts and those between replicates could be partly attributable to the aggregation of schizonts causing an uneven distribution in the sample.

When comparing the enumeration method to other enumeration techniques in this study, the method displayed smaller average coefficients of variation (Fig. 2.3). The sources of variation in the hemocytometer was caused by the starting volume whereby the quantification of a single schizonts within the 10 µL lead to a final concentration of 2500 cells.mL^{-1}, demonstrating its limited operating range. Since only schizonts localized to a specific field were enumerated this resulted in larger variability between recorded measurements when concentrations in the samples were low. The variation obtained from the Mc Masters counting chamber were obtained due the thickness of the Perspex plastic and large volume of suspension reducing the amount of transmitted light passing through the sample. This created difficulties in identifying small multinucleate spores and ability in differentiating aggregated samples. Although the lowest detectable limit of the method was low, the one millilitre volume required to fill the chamber restricted the analyses of samples in very small volumes or samples with very small numbers. The variation between sample counts using image analysis were less than the counting chamber techniques, with a CV of 29.62 % (Fig. 2.3). Since image analysis is the extraction of meaningful information from digital images, the images were sometimes found to be complex and noisy and consequently required appropriate multiple image processing steps for the extraction of meaningful quantitative information.

The enumeration method described in this paper as a qualitative test was able to recognize the morphological characteristics of *I. hoferi* (Fig. 2.6). When assessing the growth of laboratory grown cultures of *I. hoferi*, we found a predictable sequence of development similar to that observed by Okamoto *et al.* (1985). Initially when cultures were seeded into the culture media, large resting spores began to germinate and produce hyphae with rounding up in the tips of the hyphae. This was also similar to i*n vitro* studies done by Kocan *et al.* (1999) which showed that hyphal-like structures were evident growing from the schizonts by four days in culture and by eleven days in culture spherical structures developed at the hyphal tips. The dissolution of hyphae we observed was similar to those reported by Okamoto *et al.* (1985) and Spanggaard (1995). These observations concur with

the normal developmental process following the death of infected fish (Mcvicar, 1999). This cycle was not repeated unlike Okamoto *et al.* (1985) who found continuous cycles at a constant pH. This explains the accentuated levelling out of culture numbers after day 15 - 18 (Fig. 2.4) and that manipulation of the culture medium is necessary to initiate a new cycle of development (Spanggaard *et al.*, 1994). Simulating the natural cycle conditions of *I. hoferi* by varying pH levels between 3, 5 and 7 would allow for reproducible and continual growth reported in Spanggaard *et al*. (1994) and Spanggaard (1995).

According to the results as above obtained from this work, we observed that the direct counting method is a suitable, simple and easy method requiring minimum equipment to enumerate *I. hoferi* and is successful in determining the concentration of *I. hoferi* in a sample. Determining the concentration of *I. hoferi* can aid in standardizing sample preparation for downstream applications such as qPCR to allow the analytical sensitivity of qPCR to be determined by the production of a standard curve based on known schizonts concentrations. The technique will allow the growth of *I. hoferi* at various conditions to be quantified *in vitro*. Although the method can be employed due to the quality of the results, the disadvantage of the method is the time taken to enumerate and the practice required before the method can be carried out easily.

CHAPTER 3

The development and evaluation of a real-time quantitative PCR method for detection of *Ichthyophonus hoferi* in *Rhabdosargus globiceps* fish tissue samples

Abstract

Molecular based diagnostic techniques, such as the polymerase chain reaction (PCR), are increasingly used for the diagnosis of infectious disease agents in cultured marine and freshwater fish and shellfish. Due to their high analytical sensitivity, specificity and ability for rapid detection, these techniques are favoured over conventional methods, such as histopathology and culture-based techniques, and require strict accordance to validation requirements. We developed and validated a real-time SYBR green quantitative PCR assay for species-specific detection and quantification of *Ichthyophonus hoferi* which followed MIQE guidelines. The quantitative PCR was developed for amplification of a 299 bp fragment of the small subunit ribosomal DNA gene from *I. hoferi* extracted DNA from laboratory grown cultures, as well as from spiked uninfected *Rhabdosargus globiceps* tissues. The specificity of the *I. hoferi* primer set was initially demonstrated by conducting a BLAST search on the GenBank database and only showed 100 % homology to *I. hoferi*. The specificity of the primer set was also assessed at the *in vitro* level by testing for cross-reactivity with specifically selected fungal and coccidian organisms that were available at the time of assay development. The primer set did not show cross-reactivity with any of the tested isolates and only amplified DNA from *I. hoferi*. When performing the qPCR assay in a *Rhabdosargus globiceps* tissue matrix, an environment designed to be a non-sterile simulation of environmental conditions, no amplification occurred in the negative controls or non-template control. The qPCR assay developed in this study was optimised to accurately detect as little as 0.005 schizonts in a 25 µL reaction volume. By comparing the qPCR results from two separate dilution series, one made in a sterile matrix (phosphate buffered saline) and one in liver tissue matrix, we demonstrated that compounds present in the fish tissue matrix interfere with the qPCR reaction, resulting in an overall reduction of 9 % in qPCR sensitivity. The protocol used in the present study has the potential for utilisation as a routine diagnostic tool for *I. hoferi* surveillance and management.

3.1) Introduction

Molecular based diagnostic techniques have increasingly been used for the diagnosis of infectious disease agents in cultured marine and freshwater fish and shellfish. These techniques are favoured over conventional methods, such as histopathology and culture based techniques, due to their high analytical sensitivity and specificity and rapid turnaround time, when screening for the presence of pathogen DNA (Walker and Subasinghe, 2000; Cunningham, 2002). These techniques are built on the premise that every organism contains a unique species specific DNA (or RNA) target sequence that can be used to differentiate it from closely related species. Of the available molecular based diagnostic techniques, the polymerase chain reaction (PCR) technique, first described by Mullis *et al*. (1986), has since become the most common method for routine detection and identification of pathogens in infected animals (Cunningham, 2002; Adams and Thompson, 2011; Hunt, 2011). Conventional PCR involves the amplification of a unique nucleotide sequence, specific to the infectious agent, using a single pair of oligonucleotide primers. The conventional PCR method is well established and several variations of this techniques now exist, including nested PCR and real-time quantitative PCR (qPCR) (Walker and Subasinghe, 2000; Cunningham, 2002; Adams and Thompson, 2011; Hunt, 2011). Real-time quantitative PCR (qPCR) uses fluorescently-labelled probes or DNA intercalating dyes (such as SYBR green) to directly detect and monitor the amplification of the target gene in real-time. Since qPCR uses fluorescence, the measured fluorescence can reflect the amount of amplified product in each cycle, thereby allowing for the starting amount(s) of DNA to be accurately quantified (when compared to a standard curve).

PCR, as with other methods for detection and quantification of pathogens, have potential shortcomings. PCR methodologies are susceptible to contamination and PCR assays must therefore be conducted in well managed, clean laboratories using good aseptic technique(s), as contamination during processing may result in false positive or negative results (Walker and Subasinghe, 2000). PCR cycling conditions that have not been optimised correctly and/or poorly designed PCR primers that lack specificity can cause false positive results (Bott *et al.*, 2010). Conversely, co-purification of PCR inhibitors, during the DNA extraction process, can lead to complete inhibition of the PCR, causing false negative PCR results, or an underestimation of the prevalence of the pathogen in a sample. Inhibitors can include an over-abundance of non-target DNA, organic and phenolic compounds and salts (Opel *et al.*, 2009; Bott *et al.*, 2010). False negative results can also be caused by the

incorrect choice of DNA extraction method and low pathogen intensity in the host sampled (Walker and Subasinghe, 2000; Purcell *et al.*, 2011). Furthermore since amplification will generally occur provided that the correct fragment of DNA is present, distinguishing between microorganisms that are viable and non-viable is not always possible as the test is not dependent on the viability of the target microorganism.

To prevent false negative and positive results in PCR assays, validation of the PCR assay is required to ensure the assay is suitable for the purpose it is intended for (Hiney and Smith, 1998; Walker and Subasinghe, 2000; OIE, 2017). Validation is a process that involves development and optimization of a technique, method or assay and subsequent assessment of its performance under the conditions or within the matrix in which it will be employed (Hiney and Smith, 1998; Purcell *et al.*, 2011). Hiney and Smith (1998) provide a framework for the validation of PCR-based detection techniques that outline three major criteria, namely qualitative, quantitative and reliability criteria, which should be evaluated at increasing levels of experimental complexity. The levels of experimental complexity proposed in the framework are; *in vitro* studies, studies of seeded matrices, studies of incurred matrices and analysis of naturally occurring environmental samples which dictate field studies. During the first level, studies are performed *in vitro* so that the basic properties of the technique can be established. In the second level, seeded studies are performed in a matrix designed to be a sterile simulation of the environment. In these studies samples are introduced into selected sterile matrices which provide information concerning the impacts that the physical and chemical properties of the matrices have on the basic properties of the technique. The third level, incurred studies are performed in a matrix composed of non-sterilised samples of naturally occurring environments. In incurred studies, laboratory grown cultures of the target organism are introduced to the matrix and are allowed to establish themselves within the matrix before any analyses are attempted. Incurred matrix studies provide information concerning the possible impact the physiological changes in the target cell has on the performance of the technique. The final level of the framework is the non-sterile field study which evaluates the performance of the technique in a naturally infected environment. These four levels represent a logical sequence and at each level there is progressive loss of control over some important experimental parameters (Hiney and Smith, 1998).

A reliable PCR assay for species-specific detection involves the identification of a suitable DNA target region for species-specific primer design (Bott *et al.*, 2010). Target regions

that are routinely used for this purpose are genes that contain highly conserved sequences, which exhibit minimal genetic variation within a species but also vary sufficiently between species. Genes routinely used for this purpose include the small subunit (SSU) and large subunit (LSU) nuclear ribosomal DNA (rDNA) genes. Ribosomal DNA genes are comprised of an intergenic spacer region, an external transcribed spacer region and a transcription unit comprising three rRNA genes (the SSU, 5.8S and LSU) separated by the first and second internal transcribed spacers (Hillis and Dixon, 1991; Bott *et al.*, 2010). Nuclear ribosomal DNA (rDNA) genes are highly abundant in eukaryotic cells and contain highly conserved sequences that provide suitable regions for the design of species-specific primers (Criscione *et al.*, 2002; Bott *et al.*, 2010). The development of PCR primers specific to the target organism is important to achieve the best possible amplification and will influence both the analytical specificity and sensitivity of the reaction (Cunningham, 2002). Analytical specificity refers to the ability of an assay to measure a particular organism, rather than other non-target organisms in a sample (Saah and Hoover, 1997). Analytical specificity avoids generation of false positive results, which occur due to cross-amplification among heterologous species. In addition to identifying a suitable DNA target region, other criteria such as optimum primer length, correct composition of bases (GC %), optimum annealing and melting temperatures, 5' end stability and 3' end specificity should also be considered (Dieffenbach *et al.*, 1993). Analytical sensitivity is known as the limit of detection or the smallest amount of substance an assay can accurately measure within a particular matrix (Saah and Hoover, 1997; Cunningham, 2002; Adams and Thompson, 2011). Poor analytical sensitivity can arise from incorrect annealing/elongation times and temperatures, improper primer/probe design and the presence of polymerase inhibiting substances co-purified with the target DNA (Opel *et al.*, 2009; Bott *et al.*, 2010; Hunt, 2011). Primer design and the optimisation of DNA extraction protocols and PCR primer cycling conditions are therefore essential for the development of a reliable PCR assay with high analytical specificity and sensitivity.

The extraction of DNA is a critical step in the process of detecting and identifying pathogens using DNA based detection methods, like PCR. DNA extraction methods should be qualitatively and quantitatively optimised. The quality of extracted DNA can be adversely affected by co-purification of contaminants, which can inhibit the PCR (Cunningham, 2002; Altinok and Kurt, 2004). When attempting to detect small quantities of DNA from a single sample, the DNA extraction step becomes even more important, highlighting the need for an optimized DNA extraction method(s) ensuring the highest

quantity and quality of DNA possible. When template DNA preparations are optimised correctly, the analytical sensitivity of the overall PCR technique can be enhanced (Cunningham, 2002). A DNA extraction method that is frequently published and utilized on a variety of biological samples, including fish tissue samples, is the QIAGEN DNeasy blood and tissue extraction kit (Cat No./ID: 69504) (Criscione *et al.*, 2002; Arkush *et al.*, 2003; Guy *et al.*, 2003; Gregg *et al.*, 2014; Lear *et al.*, 2018). This method has been effective in retrieving *I. hoferi* DNA from *I. hoferi* infected fish tissue (Criscione *et al.*, 2002; White *et al.*, 2013; Gregg *et al.*, 2014), but includes several tube transfer steps which could possibly lead to the loss of DNA. This could be particularly problematic in samples where there is only a small quantity of starting material or target DNA. Greeff *et al.* (2012) reported a heat-lysis extraction method that has been developed to allow for direct quantification of a target DNA fragment in tissue samples. The method has also been used for the extraction of total genomic DNA from tissue of numerous invertebrates, including oysters and crabs (Macey *et al.*, 2008a; b). This method involves the lysis of DNA in the presence of Chelax-100 beads; anionic beads that chelate metal ions and help prevent DNA degradation. This extraction method does not require multiple tube transfer steps which could lead to the possible loss of DNA, or the use of any toxic chemicals.

Effective identification, control and treatment of diseases of aquatic animals requires diagnostic tests that are rapid, specific, highly sensitive and reproducible. Histopathology and culture based techniques are traditional methods used for the diagnosis of fish and shellfish diseases; however, these methods often lack specificity and many pathogens are difficult to detect when present in low numbers or when there are no clinical signs of the disease (Walker and Subasinghe, 2000). Molecular based diagnostic approaches are therefore increasingly being applied as alternatives to conventional pathogen detection techniques and routinely used alongside conventional microbiological and histopathological techniques for the confirmation of pathogens. Conventional PCR methods however present some technical limitations, for example, they do not allow quantification, which is important to estimate pathogen load. Real-time quantitative PCR (qPCR) techniques therefore represent an alternative to the conventional PCR method as it provides simultaneous amplification and visualization of newly formed DNA amplicons and quantification of target DNA sequences in different matrices. While qPCR techniques are highly sensitive and allow for species-specific detection together with quantification, the published "Minimum information for the publication of real-time quantitative PCR experiments" (MIQE) guidelines by Bustin *et al*. (2009) states that the performance of

PCR-based assays in general has been compromised by insufficient optimisation of assay performance as well as lack of transparency in reporting experimental details. To make PCR-based assays more reliable the MIQE constitutes a reference framework to deliver guidelines that promote transparency of experiments and confidence in results and provided a blueprint for good PCR assay design which should be followed to ensure unambiguous reporting of experiments. Furthermore to ensure correct validation of diagnostic techniques, the OIE has listed specific criteria that must be fulfilled during assay development and validation of all assay types to ensure the development of high quality protocols, to ensure that the system is working properly and to confirm data quality (OIE, 2017). Laurin *et al.* (2018) also identified and addressed important metrics for consideration at the design phase that should be taken into consideration. The above papers and the framework proposed by Hiney and Smith (1998) and Bott *et al.* (2010) are intended to mitigate design shortcomings resulting in the introduction of biases and inappropriate interpretation or generalization. Adhering to these recommendations will help ensure that laboratories maximize the likelihood of detection of infectious agents.

In this chapter we describe the development of a new SYBR green qPCR assay that is highly specific to the SSU rDNA sequence of *I. hoferi,* that has been designed, optimised and validated in observance of the MIQE guidelines, and validated based on the *in vitro* and seeded matrices criteria proposed by Hiney and Smith (1998) and Bott *et al.* (2010) , for use in *I. hoferi* identification and quantification from culture and *I. hoferi* infected fish tissue samples. Two different DNA extraction methods were compared to identify the most effective method to extract and recover high quality *I. hoferi* DNA. Spectrophotometry was used to determine DNA yield and purity. The optimal method was chosen based on its practicality, efficiency and repeatability in extracting high quality DNA. Post extraction a qPCR assay was developed for sensitive, specific and rapid diagnosis of *I. hoferi* in experimentally infected fish tissue samples and evaluated at the *in vitro* and sterile seeded level. Besides the ability for the assay to be used for monitoring of infection, it is intended that the validation of this assay will estimate the techniques lowest detectable limit to detect *I. hoferi* and to quantify the amount of PCR inhibition, if any, obtained from sampling environmentally *I. hoferi*-infected tissue samples.

3.2) Materials and methods

3.2.1) Microorganisms and sample preparation

Ichthyophonus hoferi was isolated from the infected liver of euthanized White Stumpnose, *Rhabdosargus globiceps* at the Two Oceans Aquarium in Cape Town South Africa and cultured (as described in 2.2.1). For the evaluation of the DNA extraction methods chosen for this study, five independent *I. hoferi* cultures were enumerated (as described in 2.2.2) and prepared at a concentration of ~ 250 schizonts.mL^{-1} in duplicate (n=10) by diluting the number of schizonts in each culture vessel, using fresh sterile culture media. Thereafter from each sample, 0.5 mL (~125 schizonts) aliquots were aseptically transferred to clean 1.5 mL microcentrifuge tubes and centrifuged for 5 min at 6000 x g. The supernatants were carefully removed and examined under an Olympus BX51 compound light microscope to determine whether any *I. hoferi* schizonts remained in the culture supernatant following centrifugation (no schizonts were visualized). The pelleted samples were then rinsed three times with autoclaved deionised water and centrifuged for 5 min at 6000 x g after each rinse step to pellet the cells. After each rinsing step the supernatant was carefully removed and examined for *I. hoferi* schizonts. Samples were kept on ice until needed.

3.2.2) DNA extraction from laboratory-grown cultures

Total genomic DNA was extracted from each pelleted sample of *I. hoferi* (~125 schizonts) using two DNA extraction methods; the QIAGEN DNeasy blood and tissue kit and the heat-lysis method described by Greeff *et al.* (2012). For each method, the manufacturer or author recommendations were followed. The final elution volume for the QIAGEN method was 100 µL. The DNA concentration and quality (OD_{260}/OD_{280}) of each sample was quantified using a Jenway Genova Nano spectrophotometer and the mean DNA concentration per schizont ± standard error for each DNA extraction method was recorded (Bustin *et al.*, 2009). To test for significant differences in the yield and purity of DNA obtained using the two DNA extraction methods, a One-Way ANOVA was performed. Significance was assigned to p-values <0.05.

3.2.3) Ichthyophonus hoferi *SSU gene primer design*

The small subunit (SSU) rDNA gene was selected for the development of qPCR primers specific for *I. hoferi.* Primer sets were designed to amplify regions of this gene which are consistently conserved within *I. hoferi* isolates/strains, yet showed variation between *I. hoferi* and other closely related mesomycetazoans. Two regions of the SSU rDNA gene of *I. hoferi* were selected for designing species specific primers, based on region A (producing a ~640 bp fragment) and B (a ~673 bp fragment) of Criscione *et al.* (2002). These regions correspond to base pairs 1-642 and 642-1335 of the *I. hoferi* (GenBank Accession number U25637) SSU rDNA gene (Criscione *et al.*, 2002). CLC workbench V6.8.4 (CLC bio, a QIAGEN company) was used to align each fragment with published SSU rDNA gene sequences of members of the genus *Ichthyophonus*, isolated from multiple hosts and from various geographical regions, and with sequences of other related species (Table. 3.1 for list of species used and their corresponding GenBank accession numbers). The multiple sequence alignments were conducted independently for each region (A & B) of the SSU rDNA gene in order to identify conserved regions for designing species specific primers. Primers were selected to regions 20 to 21 nucleotides in length, showing complementarity to *I. hoferi* SSU rDNA, but compared to all of the other sequences in our alignment had at least 2 mismatches overall. Primers were designed using Primer3 (Version 0.4.0) using standard criteria (Dieffenbach *et al.*, 1993). To ensure species specificity, candidate primers were blasted against the GenBank database (Benson *et al.*, 2005). Primer set BW1-F/R, yielding a 250 base pair (bp) fragment from region A, and primer set BW2-F/R, yielding a 299 bp fragment from region B, were selected for further assay development (Table. 3.2).

3.2.4) Optimization of real-time PCR cycling conditions

Quantitative real-time PCR cycling conditions were optimised, to ensure optimal detection specificity for each primer set by varying the annealing temperatures. All qPCR reactions were conducted using the Bio-Rad CFX96™ real-time PCR detection system and C1000™ thermal cycler; which was used for all subsequent qPCR assays. All qPCR reactions were carried out in a final volume of 25 µL and each reaction contained 50 ng of *I. hoferi* genomic DNA, 1 x KAPA SYBR fast qPCR master mix, 10 µM of each primer and PCR grade H_2O. Amplification consisted of an initial denaturation at 95 °C for 10 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing gradient from 55 – 65 °C for 30 seconds, and elongation at 72 °C for 45 seconds, melt curve from 50 °C for

Table 3.1: Species used in sequence alignment of members of the genus *Ichthyophonus* and other related species using region A & B.

GenBank Accession no.	Species	Host	Reference	Region
U25637	*I. hoferi*	*Limanda ferruginea*	(Ragan *et al.*, 1996)	Region A & B
AF232303	*I. irregularis*	*Limanda ferruginea*	(Rand *et al.*, 2000)	Region A & B
AF274051	*Amoebidium parasiticum*		(Benny and O'Donnell, 2000)	Region A & B
AF070445	*Anurofeca richardsi*		(Baker *et al.*, 1998)	Region A & B
AY336708	*Paramoebidium* sp.	*Ephemeroptera*	(Cafaro, 2005)	Region A & B
AF467785	*I. hoferi*	*Sebastes flavidus*	(Criscione *et al.*, 2002)	Region A
AF467789	*I. hoferi*	*Sebastes alutus*	(Criscione *et al.*, 2002)	Region A
AF467791	*I. hoferi*	*Sebastes alutus*	(Criscione *et al.*, 2002)	Region A
AF467793	*I. hoferi*	*Clupea pallasi*	(Criscione *et al.*, 2002)	Region A
AF467799	*I. hoferi*	*Oncorhynchus tshawytscha*	(Criscione *et al.*, 2002)	Region A
KC500080,83,87	*Ichthyophonus* sp.	*Theragra chalcogramma*	(White *et al.*, 2013)	Region A
Y19155	*Amoebidium parasiticum*		(Ustinova *et al.*, 2000)	Region A

Table 3.1 cntd: Species used in sequence alignment of members of the genus *Ichthyophonus* and other related species using region A & B.

GenBank Accession no.	Species	Host	Reference	Region
AY336698	*Eccrinidus flexilis*	*Glomeris* sp.	(Cafaro, 2005)	Region A
EU124916	*Creolimax fragrantissima*	*Leptosynapta clarki*	(Marshall *et al*., 2008)	Region A
EF024885	*Eimeriidae*		(Lesaulnier *et a*l., 2008)	Region A
AF533950	*Dermocystidium* sp.	*Perca fluviatilis*	(Pekkarinen *et al*., 2002)	Region A
DSU21336	*Dermocystidium salmonis*	*Onchorhynchus tschawytscha*	(Ragan *et al*., 1996)	Region A
AF192386	*Pseudoperkinsus tapetis*	Carpet Shell Clam	Figueras *et al*., 2000	Region A
AF118851	*Rhinosporidium seeberi*		(Herr *et al*., 1999)	Region A
EF023186	*Caprifoliaceae*		(Lesaulnier *et al*., 2008)	Region A
GQ995375	*Chytridiomycota*	Soil	Freeman et al., 2009	Region A
AF467786	*I. hoferi*	*Sebastes flavidus*	(Criscione *et al*., 2002)	Region B
JX509917	*Ichthyophonus* sp.	*Theragra chalcogramma*	(White *et al*., 2014)	Region B
AF467790	*I. hoferi*	*Sebastes alutus*	(Criscione *et al*., 2002)	Region B

Table 3.1 cntd: Species used in sequence alignment of members of the genus *Ichthyophonus* and other related species using region A & B.

GenBank Accession no.	Species	Host	Reference	Region
AF467794	*I. hoferi*	*Clupea pallasi*	(Criscione *et al.*, 2002)	Region B
AF467800	*I. hoferi*	*Oncorhynchus tshawytscha*	(Criscione *et al.*, 2002)	Region B
AY336700	*Eccrinidus flexilis*	*Glomeris* sp.	(Cafaro, 2005)	Region B
EU124915	*Creolimax fragrantissima*	tunicate	(Marshall et al., 2008)	Region B
AY336711	*Enterobryus sp.*	*Iulidae*	(Cafaro, 2005)	Region B
Y16260	*Sphaeroforma arctica*		(Jostensen, 2002)	Region B
GU727528	*Pseudoperkinsus tapetis*	*Crassostrea gigas*	(Marshall and Berbee, 2010)	Region B
HQ219468	*Uncultured ichthyosporea*		(Monchy *et al.*, 2011)	Region B
AY336710	*Enterobryus oxidi*	*Diplopoda*	(Cafaro, 2005)	Region B
AY336701	*Enterobryus sp.*	*Diplopoda*	(Cafaro, 2005)	Region B

10 seconds with an increment of 0.5 °C per cycle. Data collection and real-time analysis occurred at each annealing step and melt curve data collection and analysis occurred at each increment. The optimal annealing temperatures for the BW1-F, BW1-R, BW2-F and BW2-R primers are listed in Table 3.2. Subsequently PCR amplification was conducted using the Labnet Multigene Thermal Cycler (Labnet International) using the same qPCR cycling conditions and amplified PCR products (analysed by agarose gel, 0.8 %, electrophoresis) were sequenced to further confirm reaction specificity.

Table 3.2: Primers developed in this study and used for the validation of an *Ichthyophonus hoferi* qPCR assay.

Set no.	Primer name	Sequence 5' – 3'	Product size (bps)	Melting temp. (°C)	% GC content	Annealing temp. (°C)
1	BW1-F	AAGGCAGTGG GTGCTCTTAAT	250	60.1	47.6	61.4
	BW1-R	GCAAATGCTTT CGCAGAAGT		60.5	45.0	
2	BW2-F	GCAGTGGGTG CTCTTAATTG	299	58.4	50.0	59
	BW2-R	TGATCATCTTC GATCCCCTAA		59.5	42.9	

3.2.5) Specificity of SSU primer sets

Following optimization of cycling conditions, primers for region A & B were tested for cross-reactivity using DNA extracted from closely related fungal and coccidian species that were available at the time of assay development (Table 3.3). DNA from each of the tested isolates were obtained opportunistically from the laboratory. The sources and origins of the extracted DNA from the closely related fungal and coccidian species that were tested for cross-reactivity in this study are listed in Table 3.3. Each qPCR assay included positive controls, consisting of *I. hoferi* genomic DNA extracted from a pure culture and a negative/non-template control (NTC), which substituted PCR-grade water for DNA to confirm that the reagents were not contaminated. All reactions were performed in duplicate and contained 50 ng of the respective genomic DNA, 1 x KAPA SYBR fast qPCR master mix, 10 µM of each respective primer and PCR grade H_2O. PCR cycling conditions were

as described in section 3.2.4, with the exception that the optimised annealing temperature of each primer set was utilized. The optimal annealing temperatures for the different primer pairs are listed in Table 3.2.

3.2.6) Sensitivity of the Ichthyophonus hoferi *SSU primer set*

I. hoferi specific primers BW2-F/R were chosen for all subsequent experiments as they showed no cross-reactivity to any of the closely related species tested in this study and was shown to be the most species specific, with the most base pair differences present in both the forward and reverse primer, when compared with the SSU rDNA sequences of *I. hoferi* determined in this study and downloaded from the GenBank database (Fig. 3.2.A & Fig. 3.2.B). The analytical sensitivity or lowest detection limit for the *I. hoferi* specific primer set, BW2-F/R, was determined by running a qPCR on serial dilutions of known concentration of *I. hoferi* genomic DNA in PCR-grade water (*in vitro* study). Three axenic cultures of *I. hoferi* were prepared, maintained and diluted to a concentration of 500 schizonts.mL^{-1}; enumerated five times (as described in Chapter 2). From each of the three diluted samples, 1 mL was aseptically transferred to a separate sterile 1.5 mL microcentrifuge tube. DNA was extracted from each sample following the heat-lysis method described by Greeff *et al*. (2012). Following DNA extraction, each sample (1.67 schizonts.µL^{-1}) underwent ten-fold serial dilutions, up to 10^{-4}, prepared in PCR-grade water (*in vitro* study). Real-time qPCR reactions were then run on all samples in triplicate using 3 µL of each sample (DNA) per 25 µL reaction. Sensitivity testing therefore ranged from approximately 5 schizonts to 0.0005 schizonts (10^{-4}) per 25 µL qPCR reaction. The quantification cycle (Cq) value derived from each tenfold serial dilution served to determine the lowest detection limit for the *I. hoferi* specific primer set. The standard curve was established by plotting Cq values for each dilution versus the log value of the corresponding cell (schizont) number. The amplification efficiency percentage was calculated from the slope of the standard curve using the following formula: $E = (10^{(-1/slope)} - 1) \times 100$. Efficiencies between 90 and 110 % were considered acceptable. Standard curve precision was evaluated via coefficients of determination (R^2); an R^2 value greater than 0.985 was considered acceptable (Stratagene, 2004).

Table 3.3: Species used to test for cross-reactivity of the *Ichthyophonus hoferi* primers designed in this study.

Species	Host	Sample location	Accession number	Source
Unidentified Pleosporales	*Palinurus gilchrist*	Western Cape	JN397388	Greeff *et al.*, (2012)
***Acremonium* sp.**	*Palinurus gilchrist*	Western Cape	JN397389	Greeff *et al.*, (2012)
***Hypocreales* sp.**	*Ecklonia maxima*	Western Cape	JN397391	Greeff *et al.*, (2012)
Aphanomyces astaci	Crayfish	United Kingdom	DQ403202.1	Oidtman *et al*., (2004)
Halioticida noduliformans	*Haliotis midae*	Western Cape	GU289906	Macey *et al*., (2004)
F. falciforme		Western Cape	NRR54219	This study
F. keratoplasticum		Western Cape	JN585993	This study
***Devriesia* sp.**		Western Cape		This study
Unidentified Coccidia		Western Cape		This study

To quantify the amount of PCR inhibition for the *I. hoferi* specific primer set, BW2-F/R, under environmental conditions (seeded study), a qPCR was performed on dilutions of known concentration of *I. hoferi* genomic DNA prepared in PBS and in *Rhabdosargus globiceps* liver tissue matrix. This seeded study served to further validate specificity and quantify the amount of PCR inhibition which was used to determine the lowest detection limit for the *I. hoferi*-specific primer set in an environment or matrix that is designed to be a non-sterile simulation of the environmental conditions. Two sets of *I. hoferi* cultures were prepared at 500, 400, 200 and 100 schizonts.mL^{-1} in triplicate (n=24). The samples were then centrifuged for 5 min at 6000 x g, the supernatants discarded and the pellets were washed (as described in 3.2.1). Following preparation, a 0.03 g piece of uninfected *R. globiceps* liver tissue and a millilitre of PBS supplemented with 0.1 % Tween 20 and 0.05 % Antifoam A was aseptically transferred to the first set of cultures (seeded matrix). Millilitre of PBS supplemented with 0.1 % Tween 20 and 0.05 % Antifoam A was then aseptically transferred to the second set of cultures (sterile matrix). All samples were then homogenised for 1 min 30 sec at 22,000 rpm using a Polytron PT 2100 tissue homogeniser before being centrifuged at 14,000 x g for 10 min at 4 ˚C. The supernatant in each tube was carefully removed and DNA extracted according to the heat-lysis method described by Greeff *et al.* (2012). The qPCR cycling conditions were as described in 3.2.5 above. The differences in Cq values observed at each culture dilution between the samples homogenized with *R. globiceps* liver tissue and PBS and in PBS only were recorded and the average increase in qPCR cycle number was recorded as percentage change in PCR sensitivity.

3.3) Results

3.3.1) DNA extraction methods

Two DNA extraction methods were tested and compared, the heat-lysis method described by Greeff *et al.* (2012) and QIAGEN DNeasy Blood and Tissue kit. Significantly higher amounts of DNA were extracted from an equivalent number of *I. hoferi* schizonts using the heat-lysis method, when compared with QIAGEN DNeasy Blood and Tissue kit extraction method (Fig. 3.1; One-way ANOVA, p=0.0005). The average DNA yield obtained using the heat-lysis method was 0.124 ± 0.005 (0.1059 - 0.1361) µg DNA.schizont^{-1}, compared to an average of 0.017 ± 0.002 (0.0116 - 0.0251) µg DNA.schizont^{-1} for samples subjected to the kit extraction method. DNA purity was estimated by determining the A260/A280

ratio. Similar ratios was obtained using each method, with DNA extracted using the heat-lysis method yielding a mean ratio of 1.639 ± 0.027 (1.566 - 1.721), compared to 1.784 ± 0.018 (1.739 - 1.822) using the QIAGEN DNeasy Blood and Tissue kit.

3.3.2) Specificity of SSU primer sets and optimization of real-time qPCR conditions

A BLAST search of the GenBank database revealed that the qPCR primers designed for region A and B of the *I. hoferi* SSU rDNA gene showed homology only to *I. hoferi* strains that had been deposited in the GenBank database at the time of the BLAST search. The specificity of primers BW1 F&R and BW2 F&R were further confirmed by aligning them to closely related species (Table 3.1). Primer BW1-F showed 100 % homology to *I. hoferi* and differed by at least 2 base pairs (bp) from closely related species. Conversely, primer BW1-R showed 100 % homology to *I. hoferi*, but also to a selected number of the phylogenetically closely related species (Fig. 3.2.A). Primer BW2-F showed 100 % homology to *I. hoferi* and differed by at least 2 bp from closely related species. Unlike primer BW1-R, primer BW2-R showed 100 % homology to *I. hoferi* and differed by at least 1 bp from the sequences of phylogenetically close relatives (Fig. 3.2.B).

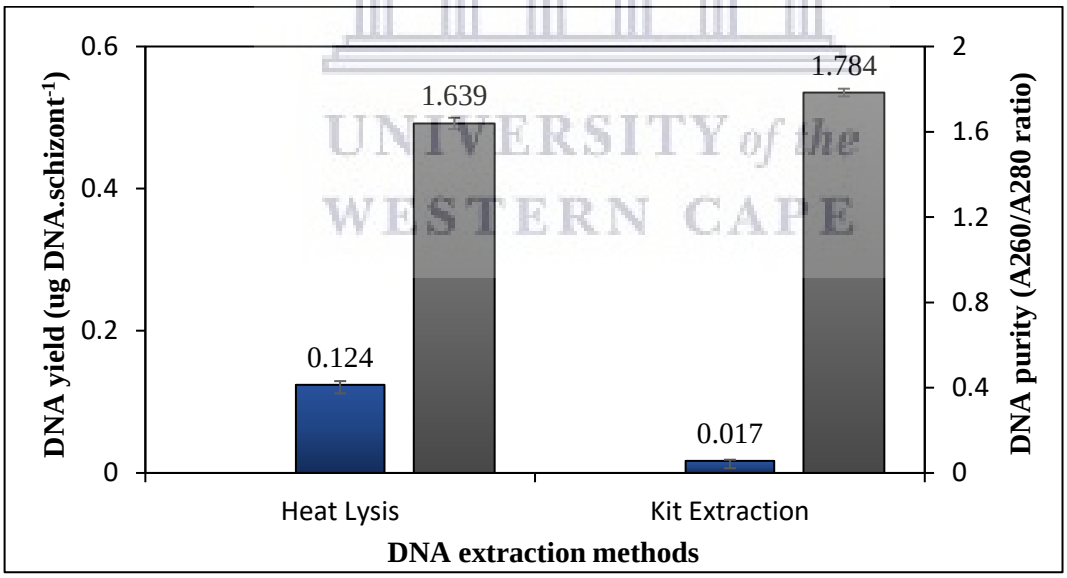

Fig. 3.1: Assessment of two different DNA extraction methods, heat-lysis and QIAGEN DNeasy Blood and Tissue kit extraction. Left Axis: The average yield of DNA extracted from 1 schizont of *I. hoferi*, expressed in micrograms of DNA.schizont^{-1}. Right Axis: The A260/A280 ratios. Values represent the mean (±SE) of five samples for each extraction method.

The BW1-F/R primer set had an optimum annealing temperature of 61.4 °C and amplified a 250 bp fragment from region A of the SSU rDNA gene of *I. hoferi* (Table 3.2). The BW2-F/R primer set had an optimum annealing temperature of 59 °C and amplified a 299 bp fragment from region B of the SSU rDNA gene of *I. hoferi* (Table 3.2). When tested for cross-reactivity to DNA extracted from marine fungi and a single coccidian isolate obtained from the environment, both primer sets did not show cross-reactivity with any of the tested isolates and only amplified DNA from *I. hoferi* (Fig. 3.3). The non-template control did not produce any fragments, confirming that the reagents used for PCR were not contaminated. Amplified DNA was also sequenced to confirm the specificity of the PCR reactions and subsequent analysis of the nucleotide sequences showed that the PCR products were identical to *I. hoferi*. Based on these findings, primer set BW2-F/R was selected for further optimization and validation. Real-time PCR amplification using the *I. hoferi* specific primers BW2-F/R produced a melting temperature of 80 ± 0.5 °C for the 299 bp product, with an optimum annealing temperature of 59 °C. A reduction in the cycle threshold number from 35 to 32 had no influence on primer specificity. Any samples that recorded a quantification cycle (Cq) value after setting the PCR cycle number to 32 and the baseline threshold value to 200, with a melt peak of 80 ± 0.5 °C on the melt curve graph were considered to be positive.

3.3.3) Sensitivity of the Ichthyophonus hoferi *specific BW2-F/R primer set*

The lowest detectable limit for the BW2-F/R primer set in PCR-grade water following real-time PCR amplification was 0.005 schizonts in a 25 µL reaction at a Cq value of 29 ± 0.5 (Fig. 3.4). The amplification efficiency percentage calculated from the slope of the standard curve was 101.07 %. Standard curve precision evaluated via coefficients of determination was 0.99. To assess non-target DNA in PCR inhibition, DNA extraction and subsequently qPCR was performed on diluted samples in the presence of a liver tissue matrix. This was compared to samples that were prepared in PCR-grade water. Overall the increase in Cq values at each corresponding dilution demonstrates a reduction in qPCR sensitivity by 3.34 cycles (Fig. 3.5).

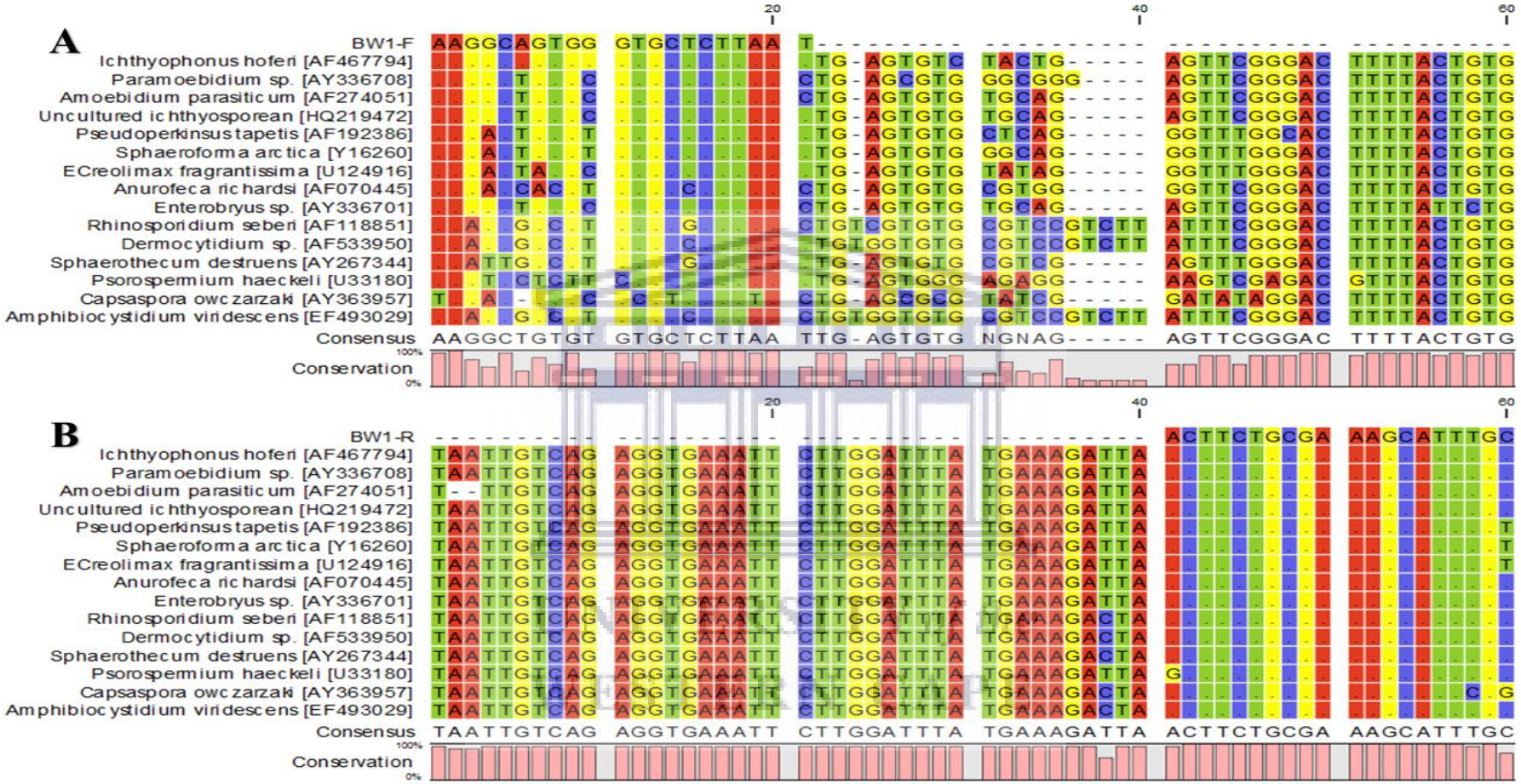

Fig. 3.2.A: Alignment of the (A) BW1-F and (B) BW1-R primers to the SSU rDNA gene of *Ichthyophonus hoferi* and closely related organisms. Identical base pairs are indicated as dots.

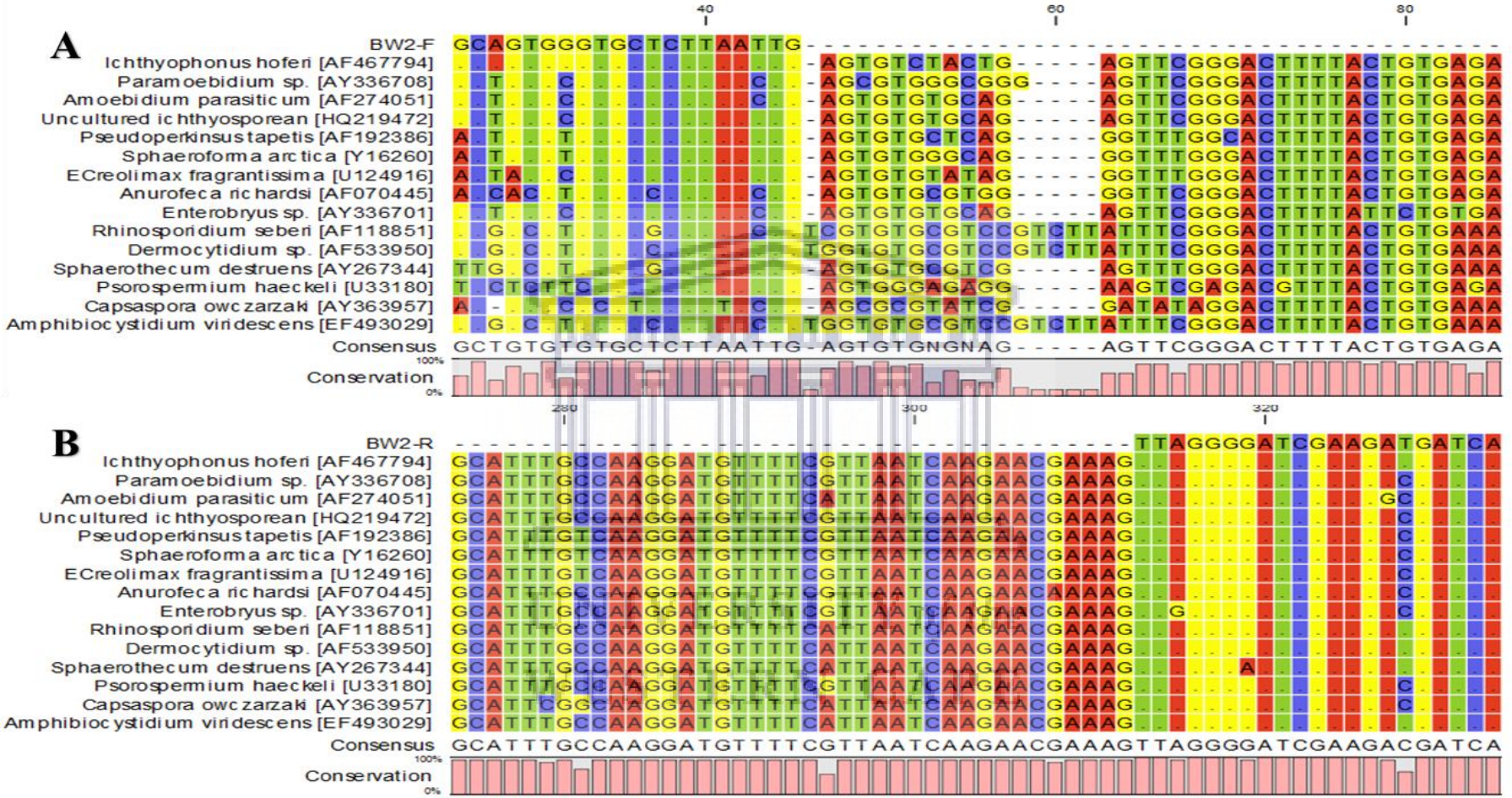

Fig. 3.2.B: Alignment of the (A) BW2-F and (B) BW2-R primers to the SSU rDNA gene of *Ichthyophonus hoferi* and closely related organisms. Identical base pairs are indicated as dots.

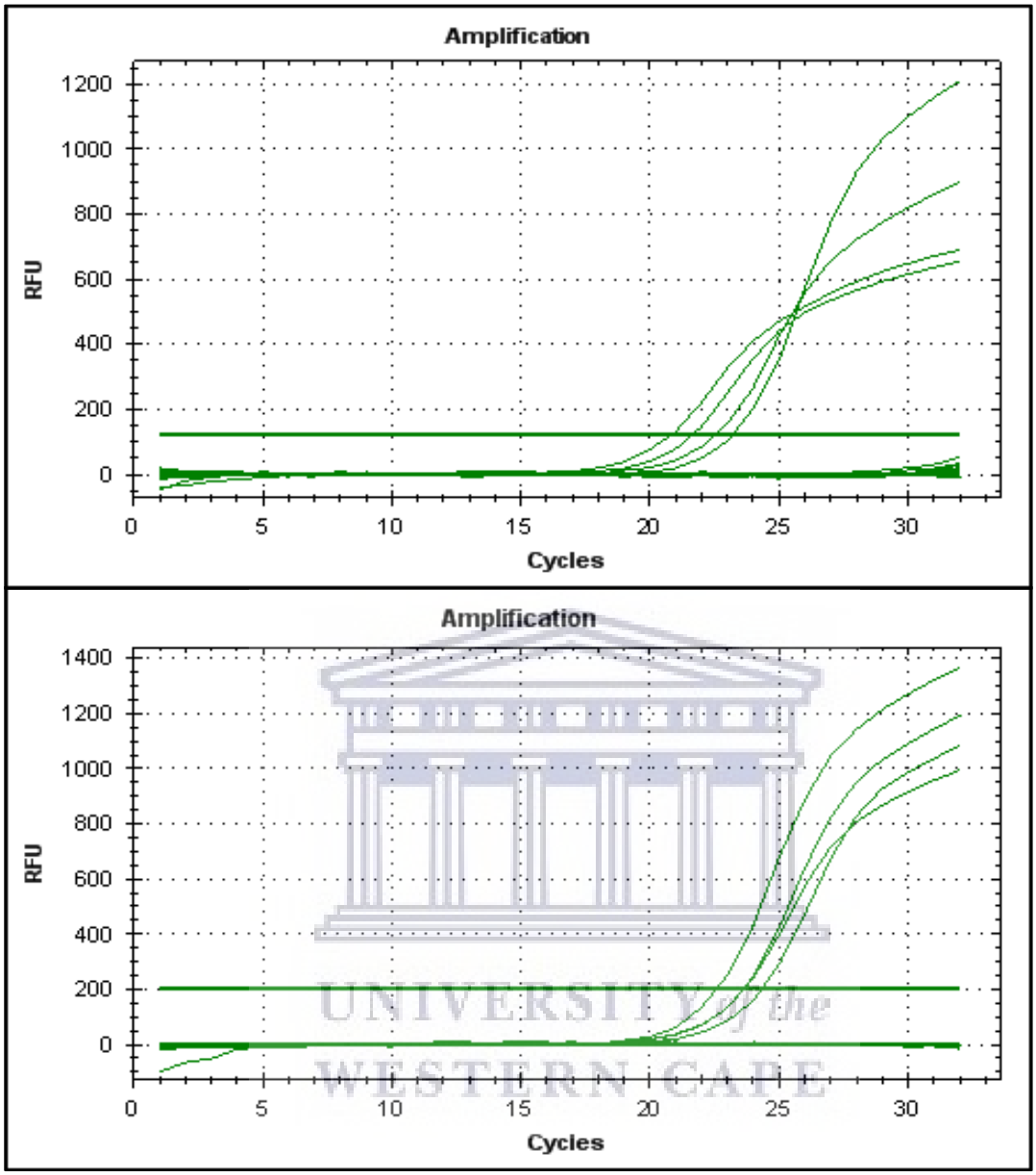

Fig. 3.3: Real-time PCR amplification curves following amplification with primer sets (A) BW1-F/R and (B) BW2-F/R. Genomic DNA isolated from *I. hoferi* and other fungal and coccidian species was added for specificity testing. These included unidentified Pleosporales, *Acremonium* sp., *Hypocreales* sp., *Aphanomyces astaci*, *Halioticida noduliformans*, *F. falciforme*, *F. keratoplasticum*, *Devriesia* sp. and unidentified Coccidia. A non-template control (NTC) was also included. Amplification only occurred for *I. hoferi* samples.

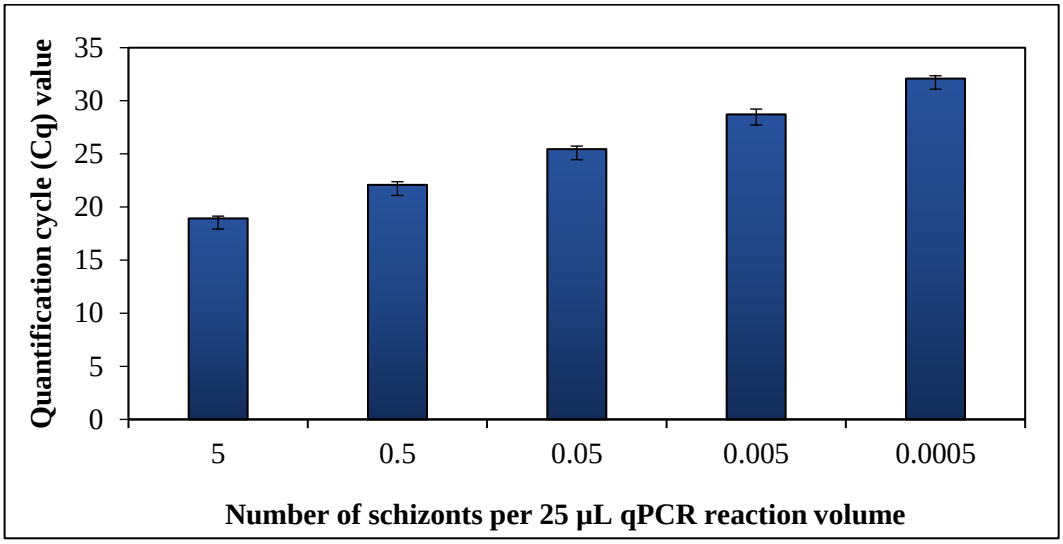

Fig. 3.4: Quantification cycle (Cq) value obtained following qPCR on serial dilutions of *I. hoferi* DNA extracted from a known quantity of schizonts (n=5). Three separate serial dilutions were prepared and each reaction was done in triplicate. All reactions were conducted using the *I. hoferi* specific primers BW2-F and BW2-R. Results are presented as the mean (±SEM) Cq value for each respective schizont concentration and demonstrate that the *in vitro* test is capable of detecting as little as 0.005 schizonts in a 25 µL reaction at a Cq value of 29 ± 0.5.

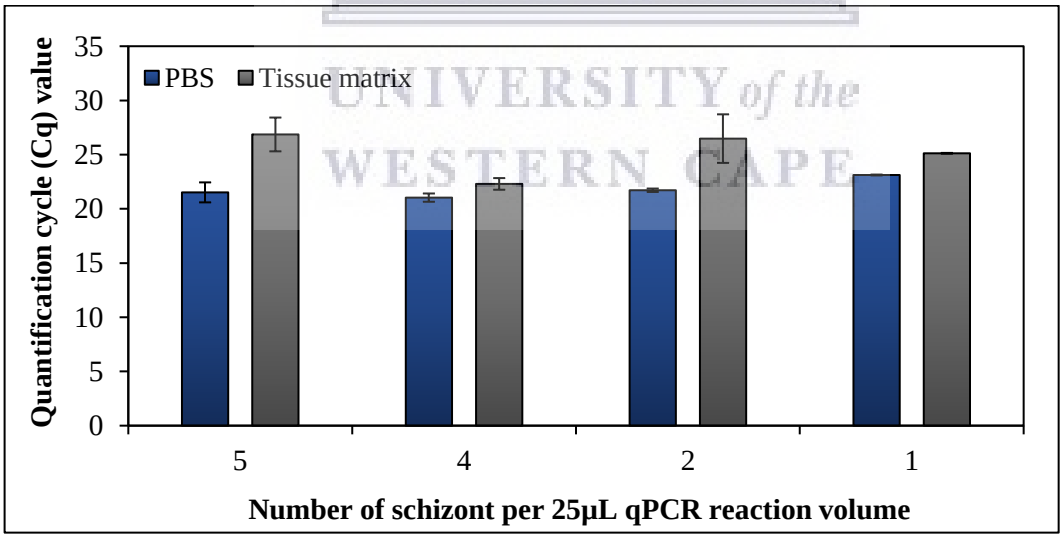

Fig. 3.5: Quantification cycle (Cq) value obtained following qPCR analysis of *I. hoferi* DNA obtained from various known schizonts quantities prepared in a sterile (PBS) and seeded matrix (uninfected *Rhabdosargus globiceps* liver tissue). The values represent the mean (±SEM) Cq value for the varying schizont concentrations. The seeded study served to quantify the amount of qPCR inhibition in a matrix designed to be a non-sterile simulation of the environmental conditions.

3.4) Discussion

The recovery of high quality genomic DNA is an essential prerequisite for ensuring both sensitive and accurate PCR quantification of specific DNA fragments within a sample. DNA extraction and recovery methods need to be carefully assessed and optimised to enhance the recovery of DNA from samples and to reduce the co-purification of PCR inhibitors. PCR assays can be inhibited by substances co-purified with DNA extracted from environmental and animal tissue samples, causing false negative results (Bott *et al.*, 2010; Hunt, 2011; Purcell *et al.*, 2011). These inhibitors can include bacterial constituents, organic and phenolic compounds, an over-abundance of non-target DNA, heavy metals and salts that are high in abundance in marine samples; as well as other contaminants (Cunningham, 2002; Bott *et al.*, 2010). In this study two DNA extraction and recovery methods were tested, namely the QIAGEN DNeasy blood and tissue kit and the heat-lysis method reported by Greeff *et al.* (2012). The heat-lysis method described by the latter authors has also been shown to successfully extract high quality genomic DNA, allowing for accurate determination of specific target organisms (e.g. the bacterium *Vibrio campbelii*), and from tissues of marine invertebrates, including the Atlantic blue crab, *Callinectes sapidus* (Macey *et al.*, 2008a), and the Eastern Oyster, *Crassostrea virginica* (Macey *et al.*, 2008b). In this method the Chelax-100 beads chelate metal ions that are required as catalysts or cofactors in enzymatic reactions and help to prevent DNA degradation. Advantages of the heat-lysis extraction method are its simplicity, lack of phase extractions and use of harmful chemicals, such as phenol and chloroform, which can lead to loss of DNA and cause inhibition during PCR (Cunningham, 2002). We have demonstrated that the heat-lysis method can yield high amounts of DNA (Fig. 3.1) in less than 3 hours, which is highly advantageous for a diagnostic tool. The kit extraction is more labour intensive than the heat-lysis method and involves several tube transfers and washing steps, potentially resulting in the loss of genomic DNA. The kit extraction yielded significantly lower amounts of genomic DNA, average yield of 0.157 ± 0.001 µg DNA.schizont^{-1}, compared with the heat-lysis method that yielded an average of 0.497 ± 0.002 µg DNA.schizont^{-1} (Fig. 3.1). The lower yield of genomic DNA obtained with the kit could be particularly problematic for samples where there is only a small quantity of starting target DNA. Although this kit extraction method has been widely used with great success for extracting DNA, results presented here suggest that the heat-lysis method can also be used as a method for extracting genomic DNA from tissue and environmental samples and especially for the extraction of *I. hoferi* DNA. We further demonstrated that

the heat-lysis extraction method is reproducible. Based on the above findings, we selected the heat-lysis DNA extraction as the method of choice for further validation in this study.

The approach for real-time quantitative PCR (qPCR) assay development and validation in this study is based on specific criteria proposed by Hiney and Smith (1998) and Bott *et al.* (2010) and followed MIQE guidelines (Appendix A.1). The qPCR primers were designed to specifically target a conserved region of the *I. hoferi* small subunit rDNA gene within a mixed genomic DNA background, including DNA of closely related non-target organisms and liver tissue of *Rhabdosargus globiceps*. To ensure high analytical specificity, DNA regions selected for primer design should exhibit little or no genetic variation within a species, but should differ sufficiently between species to allow for unambiguous delineation of the target organisms DNA (Bott *et al.*, 2010). Of the two primer sets designed in the present study, BW2-F/R was shown to be the most species specific, with more base pair differences present in both the forward and reverse primer, when screened against a panel of closely related species (Fig. 3.2.A & B). The specificity of this primer set was further demonstrated following a BLAST search on the GenBank database, exhibiting 100 % similarity to *I. hoferi* sequences and no similarity to sequences of closely related organisms that were available on the GenBank database at the time of this study. *In vitro* qPCR assays conducted in PCR-grade water demonstrated that primer pair BW2-F/R is specific to *I. hoferi* and does not cross-react to the DNA extracted from the other fungal and coccidian organisms tested in the present study (Table 3.3 and Fig. 3.3). Although the organisms used to test for cross amplification were not those closely related due to the difficulty in obtaining a diverse set of phylogenetically or phenotypically related pathogens for the specificity testing, the fungi and coccidian tested in the study were from the environment in which fish, and specifically *R. globiceps*, naturally occur. The validation approach discussed above addressed the recommendation of Hiney and Smith (1998) to screen primers against DNA isolated from organisms occurring in the immediate environment within which the host organisms occurs naturally.

The qPCR assay developed in this study was able to detect as little as 0.005 schizonts in a 25 µL reaction volume, when conducting the assay in a 'test-tube' matrix of PCR-grade water. The level of sensitivity reported here is similar to the results reported by Whipps *et al.* (2006). The authors estimated the lowest limit of PCR as 10^{-5} spores through estimation of the number of parasite spores in histologically infected heart tissue. White *et al.* (2013) provided a qPCR assay to quantify *I. hoferi* and identified the lowest limit of detection

(analytical sensitivity) using plasmid vectors as 1 copy per reaction. There are however a variety of inhibiting substances that are often co-purified from samples obtained from the environment that can lead to a reduction in qPCR sensitivity. Therefore, conducting seeded studies, where known concentrations of the target organisms are introduced into a matrix that simulates the environmental conditions, is essential for evaluating the effects of the physical and chemical properties of a tissue matrix on the performance of the PCR assay (Hiney and Smith, 1998). In this study, the inhibitory substances present in environmental samples were quantified by comparing qPCR results from two dilution series, one made in a sterile matrix of PBS and another in a *R. globiceps* liver tissue matrix. We demonstrated that the substances present in the fish tissue matrix adversely interfere with the qPCR reaction, resulting in an overall increase in the qPCR Cq value of 3.34 cycles. This equates to a 9 % reduction in PCR sensitivity. Previous studies by Byers *et al.* (2002) and Gonzalez *et al.* (2003) have also reported reduction in PCR sensitivity caused by substances from the environment or from the host matrix. Gonzalez *et al.* (2003) reported that the detection limit of the PCR was raised from 1 to 10 bacterial cells per reaction to 10 to 100 when tissue or blood samples of infected turbot, *Scophthalmus maximuswas*, were added to the reaction using *Listonella. anguillarum* pure cultures. Byers *et al.* (2002) reported that the addition of either fish tissue or fish tissue DNA to the PCR mix was found to have an inhibitory effect on all of the PCRs they conducted. Although interference has been reported, reports from Greeff *et al.* (2012) had demonstrated a minimal overall reduction in real-time PCR sensitivity of <2 % with their assay and Mendonca and Arkush (2004) mentioning that the addition of 300 ng fish DNA prior to amplification did not affect the detection thresholds of the PCR assays.

In this study the qPCR assay was validated based on two of the criteria proposed by Hiney and Smith (1998) and Bott *et al.* (2010) for the validation of molecular diagnostic tools, the *in vitro* level and the sterile seeded level. The third stage, the incurred level, and the final stage of validation, field study are presented in Chapter 4 and Chapter 5 respectively. Unlike seeded matrices which provide information on how the physical and chemical properties of a matrix may affect the basic properties of a technique, non-seeded matrices utilize experimentally infected animal tissues or tissues from naturally infected animals to provide information such as the effect the potential physiological adaptations incurred by cells has on the assay. Their interaction with the host organism nutrients and immune system encountered in the host tissues may induce significant physiological changes in the target cell (Hiney and Smith, 1998). Furthermore it is suggested that an independent non-

sterile field test be conducted to compare the results of the diagnostic tool developed in the present study to the results of current methods for the diagnosis of *I. hoferi*, namely microscopic visualization of wet-mount squash preparations of tissues (Paperna, 1986); histological evaluations of tissues (Kocan *et al.*, 2011); and *in vitro* culture of *I. hoferi* from infected tissues (Hershberger *et al.*, 2002) as this will fulfil the last criteria proposed by Hiney and Smith (1998) as part of a complete validation process of a PCR based technique. This is further explained in Chapter 4. Adhering to these recommendations will help ensure that laboratories maximize the likelihood of detection of *I. hoferi*, if present in samples. In conclusion, the molecular based diagnostic tool developed in this study was shown to be practical, specific, and sensitive and is a tool that can be used for future research on *I. hoferi* prevalence estimations and targeted surveillance.

CHAPTER 4

The evaluation of various diagnostic techniques for the detection of *Ichthyophonus hoferi* in fish tissue samples

Abstract

A variety of methods are currently available to detect *Ichthyophonus hoferi* in infected fish hosts. These include macroscopic examination of tissues, microscopic examinations of wet-mount squash preparations of tissue, histological examination of tissue sections, *in vitro* culture of tissue explants, the polymerase chain reaction (PCR) and real-time quantitative PCR (qPCR) using *I. hoferi*-specific primers. It is apparent that these different diagnostic methods when evaluating infection prevalence of the same tissues produce different results. This study was conducted to evaluate the comparability of wet-mount squash preparation, histological examination, *in vitro* culture and real-time quantitative PCR (qPCR) for detecting *I. hoferi* in *Rhabdosargus globiceps* fish samples collected from the Two Oceans Aquarium, and to test whether homogenization of the fish tissues can improve the overall diagnostic sensitivity of *in vitro* culture and qPCR. Assuming diagnosis by more than one test as the 'true' infection status of the fish, we calculated the diagnostic sensitivity, the diagnostic specificity, the positive predictive value (PPV), the negative predictive value (NPV) and the apparent prevalence of each diagnostic method. The test with the highest diagnostic sensitivity and NPV was the wet-mount squash preparations of all organ tissues (95.24 % and 88.89 % respectively) followed closely by qPCR of homogenised liver tissue (90.48 % and 87.50 % respectively). The wet-mount squash preparations of all organ tissues conversely had the lowest diagnostic specificity (57.14 %) and PPV (76.92 %). Real-time qPCR, *in vitro* culture and histology of liver had diagnostic specificities of 100 % with positive predictive values of 100 %. Examination of histological liver sections was the least sensitive (9.52 %) of the methods tested. We observed a 14.28 % increase in diagnostic sensitivity of *in vitro* culture from 71.43 % to 85.71 % and a 9.53 % increase in diagnostic sensitivity of qPCR from 80.95 % to 90.48 % after the liver tissues were homogenized in buffer suggesting the disparities observed between the tests can result from an uneven distribution of the parasite within the infected host tissues. Before the selection of a diagnostic method, considerations such as purpose, sampling cost and logistics should be acknowledged and that the concentration of the pathogen within the

host and the amount and type of tissue sampled play an important role in the diagnostic sensitivity of the selected test.

4.1) Introduction

Utilising diagnostic techniques to estimate parasite prevalence is important for describing infections in epidemiological studies (Thrusfield, 2005; Altinok and Kurt, 2004; Banoo *et al.*, 2010; Hunt, 2011; Peeler and Taylor, 2011). Accurate estimates of prevalence can however be constrained by the differences in the analytical and diagnostic specificity and sensitivity of the techniques used (Cunningham, 2002; Gozlan *et al.*, 2006; Banoo *et al.*, 2010; Adams and Thompson, 2011). Analytical specificity refers to the ability of an assay to measure a particular organism rather than others in a sample and analytical sensitivity represents the ability of an assay to accurately measure the smallest amounts of substance present in a sample (Saah and Hoover, 1997). Diagnostic specificity is the ability of the test to correctly identify the uninfected individuals in the sample population (true negatives) whereas diagnostic sensitivity is the ability of the test to correctly identify the infected individuals in the sample population (true positives) (Saah and Hoover, 1997). Although there are a variety of methods currently available to detect pathogens in the aquatic environment, each with their own level of analytical and diagnostic sensitivity and specificity (Bott *et al.*, 2010; Adams and Thompson, 2011), the selection of an appropriate diagnostic technique or combination of techniques may vary and can depend on a number of factors and practical constraints. These can include and are not limited to; the cost of the test per sample; the resources at sample locations; the technology of the laboratory; the nature of the intended results such as for diagnosis, screening or surveillance; the number of tests to be performed; test performance and turnaround time for results (Thrusfield, 2005; Gozlan *et al.*, 2006; Kocan *et al.*, 2011; OIE, 2016).

Currently the methods available to detect *I. hoferi* include macroscopic examination of tissues (Rahimian and Thulin, 1996), microscopic visualization of wet-mount squash preparations of tissues (Paperna, 1986), histological evaluations of tissues (Kocan *et al.*, 2011), *in vitro* culture of *I. hoferi* from infected tissues (Hershberger *et al.*, 2002), the polymerase chain reaction (PCR) (Whipps *et al.*, 2006) and real-time quantitative PCR (qPCR) using *I. hoferi*-specific primers. Macroscopic examination of fish infected with *I. hoferi* externally reveal a rough sandpaper skin texture with occasional skin ulcerations (Rahimian and Thulin, 1996; Mcvicar, 1999) and internally gross nodular pigmented

lesions are visible throughout infected tissues, usually surrounded by granulomatous tissue reaction (Mcvicar, 1999). Macroscopic examination of tissues can grossly underestimate infection prevalence (Rahimian and Thulin, 1996; Mcvicar, 1999) and is non-specific as it can be associated with mechanical damage, or other infections and should therefore be used and verified in conjunction with other more specific techniques (Rahimian and Thulin, 1996; Mcvicar, 1999). Histopathology such as microscopic visualization of wet-mount squash preparations of tissues (Paperna, 1986) and histological evaluations of tissues (Kocan *et al.*, 2011) can reveal the presence of thick-walled spherical schizonts usually surrounded by host granulomatous tissues, with germination tubes typically observed after the infected host has been dead for a period of time (Mcvicar, 1999). Microscopic examinations of tissue preparations often detect higher infection prevalence than macroscopic examination of the same tissues. Rahimian and Thulin (1996) showed that when evaluating infection prevalence in a population of *Clupea harengus* (Atlantic herring), 1.06 % (269/25156) tested positive for infection using macroscopic examination whereas 3.79 % (573/15120) tested positive for microscopic examination. Caution however should be exercised in diagnosing the disease solely from the observation of histopathology. There is a lack of distinguishing features in the schizonts of *Ichthyophonus* sp. (Mcvicar, 1999) and although some infections of the organs and muscle tissue can lead to granulomas with spherical bodies, some granulomas do not always contain the spherical bodies one associates it with (Rahimian, 1998).

A more specific and sensitive technique for detecting *I. hoferi* infections involves the *in vitro* culture of tissue explants (Kocan *et al.*, 2011), which allows for live *I. hoferi* schizonts to reproduce during incubation. Kocan *et al.* (1999) showed that the *in vitro* culture of herring tissue was more sensitive than histological examination with 70 % (21/70) of wild adult *Clupea pallasii* testing positive for infection using *in vitro* culture and only 7 % of the same fish testing positive for histology. Kocan *et al.* (2011) expanded on this and showed that of the 104 culture-confirmed *Ichthyophonus*-infected Chinook salmon, only 74 % (77/104) were detected using histology of the cardiac tissue. The main advantage of *in vitro* culture is that it allows live *I. hoferi* schizonts to reproduce during incubation thereby allowing for the multiplication of the microorganism in the sample thus increasing the probability of detection in low intensity infections. This is possibly a reason for the discrepancies observed between the prevalence levels recorded in *in vitro* culture after 14 days and histology. Furthermore, by sampling a small sample from a host exhibiting low intensity infections, the parasite may not be sampled and therefore not detected thus also

contributing to the low detection sensitivity recorded using histology. Although *in vitro* culture of the pathogen allows the examiner to observe all the stages of parasite development, *in vitro* culture has the limitation that it relies upon growth using specific culture conditions which can be impractical when samples are collected at remote sites with no means of transportation and/or refrigeration and when a short presumptive diagnosis is required, or when frozen fish are sampled. These limitations require alternative diagnostic methods such as histology and/or polymerase chain reaction (PCR) to be considered which allow for chemical preservation of samples and long-term storage. Laboratory detection and identification of infections using histology can reveal the organisms within infected tissues and the diagnosis of subcutaneous and systemic infections and allow for the rapid screening of samples, however require a skilled laboratory technician to recognize the distinct appearance of the organism in tissues. These limitations of phenotypic based methods as diagnostic tools has led to the increase in supplementary genotypic or molecular approaches such as the polymerase chain reaction (PCR), which have been shown to improve the detection and identification of infections (Hamazaki *et al*., 2013a; White *et al*., 2013).

In detecting *I. hoferi* infections, the polymerase chain reaction (PCR) has been reported as being more specific and sensitive than histopathology. White *et al*. (2013) compared histology and PCR and showed that when they examined *Theragra chalcogramma* heart muscle, 4.8 % (4/83) tested positive using histology and 10.8 % (9/83) tested positive using PCR. PCR methods have become a routine diagnostic tool for many fish pathogens (Cunningham, 2002; Bott *et al.*, 2010) and are as accurate (or more so) at detecting tissue-level *Ichthyophonus*-infection as explant culture (Hamazaki *et al*., 2013a; b). Hamazaki *et al*. (2013) showed that when evaluating infection prevalence using PCR and *in vitro* culture, PCR had detected *I. hoferi* infection in 12.7 % of the sample population compared to 8.6 % for *in vitro* culture. The high analytical sensitivity of PCR methodologies which are proxy indicators of pathogen presence, predisposes them to contamination and consequently require extensive validation and testing to ensure confidence in the results (Cunningham, 2002; Purcell *et al.*, 2011; Lapatra and Kocan, 2013; White *et al*., 2013). Furthermore as with *in vitro* culture the PCR technique does not provide any information regarding the actual pathology of the infected tissues. PCR amplification generally occurs provided that the correct fragment of DNA is present. The test is not dependent on the viability of the target microorganism. An advancement of the PCR technique is the quantitative real-time PCR (qPCR) technique which provides simultaneous amplification

and visualization of newly formed DNA amplicons through measuring fluorescence which can allow for the starting amount(s) of DNA to be accurately quantified (when compared to a standard curve).

Apart from the performance of the respective diagnostic tests, other factors can also influence diagnostic results between tests when evaluating infection prevalence within the same population and within the same individual tissues. Mcvicar (1999) postulated that the differences in determining the true prevalence of *Ichthyophonus*-infection in natural populations could be due to the non-uniform distribution of the parasite within populations and between geographic areas. Mcvicar (1999) however did not consider the distribution of the parasite within the host and that the disparities observed between the evaluations of the same tissues might result from a) an uneven distribution of the parasite within the host tissues (Kocan *et al.*, 2011) and b) the amount of the tissue sampled relative to the infected organ and the concentration of the pathogen within the tissue (Gozlan *et al.*, 2006). Since infections can vary greatly between individuals and host species, as seen in *Ichthyophonus*-infected fish (Mcvicar, 1999), this can lead to inaccuracies in estimating parasite prevalence at the population level. Diagnostic assays used for surveillance or diagnoses therefore must be sensitive and specific, perform consistently, be repeatable and be reliable in order to obtain accurate prevalence estimates for epidemiological studies.

This study was conducted to evaluate the comparability of several diagnostic techniques for detecting *I. hoferi* and for their suitability to accurately detect *I. hoferi*-infections in wild fish populations. We attempted to compare examinations of wet-mount squash preparations (Rahimian and Thulin, 1996), histological evaluation (Kocan *et al.*, 2011), *in vitro* tissue explant culture (Mcvicar, 1999; Hershberger *et al.*, 2002), and qPCR (as described previously in Chapter 3), by calculating the diagnostic sensitivity (correctly identifying fish positive), diagnostic specificity (correctly identifying fish negative), positive predictive value (probability that subjects with a positive screening test truly have the disease) and negative predictive value (probability that subjects with a negative screening test truly don't have the disease) of each diagnostic technique. However, because of the discrepancies in parasite detection sensitivity between different diagnostic techniques due to focal or multifocal tissue infections (Mcvicar, 1999) some assays, namely culture and qPCR were also then compared after the homogenization of the same tissue samples. This was done to determine whether the distribution of the parasite within the host tissues influenced the ability of the techniques to detect the organism and whether

homogenization could improve the overall analytical and diagnostic sensitivity of culture and qPCR respectively. Liver tissue was selected as the reference tissue for homogenization for culture and qPCR because of the size of the organ relative to the host. Diagnostic sensitivities and specificities for each technique were determined by comparing the results to the overall infection, and target sites for infection were determined by examining tissue samples of liver, spleen, heart, kidney and gonad using wet-mount squash preparations and histological evaluation. The data represented here further contributes preliminary data towards the validation of our qPCR technique (described in Chapter 3) in an incurred matrix which fulfils the last recommendation by Hiney and Smith (1998) for the validation of the qPCR technique.

4.2) Materials and methods

4.2.1) Host samples and sample preparation

Thirty five naturally infected Cape white stumpnose, *Rhabdosargus globiceps* that were being held together as part of a study investigating *I. hoferi*, were opportunistically collected from the Two Oceans Aquarium in Cape Town, South Africa over four days. Fish were captured, euthanized with an overdose of 2-phenoxyethanol and laid on ice to impede post-mortem changes before being necropsied. The euthanasia of these fish was part of the aquarium's disease control strategy which was conducted by their animal health team under the supervision of their veterinarian. Fish weight and length, recorded as mean ± standard error (SE) (range), and sex and signs of clinical disease, if any, were recorded prior to dissection. Subsequently the liver, spleen and gonad were excised and weighed to the nearest 0.001 g. Using a sterile scalpel and forceps, small pieces of liver, spleen, heart, kidney and gonad tissues were each placed on separate microscope slides in duplicate for analysis using wet-mount squash examination and placed inside histology cassettes fixed in 10 % buffered neutral formalin for histological processing. Small pieces of liver tissue were then aseptically excised from the rest of the liver tissue and fixed in 1.5 mL microcentifuge tubes containing 100 % ethanol for qPCR analysis and 0.5 g in HEPES-buffered (pH 7) Eagles minimum essential medium (EMEM) (Sigma Aldrich, Cat# M0268) supplemented with 10 % foetal bovine serum, 100 $IU.mL^{-1}$ penicillin (Sigma Aldrich, Cat# P3032), 100 $\mu g.mL^{-1}$ streptomycin sulphate (Sigma Aldrich, Cat# S9137), and 100 $\mu g.mL^{-1}$ gentamicin sulphate (Sigma Aldrich, Cat# G1264) for *in vitro* culture. The remaining liver tissue was then weighed to the nearest 0.01 g and placed in HEPES

buffer (0.15 g.mL^{-1}, pH 7) (Sigma, Cat. #H3375) for homogenization. The remaining liver tissues that were placed in HEPES buffer (0.15 g.mL^{-1}, pH 7) were blended using a hand blender for 1 min 30 sec in the order of dissection. After each sample homogenization step the blender and container used to homogenize the remaining liver tissue in HEPES buffer were decontaminated by washing and blending in 10 % bleach, 100 % ethanol, 70 % ethanol and dH_2O respectively. All samples that were not being used were held on ice. Thereafter once all tissues were homogenized, samples were vortexed briefly to distribute the cells evenly. Before the cells had a chance to settle, the equivalent of 0.5 g of tissue homogenate for culture (3.3 mL), and 0.03 g of tissue homogenate for qPCR (0.2 mL) were then pipetted into EMEM culture media for *in vitro* culture and into 1.5 mL microcentrifuge tubes which were then stored at -20 ˚C for qPCR analysis respectively. The specific diagnostic techniques are described below.

4.2.2) Diagnostic techniques

4.2.2.1) Microscopic examinations of wet-mount squash preparations of tissue

The small pieces of liver, spleen, heart, kidney and gonad tissues each placed on separate microscope slides in duplicate were gently squashed between a coverslip and the slide. The preparations were examined microscopically using an Olympus CX41 light microscope for the presence of spherical schizonts and/or the development of hyphae usually surrounded by varying amounts of host granulomatous tissue reaction (Fig. 4.1. C - F). Schizonts were enumerated in infected liver tissue by quantifying the number of schizonts per random field of view at 200 X magnification in triplicate.

4.2.2.2) Histological evaluation of haematoxylin-eosin stained tissue sections

Tissue sections from liver, spleen, heart, kidney and gonad that were fixed in 10 % buffered neutral formalin were processed using standard histological methods (Austin and Austin, 1989). Subsequently histological sections were stained with Harris's haematoxylin and eosin and observed using a Nikon eclipse N1 compound microscope fitted with a Nikon Digital sight DS-U3 camera system and interfacing with NIS-Elements BR software and were examined at 200 X magnification for the presence of spherical schizonts and/or the development of hyphae (Paperna, 1986; Franco-Sierra and Alvarez-Pellitero, 1999; Kocan *et al.*, 2013). Infection intensity was quantified by counting the number of schizonts per

random field of view at 200 X magnification in triplicate. Schizont diameter of *I. hoferi* in the tissues was also examined from live images with a Nikon eclipse N1 compound microscope fitted with a Nikon Digital sight DS-U3 camera system and interfacing with NIS-Elements BR software.

4.2.2.3) In vitro *culture in Eagles Minimal Essential Medium*

Liver tissues were cultured (as described in 2.2.1) and examined microscopically for *I. hoferi* on day 7 and 14. Cultures identified as positive were physically enumerated (as described previously in 2.2.2).

4.2.2.4) Detection of Ichthyophonus hoferi *SSU rDNA in* Rhabdosargus globiceps *liver tissue using qPCR*

Liver tissue fixed in 1.5 mL microcentifuge tubes containing 100 % ethanol (0.03 g) was aseptically transferred to a clean 1.5 mL microcentrifuge tube and rinsed three times by centrifuging the samples in autoclaved deionised water for 5 min at 6,000 x g. Using a Polytron PT 2100 tissue homogeniser samples were then homogenized in 1 mL phosphate buffer saline (PBS) supplemented with 0.1 % Tween 20 and 0.05 % Antifoam A for 1 min 30 sec at 22,000 rpm. Samples were then spun for 10 min at 14,000 x g at 4 °C and the supernatant was removed. The remaining pellet then underwent the same procedure as described by Greeff *et al.* (2012). During extraction two positive and two negative reference tissue samples were included during each extraction process. The positive reference tissue samples were included to account for the efficacy of the DNA extraction process. The negative reference tissue samples, obtained from non-infected *R. globiceps* liver tissue, were homogenized between each sample to ensure that no contamination occurred during sample preparation. real-time qPCR reactions were run on all DNA extracted samples and each qPCR reaction contained 50 ng of the respective genomic DNA, 1 x KAPA SYBR fast qPCR master mix, 10 µM of each respective primer (BW2-F and BW2-R) and PCR grade H_2O. PCR cycling conditions consisted of an initial denaturation at 95 °C for 10 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing temperature of 59 °C for 30 seconds, and elongation at 72 °C for 45 seconds, melt curve from 50 °C for 10 seconds with an increment of 0.5 °C per cycle, to amplify a 299bp product. Reactions were run in triplicate and included; two non-template controls which substituted PCR-grade water for DNA to confirm that the reagents were not

contaminated; a positive PCR control of *I. hoferi* DNA extracted from pure cultures; a negative PCR control of DNA from uninfected *R. globiceps* liver tissue, DNA from two positive extraction reference samples, DNA from two negative extraction reference samples and two standard curves. Standard curves involved running qPCR on tenfold serial dilutions of the DNA prepared for sensitivity testing in Chapter 3. The standard curve was established by plotting quantitation cycle (Cq) values for each dilution versus the log value of the corresponding cell (schizont) number. The amplification efficiency percentage was calculated from the slope of the standard curve using the following formula: $E = (10^{(-1/slope)} - 1) \times 100$. Efficiencies between 90 and 110 % were considered acceptable (Stratagene, 2004). Standard curve precision was evaluated via coefficients of determination (R^2); an R^2 value greater than 0.985 was considered acceptable. All standard curves run throughout all the assays were consolidated to obtain an average standard curve. The linear regression obtained from the standard curve and the amount of tissue inhibition obtained from Chapter 3 were used to determine the intensity of *I. hoferi* in each *I. hoferi* infected tissue sample using their respective threshold cycle (Ct) value. Data collection and real-time analysis occurred at the annealing step of each cycle and melt curve data collection and analysis occurred at each increment. Any samples that recorded a cycle threshold (Ct) values after setting the PCR cycle number to 32 and the baseline threshold value to 200, with a melt peak of 80 ± 0.5 °C on the melt curve graph were considered positive. Samples that failed to record a Cq value or failed to produce a specific melt peak were considered negative.

4.2.3) Ichthyophonus hoferi disease status and statistical analysis

A fish was considered as infected with *I. hoferi* if tissues tested positive with more than one independent diagnostic test and uninfected if all test results were negative or if only one test was positive. The outputs of the different combinations of test results used for the analysis were recorded. Gonadosomatic index (GSI: gonad weight [g] / whole fish weight [g] × 100), hepatosomatic index (HSI: liver weight [g] / whole fish weight [g] × 100), organosomatic index of spleen (SSI: spleen weight [g] / whole fish weight [g] × 100) and condition factor (CF: weight × 100 / (standard length)^3) were calculated (Afonso-Dias *et al*., 2005; Rajaguru, 1992; Richter, 2000) to evaluate fish condition for both infected and non-infected genders and differences were tested by means of a t-test. Significance was assigned to p-values < 0.05. The diagnostic sensitivity (the ability of a test to identify true positives), the diagnostic specificity (the ability of a test to identify true negatives), the positive predictive value (probability that subjects with a positive screening test truly have

the disease), negative predictive value (probability that subjects with a negative screening test truly don't have the disease) as well as the apparent prevalence (percentage of fish infected with the parasite) of each diagnostic technique was calculated. Calculations were predicted using the equations and schematic in Table 4.1 (Banoo *et al.*, 2010). Diagnostic sensitivity (%) = A / (A + C) x 100, diagnostic specificity (%) = D / (D + B) x 100, positive predictive value (%) = A / (A + B) x 100, negative predictive value (%) = D / (D + C) x 100 and apparent prevalence (%) = $T_{Disease}$ / Total x 100.

Table 4.1: Determining the diagnostic sensitivity, the diagnostic specificity, the positive predictive value, negative predictive value and the apparent prevalence of the diagnostic techniques. Columns indicate the actual condition of the subjects, diseased or non-diseased and the rows indicate the results of the test, positive or negative (Banoo *et al.*, 2010).

Test under evaluation	**Reference standard / true infection status**		**Total**
	Disease	**Non-Disease**	
Positive	A (True Positive)	B (False Positive)	$T_{Test\ Positive}$
Negative	C (False Negative)	D (True Negative)	$T_{Test\ Negative}$
Total	$T_{Disease}$	$T_{Non\text{-}Disease}$	

4.3) Results

4.3.1) Host samples condition and overall diagnosis of Ichthyophonus hoferi-*infection*

Biometrical data of the fish are shown in Table 4.2. The collected fish (19 females and 16 males) had a mean standard length of 47.5 ± 1.5 cm (21.5 - 59.5) with an average weight of 2.1 ± 0.2 kg (1.0 - 3.7 kg). Females had an average weight of 2.1 ± 0.2 kg (1.0 - 3.7 kg) and length of 46.1 ± 2.2 cm (21.5 – 59.5 cm). Males had an average weight of 2.2 ± 0.2 kg (1.0 -3.7 kg) and length of 49.1 ± 1.7 cm (40.0 – 59.0 cm). Of the 35 fish, 12 females and 9 males were regarded as infected with *I. hoferi* with an apparent infection prevalence, determined by tissues testing positive with more than one independent test, of 60.0 % (21/35).

Table 4.2: Means and standard deviations of standard length (cm), Total weight (kg), gonadosomatic index (GSI), hepatosomatic index (HSI), organosomatic index of spleen (SSI) and condition factor (CF) in *Rhabdosargus globiceps* in both *I. hoferi*-infected and non-infected genders. *Significant difference within genders (P < 0.05), parenthesis: 95 % confidence interval.

	Total	**Male**		**Female**	
		Infected	**Non-infected**	**Infected**	**Non-infected**
No. of fish	35	9	7	12	7
Standard length	47.457 ± 8.661	46.556 ± 6.789	52.429 ± 6.432	43.292 ± 10.597	50.786 ± 6.217
Total weight	2.101 ± 0.940	1.791 ± 0.686	2.642 ± 0.858	1.754 ± 1.037	2.554 ± 0.926
HSI	1.264 ± 0.295	1.272 ± 0.146	1.232 ± 0.245	1.274 ± 0.259	1.271 ± 0.530
SSI	0.090 ± 0.031	0.087 ± 0.025	0.082 ± 0.036	0.104 ± 0.031	0.081 ± 0.029
GSI	0.745 ± 0.669	0.306 ± 0.287	0.327 ± 0.240	0.789± 0.539*	1.651 ± 0.637*
CF	2.119 (1.425 - 2.813)	1.711 (1.635 - 1.788)	1.778 (1.588 - 1.968)	1.760 (1.692 – 1.828)	1.865 (1.666 - 2.064)

Between infected and non-infected females there were no significant differences with regards to their SSI, HSI and CF, however there was a significant difference between infected and non-infected females GSI (p value = 0.012). There were no significant differences between infected and non-infected males with regards to their SSI, HSI, GSI and CF (Table 4.2). The outputs of the different combinations of test results used for the analysis are summarized in Table 4.3 and a detailed list of the statistical values representing diagnostic sensitivity, diagnostic specificity, positive predictive value, negative predictive value and apparent prevalence for the various diagnostic methods is presented in Table 4.4. Only 8 fish tested positive from all the methods tested. When infection detection by two or more methods was considered as the reference for overall disease status of the host, 14 out of the 35 fish were regarded as negative of which 7 of fish tested positive from only one of the available tests (Table 4.3). The test with the highest apparent diagnostic sensitivity was the collective wet-mount squash preparations of all tissues with a diagnostic sensitivity of 95.24 %. This test however had the lowest diagnostic specificity of 57.14 % suggesting a high number of false positive tests. The test with the highest combination of diagnostic sensitivity and diagnostic specificity was qPCR of homogenised liver tissue (diagnostic sensitivity of 90.48 % and diagnostic specificity of 100 %), followed closely by culture of homogenized liver tissue after 14 days (diagnostic sensitivity of 85.71 % and diagnostic specificity of 100 %). Real-time qPCR, culture and histology of liver were 100 % specific with positive predictive values of 100 %. The test with the highest negative predictive value was the collective results from individual squash mounts of all the tissues from the same individual fish (88.89 %), followed closely by qPCR of homogenised tissue (87.50 %) and the test with the lowest negative predictive value was histology of the liver only (42.42 %) (Table 4.4).

4.3.1.1) Wet-mount squash preparation examination of tissues

Fish tissue diagnosed as positive for *I. hoferi* using wet-mount squash preparations exhibited spherical schizonts in the infected organs (Fig. 4.1.C – 4.1.F). These schizonts displayed a thick wall and a granular cytoplasm and in some cases distension of the schizont wall forming germination tubes (Fig. 4.1.F). The collective wet-mount squash preparation of all tissues (liver, spleen, heart, kidney and gonad) produced the highest diagnostic sensitivity of all the methods as the proportion of fish confirmed infected with *I. hoferi* that tested positive using the test was 95.24 %. The collective wet-mount squash preparation of all tissues conversely produced the lowest diagnostic specificity as the proportion of true

negative fish that produced a negative result using this test was 57.14 % (8/14). Since a false negative result was recorded for using the test, the probability that a fish is correctly identified as negative for *I. hoferi* using the test was 88.89 % (Table 4.3 and 4.4). When compared to the results of the wet-mount squash preparation of liver alone, the collective examination of all of the tissues (spleen, heart, liver and kidney) performed better in detecting *I. hoferi* infected individuals as 4 of the 21 (19.04 %) fish that were considered to be infected produced false negative results by wet-mount squash preparation of liver alone. Among those fish considered to infected, wet-mount squash preparation of spleen detected 95.24 % of all fish considered to be infected, against 85.71 % for kidney, 80.95 % for liver, 76.19 % for heart and 0 % for gonad (Fig. 4.2).

4.3.1.2) Histological examination of haematoxylin-eosin stained tissue sections

Observed stages of *I. hoferi* in fish diagnosed as infected were the spherical schizonts varying in size, 33.1 ± 18.8 µm in diameter (9.8 - 93.6 µm in diameter) (Fig. 4.3) surrounded and enclosed by an inflammatory, granulomatous host reaction (Fig. 4.1.G and 4.1.H). The collective histological examination of all tissues (liver, spleen, heart, kidney and gonad) detected 13/21 (61.90 %) of the fish that were regarded as infected with *I. hoferi*, however because a false positive result was produced by histology (Table 4.3), the probability that a fish was correctly identified as positive for *I. hoferi* using the test was 92.86 % (13/14). The proportion of fish that for which a true negative result was attained using this test was 92.86 % (13/14) (Table 4.4). Since false negative results were attained for 8 fish using this test, the probability that a fish is correctly identified as negative for *I. hoferi* using the test was 61.90 % (Table 4.3 and 4.4). Histological examination of liver tissue alone detected *I. hoferi* schizonts in only 9.52 % (2/21) of all fish considered to be infected, against 61.90 % for spleen, 19.05 % for kidney, 4.76 % for heart and 0 % for gonad (Fig. 4.2).

Table 4.3: Summary count of the 13 combinations of positive / negative test results collected from the various diagnostic methods used in detecting *I. hoferi* in *Rhabdosargus globiceps* (n=35).

Tissue squash	**Histology**	**Culture**		**qPCR**		**# Positive tests**	**Number of fish**
		Non-homogenized Day 14	**Homogenized Day 14**	**Non-homogenized**	**Homogenized**		
+	+	+	+	+	+	6	8
+	+	+	-	+	+	5	1
+	+	-	+	+	+	5	2
+	-	+	+	+	+	5	4
+	-	+	+	-	+	4	1
-	-	+	+	+	+	4	1
+	-	-	+	-	+	3	1
+	+	-	+	-	-	3	1
+	-	-	-	+	+	3	1
+	+	-	-	-	-	2	1
+	-	-	-	-	-	1	6
-	+	-	-	-	-	1	1
-	-	-	-	-	-	0	7
						Total	**35**

4.3.1.3) In vitro examination of Ichthyophonus hoferi *in cultured liver tissue*

In vitro examination of cultures that were incubated at 15 ˚C for 14 days detected 71.43 % (15/21) of all fish considered to be infected when liver tissues were not homogenized and detected 85.71 % (18/21) of the fish when liver tissues were homogenized. The probability that fish not infected with *I. hoferi* had a negative result for the *in vitro* culture tests incubated for 14 days before homogenization was 70.00 % as 6 of the 21 (28.57 %) fish were regarded as false negatives. The probability that fish not infected with *I. hoferi* had a negative result for the *in vitro* culture tests incubated at 15 ˚C after homogenization was 82.35 % as only 3 of the 21 (14.28 %) fish were regarded as false negatives. The probability that subjects with a positive screening test truly had the disease when using *in vitro* culture was 100 % as there were no false positives produced using the *in vitro* tests (Table 4.3 and 4.4).

4.3.1.4) Detection of Ichthyophonus hoferi *SSU rDNA in* Rhabdosargus globiceps *liver tissue using qPCR*

Samples that produced a qPCR product when assayed with the SSU rDNA primers indicated the presence of amplifiable DNA and were regarded as positive for infection when using the test. Of the 35 fish sampled, qPCR after liver tissues were homogenised detected 90.48 % (19 / 21) of all *I. hoferi*-infected fish and qPCR before liver tissues were homogenized detected 80.95 % (17/21) of all fish regarded as infected. The probability that subjects with a positive screening test truly had the disease when using qPCR was 100 % as there were no false positives produced using this test. The probability that fish not infected with *I. hoferi* produced a negative result for qPCR before homogenization was 78 % (14/18) as 4 fish were regarded as false negatives, and the probability that fish not infected with *I. hoferi* that had a negative result for qPCR after the homogenization of the liver was 88 % (14/16) as only 2 fish were regarded as false negatives using the test (Table 4.3 and 4.4). When the number of schizonts from infected samples were calculated using the Cq values from qPCR before and after the liver tissues were homogenised, two samples with very low schizont counts (<1 schizont in 30 mg of tissue) were detected by the qPCR method after the homogenization of the liver tissue (Fig. 4.4). The amplification efficiency percentage calculated from the slope of the standard curve from qPCR was 96.7 % and the standard curve precision evaluated via coefficients of determination was 0.99.

Table 4.4: Diagnostic sensitivity, diagnostic specificity, positive predictive value and negative predictive value of all diagnostic tests used in detecting *Ichthyophonus hoferi* in *Rhabdosargus globiceps* (n=35). Total *I. hoferi*-infected fish were 21 out of the 35 examined with an overall infection prevalence of 60 %. Hom: Homogenised tissue

Diagnostic test	Diagnostic sensitivity (%)	Diagnostic specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Apparent prevalence (%)
Squash (All)	95.24	57.14	76.92	88.89	74.29
Squash (Liver)	80.95	71.43	80.95	71.43	60.00
Histology (All)	61.90	92.86	92.86	61.90	40.00
Histology (Liver)	9.52	100.00	100.00	42.42	5.71
Culture (Non-hom Day 7)	52.38	100.00	100.00	58.33	31.43
Culture (Non-hom Day 14)	71.43	100.00	100.00	70.00	42.86
Culture (Hom Day 7)	66.67	100.00	100.00	66.67	40.00
Culture (Hom Day 14)	85.71	100.00	100.00	82.35	51.43
qPCR Non-hom	80.95	100.00	100.00	77.78	48.57
qPCR Hom	90.48	100.00	100.00	87.50	54.29

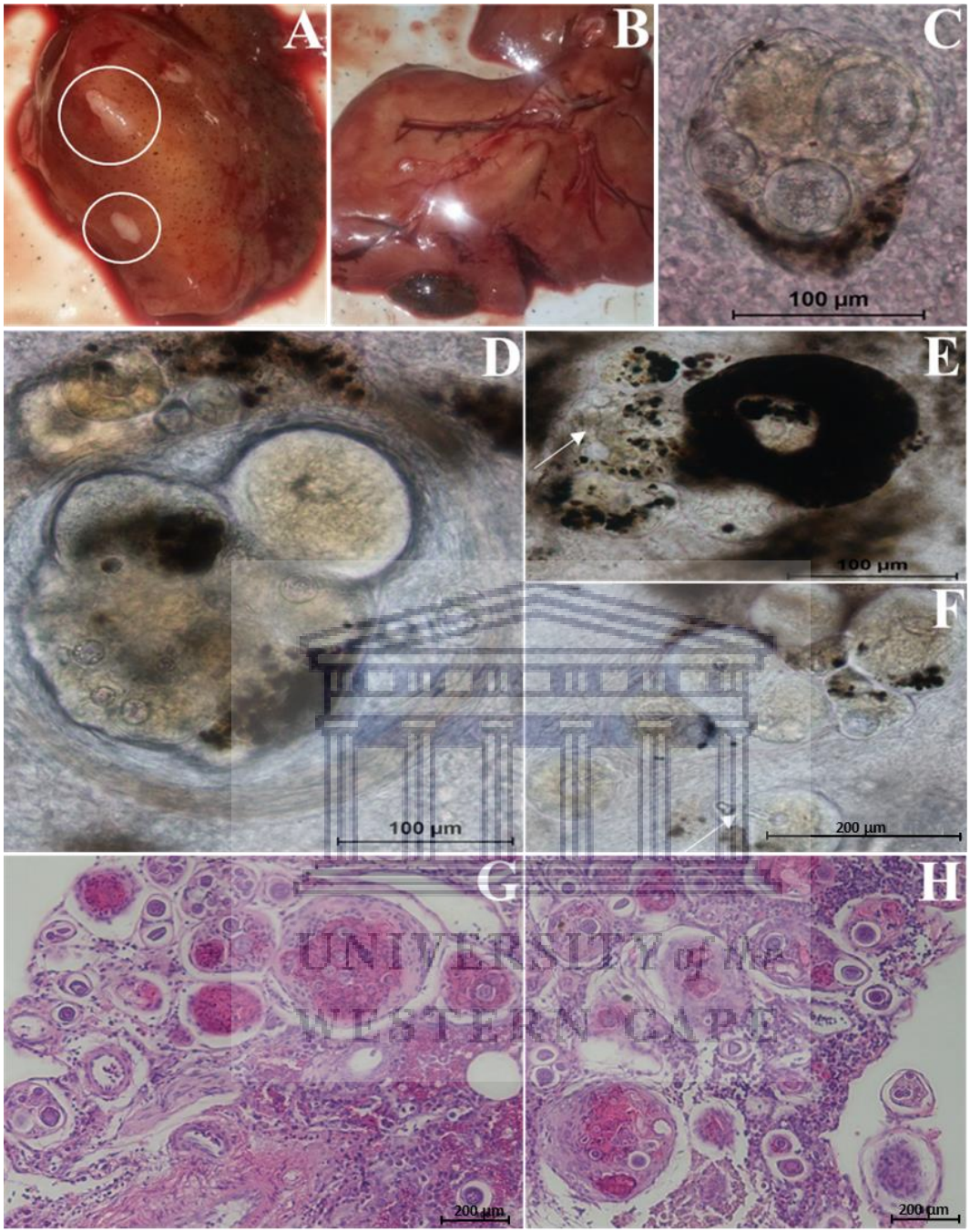

Fig. 4.1.A – 4.1.H: Macroscopic and microscopic examinations of *Ichthyophonus hoferi*-infected *Rhabdosargus globiceps* liver tissue. A: Macroscopic examination of liver tissue with white nodular lesions. B: Observed firm, darkly pigmented nodule. C & D: Squash preparation of liver tissue showing the spherical schizonts of *I. hoferi.* E & F: Distension of the schizont wall forming germination tubes surrounded by host granulomatous tissue. G & H: Histological examinations of haematoxylin-eosin stained *I. hoferi* infected tissue sections.

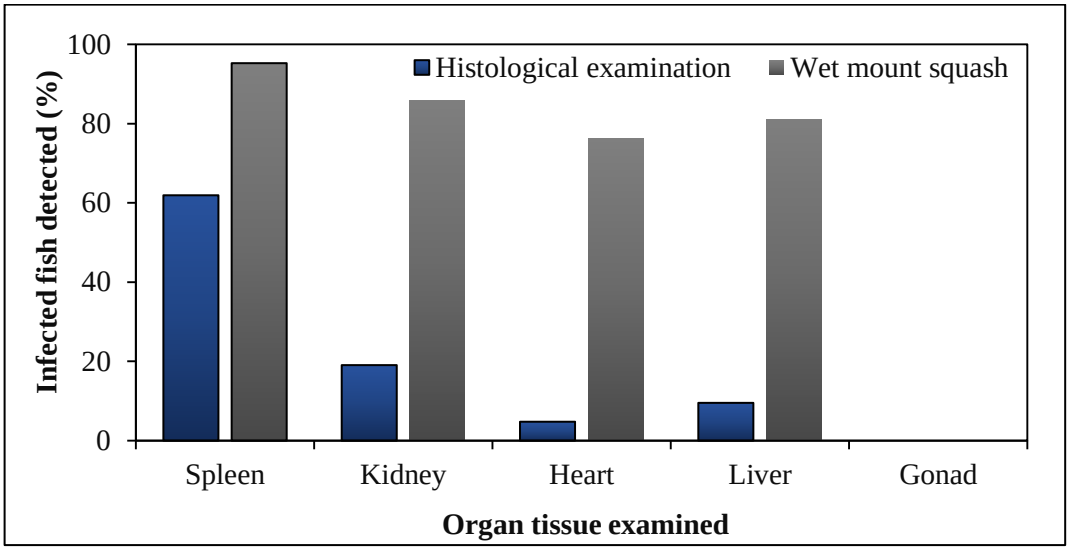

Fig. 4.2: The proportion of fish (n=35) regarded as infected with *I. hoferi* after the examination of the kidney, heart, spleen, gonad and liver using wet-mount squash preparation and histological examination.

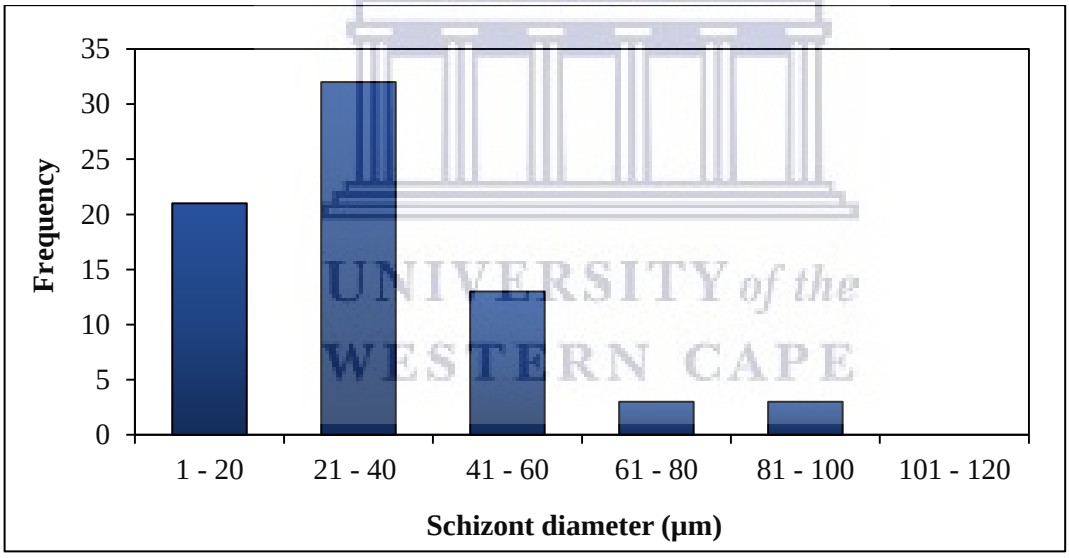

Fig. 4.3: Frequency occurrence of schizont diameter in histological examined Harris's haematoxylin and eosin stained tissue sections based on the measurement of 75 schizonts.

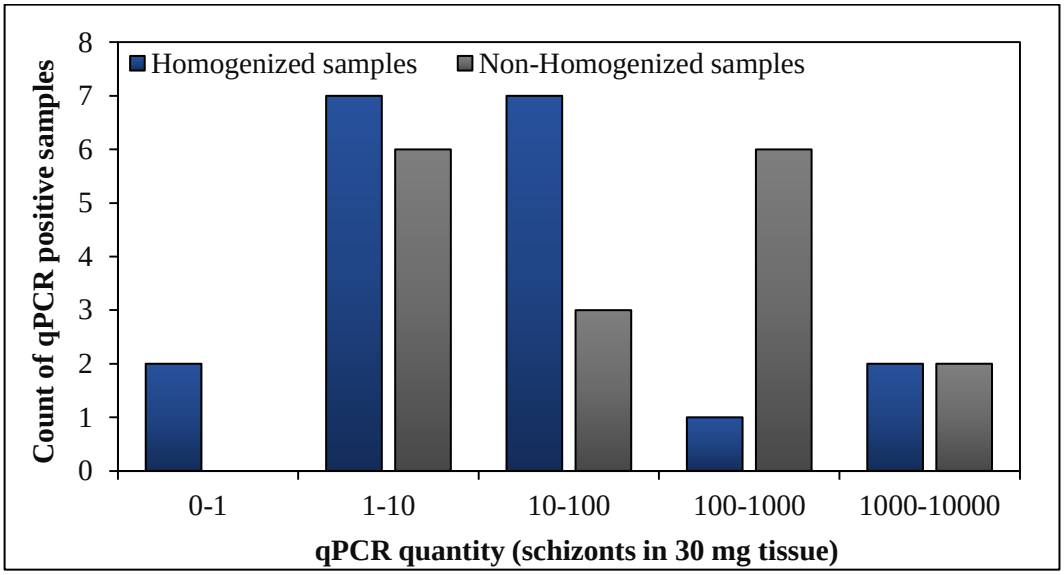

Fig. 4.4: Proportion of real-time quantitative PCR (qPCR) positive samples from *Rhabdosargus globiceps*. Groups arranged by increasing schizont count. Results indicate that samples with low schizont numbers (<1 schizont in 30 mg of tissue) were less likely to be detected by the qPCR method before the homogenization of liver tissue.

4.4) Discussion

In the present study, we tested the diagnostic techniques; wet-mount squash preparations (Paperna, 1986), histological evaluation (Kocan *et al.*, 2011), *in vitro* tissue explant culture (Hershberger *et al.*, 2002) and qPCR (Chapter 3) in detecting *I. hoferi* in *Rhabdosargus globiceps* host tissues and compared the methods ability in detecting infections to one another. We used the results from all the tests (i.e. positive or negative) to define the overall infection status according to which each individual test was evaluated to assess its respective diagnostic precision. The resultant prevalence of *I. hoferi*-infection in our *R. globiceps* samples was 60 % (21/35) when all test results were combined. Real-time qPCR was the test that had the highest combination of diagnostic sensitivity and diagnostic specificity (and NPV and PPV) in detecting *I. hoferi* infections. Real-time qPCR and *in vitro* culture were highly specific tests (100 %) with positive predicative values of 100 % as neither of these tests recorded false positive results in this study. These tests could be used to confirm presumptive diagnoses derived from some of the other tests evaluated in this study. The collective wet-mount squash preparation of all of the tissues tested was able to detect the highest proportion (20/21) of all the fish infected with *I. hoferi*. The test also had the highest proportion of false negatives (testing 6 fish positive that were regarded as negative), thus overestimating infection prevalence (apparent prevalence is 74.29 %

compared with the true prevalence of 60.0 %). The data obtained from wet-mount squash preparation of all of the tissues showed that the examination of all tissues of the *R. globiceps* samples are imperative to accurately determine the true infection prevalence as the proportion of fish confirmed infected with *I. hoferi* that tested positive using the test improved from 80.95 % to 95.24 % after the examination of all tissues using wet-mount squash preparations (Table 4.4). This was also observed in histological examinations whereby the proportion of fish infected with *I. hoferi* that were detected as positive using the test was 9.52 % after the examination of liver alone against 61.90 % after the examination of all tissues. Since the wet-mount squash preparation of spleen detected 95.2 % of all fish considered to be infected, against 85.71 % for kidney, 80.95 % for liver, 76.19 % for heart and 0 % for gonad (Fig. 4.2), sensitivity of the tests depends on the tissue being examined and selecting the appropriate organ for testing is critical in determining true infection prevalence. This was highlighted in Mcvicar (1999) which reported that the most obvious lesions occur in the white muscle in haddock and in the heart in herring and in the liver and kidney in plaice. Similarly Whipps *et al.* (2006) reported that the primary target tissue for *I. hoferi*-infection in Yukon River Chinook salmon appears to be the cardiac muscle. Although the liver, kidney and heart may be infected with *I. hoferi*, the target tissue for *I. hoferi*-infection in *R. globiceps* appears to be the spleen. To obtain the best estimate of *I. hoferi* prevalence, it is recommended that more than one tissue type is collected for analysis (Table 4.4).

Ichthyophonus hoferi commonly elicits a granulomatous response resulting in an infection which is focal rather than diffuse (Mcvicar, 1999) (Fig. 4.1). The differences therefore observed in the diagnostic test sensitivities could also be a result of the uneven distribution of the parasite in host tissues (Kocan *et al.*, 2011) and the size of the sample relative to the infected organ and host. Kocan *et al.* (2011) described the variable distribution in a 3-dimensional analysis of schizonts in Pacific herring skeletal muscle where *Ichthyophonus* sp. schizonts had variable distribution within host tissue as well as between individuals. Histological evaluation of our samples used a section of tissue approximately 5 µm thick, while squash up to 22 mm, tissue explant culture up to 0.5 g of tissue and PCR up to 0.03 g of tissue thus significantly decreasing the probability of detecting infections and possibly a reason for the low detection prevalence recorded using histology. Thus, it is possible that by sampling a small sample from a single organ, the parasite is not sampled and therefore not detected. However although the qPCR method used in this study tested a 0.03 g piece of liver tissue, we were still able to obtain a diagnostic sensitivity of 81.0 % before tissues

were homogenized. White *et al.* (2013) stated that pooling samples from different organs and homogenization of larger samples could have the potential for enhancing qPCR diagnostic sensitivities. Because the differences observed between the diagnostic techniques after evaluation of the same tissues might result from the uneven distribution of the parasite within the host tissues, we tested this hypothesis and examined whether the homogenization of tissue would improve the diagnostic sensitivity of some of the assays, namely culture and qPCR. The homogenization process involved blending the liver tissue in a HEPES buffered solution before being assayed to ensure that all fractions of the sample were equal in composition. We observed a 9.53 % increase in diagnostic sensitivity from qPCR, and a 14.28 % increase in diagnostic sensitivity from *in vitro* culture after the liver tissues were homogenized in buffer (Table 4.4). This showed that the diagnostic sensitivity of the culture and qPCR assay can be improved when tissues have been homogenized. Although PCR tests generally have low minimum detection limits, and thus are inherently sensitive diagnostic tests (Cunningham, 2002; Bott *et al.*, 2010), qPCR after the homogenization of the liver tissue also proved to be more effective than qPCR before the homogenization of the liver tissue for identifying *I. hoferi* DNA at low concentrations (Fig. 4.4).

In conclusion, the likelihood of misdiagnosing an infected fish is directly proportional to; the amount of tissue(s) sampled, the distribution of the parasite within individual tissues and the intensity of infection. The examination of all tissues using more than one diagnostic method to accurately determine the true *I. hoferi*-infection prevalence of ***Rhabdosargus globiceps*** is imperative. In this study the wet-mount squash examination technique was a viable diagnostic test to detect *I. hoferi*-infections, especially when evaluating all the visceral organs of the host (Table 4.4); however this does not necessarily imply that squash examination should replace any other method, but rather be used as a screening test (Thrusfield, 2005) and used in conjunction with another method in order to prevent misidentification, such as qPCR. Therefore individuals with a positive test results which are classified as diseased by wet-mount squash preparation (the screening test) will require further investigation for definite diagnosis through qPCR. It is important to note that a diagnostic technique is rarely 100 % accurate when testing for infection in naturally infected samples and therefore false positive and false negative results can occur and the differences observed in detection between assays may be attributed to a variety of causes such as focal infections, the size of tissues examined and/or an inability of test to detect light infections. The pros and cons of each diagnostic technique need to be acknowledged

before sampling and diagnostic technique selection has to be based on other considerations, such as the objectives of the assessment, logistics of sampling, the total cost and speed required for diagnosis. The data from this chapter further fulfils the last recommendation by Hiney and Smith (1998) for the validation of the real-time qPCR technique developed in Chapter 3.

CHAPTER 5

Testing for the occurrence of *Ichthyophonus hoferi* in local mullet and pelagic fish species in South Africa

Abstract

Ichthyophonus hoferi is a widely distributed parasite of fish and displays low host specificity. In Southern Africa *I. hoferi* has previously been identified at a prevalence of 2 % in flathead mullet (*Mugil cephalus*), from the Kowie lagoon in 1986 and more recently from multiple species on exhibit at the Two Oceans Aquarium. The aims of the current study were to determine whether *I. hoferi* is present in local *Sardinops sagax*, *Mugil cephalus*, *Merluccius capensis*, *Merluccius paradoxus* and *Engraulis capensis* subpopulations of South Africa, which are fed to exhibited fish at the Two Oceans Aquarium. Fish were opportunistically collected from bycatch received from inland surveys by the Department of Agriculture Forestry's and Fisheries (DAFF). A total of 748 fish were necropsied and screened for *I. hoferi*-infection using wet-mount squash preparations. Real-time quantitative polymerase chain reaction (qPCR) was used to confirm infection in any samples that exhibited any pathology or tissue with granulomas suspect of infection with *I. hoferi*. Of these, 438 were *Sardinops sagax* (30 Western, 341 Southern and 67 Western stocks), 159 were *Mugil cephalus* (9 Eastern and 150 Western stocks), 42 were *Merluccius capensis* (26 Southern and 16 Western stocks), 50 were *Merluccius paradoxus* (25 Southern and 25 Western) and 59 were *Engraulis capensis* (39 Eastern and 20 Southern stocks). None of the fish collected during this study tested positive for infection with *I. hoferi*. Although *I. hoferi* was not prevalent in the local populations at a detectable level at the time of this study, possibly due to the small sample sizes within the subpopulations of each species, caution must be exercised with these results and an expanded surveillance assessment is recommended to provide a better estimate of infection prevalence within and between these species subpopulations.

5.1) Introduction

Ichthyophonus hoferi is a mesomycetozoan parasite of fishes (Mendoza *et al.*, 2002) that causes a systemic granulomatous disease, Ichthyophoniasis. *I. hoferi* is globally distributed (Fig. 1.3) and displays low host specificity with infections varying among infected individuals and species (Mcvicar, 1999; Gregg *et al.*, 2014). *I. hoferi* is known to be highly pathogenic and infections with *I. hoferi* are regarded as being detrimental to the infected host (Rahimian, 1998; Franco-Sierra and Alvarez-Pellitero, 1999; Mcvicar, 1999; Kocan *et al.*, 2009). For commercial fisheries and aquaculture industries infections with *I. hoferi* can reduce the quality of the flesh of the fish (Rahimian, 1998; Mcvicar, 1999) negatively impacting the marketability of higher value products and increasing the potential of the fish to be rejected for human consumption (Lafferty *et al.*, 2015). Within South Africa, *I. hoferi* has been reported along the South African coast, from flathead mullet (*Mugil cephalus)* from the Kowie lagoon in the South Eastern Cape of South Africa at a prevalence of ~2 % (Paperna, 1986), and more recently from multiple species on exhibit at the Two Oceans Aquarium thought to have been infected through the feeding of un-processed tissues from infected marine wild caught fish.

Determining disease prevalence and distribution is important for commercial fisheries and aquaculture industries to identify high risk fishing areas to avoid the potential risks of introducing infected fish into both captive and wild fish populations through feeding of infected fish material. The natural transmission of *I. hoferi* occurs among susceptible hosts via predation or scavenging (Mcvicar, 1999) and in cultured fish the occurrence of *I. hoferi* is often traced to the feeding of un-processed tissues from infected fish (Hansen and Pethon, 1985; Kocan *et al.*, 1999; Mcvicar, 1999). There is however difficulty in determining the true prevalence of *I. hoferi*-infected hosts. Inaccurate recordings of prevalence (Patterson, 1996) can be caused by the non-uniform distribution of *I. hoferi* between geographical areas within wild fish populations, within infected individuals (Mcvicar, 1999) and due to severely infected hosts dying before being sampled (Sitja-Bobadilla and Alvarez-Pellitero, 1990; Kocan and Hershberger, 2006). Although there are difficulties in determining the true prevalence of infection in natural populations, with the development of multiple methods for detecting *I. hoferi* such as wet-mount squash preparations (Rahimian and Thulin, 1996), histological evaluation (Kocan *et al.*, 2011), *in vitro* tissue explant culture (Mcvicar, 1999; Hershberger *et al.*, 2002), molecular identification such as conventional

polymerase chain reaction (PCR) (Whipps *et al.*, 2006) and qPCR (as described in Chapter 3), prevalence estimations of *I. hoferi* are possible.

The objective of this study was to test for the occurrence of *I. hoferi* in local flathead mullet and pelagic species such as sardines, anchovies and hake around the coast of South Africa. This was accomplished through sampling opportunistically from multiple geographical areas around South Africa. Presumptive diagnosis was made by the evaluation of fish organ tissues using wet-mount squash preparations and confirmatory diagnosis by qPCR analysis. The infection prevalence from the different subpopulations of the different species around the South African coastline would improve our understanding of the distribution of *I. hoferi* around South Africa and would aid in identifying risk areas and potential pathways for *I. hoferi* transmission into captive populations. It should also be acknowledged that the prevalence levels recorded can only be related to the detection method used and should only be used as an estimate of infection prevalence in the source population.

5.2) Materials and methods

5.2.1) Sampling

Subpopulations of local sardines (S*ardinops sagax*), anchovies (*Engraulis capensis*), hake (*Merluccius capensis* and *Merluccius paradoxus*) and flathead mullet (*Mugil cephalus*) were provided from routine fisheries assessment surveys off the coast of South Africa (Fig. 5.1). 748 fish in total were necropsied and examined for *I. hoferi*-infection. Of these, 438 were *S. sagax* (30 Western, 341 Southern and 67 Western stocks), 159 were *M. cephalus* (9 Eastern and 150 Western stocks), 42 were *M. capensis* (26 Southern and 16 Western stocks), 50 were *M. paradoxus* (25 Southern and 25 Western) and 59 were *E. capensis* (39 Eastern and 20 Southern stocks). Samples were received frozen from bycatch collected from inshore and pelagic surveys by the Department of Agriculture Forestry's and Fisheries (DAFF) and were kept frozen until subsequent laboratory processing and analysis. Site locations ranged from the Durban harbour on the East coast of South Africa (Indian Ocean) to Port Nolloth on the West coast of South Africa (Atlantic Ocean) (Fig. 5.1). Fish demographic data which included length, gender, weight, organ weight and collection location (latitude, longitude) were recorded prior to dissection. To evaluate differences between fish sizes sampled between coasts, a t-test was used with significance assigned to p-values <0.05. Gonadosomatic index (GSI: gonad weight × 100 / weight), hepatosomatic

index (HSI: liver weight × 100 / weight), organosomatic index of spleen (SSI: spleen weight × 100 / weight) and condition factor (CF: weight × 100 / (standard length)^3) were calculated to evaluate sardine, anchovy and mullet fish condition. GSI, HSI, SSI and CF were calculated for both genders. To evaluate differences between genders in and between coasts a t-test was used. Significance was assigned to p-values <0.05.

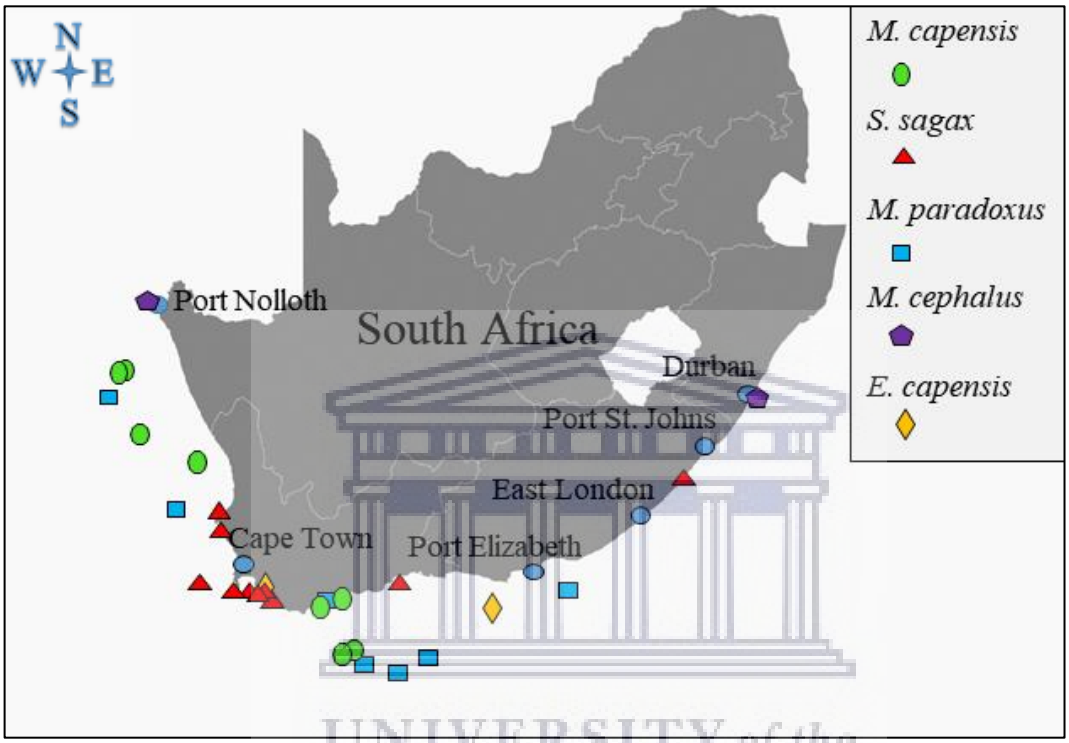

Fig. 5.1: Bycatch locations from which *Merluccius capensis*, *Sardinops sagax*, *Merluccius paradoxus*, *Mugil cephalus* and *Engraulis capensis* were collected for assessment of *I. hoferi*-infection. Some important coastal towns and cities are shown.

5.2.2) Detection of Ichthyophonus hoferi *in samples*

The liver, spleen, heart, gonad and kidney of sardines, anchovies and mullet were examined microscopically for *I. hoferi*-infection in triplicate using the wet-mount squash preparation technique as described in 4.2.2.1. Only the livers of the hake were examined due to the availability of samples. Examined samples suspicious for infection were fixed in 100 % ethanol for real-time quantitative polymerase chain reaction (qPCR) for confirmation. Additional samples for qPCR analysis were also randomly selected. To evaluate whether subpopulations were free from disease at a minimum expected prevalence of 2 % (Paperna,

1986), with a confidence interval of >95 %, and with sensitivity of 95.2 % and specificity of 100 % for tests used (Chapter 4), FreeCalc software (Cameron, 1999) was used.

5.3) Results

The sampling and biometrical data of the fish sampled are shown in Table 5.1 and 5.2. The average size and weight of *S. sagax* sampled on the South coast were significantly higher than those sampled on the East and West coast and the average size of *M. capensis* sampled on the West coast were significantly higher than those sampled on the South coast (t-test, $P < 0.05$) (Table 5.1). After examination of all samples, there were 4 *Mugil cephalus* and 2 *Merluccius paradoxus*, that were observed to contain granulomatous tissue reaction with microscopic inclusions when subjected to wet-mount squash examination. These however had no amplification when subjected to qPCR. All other samples subjected to DNA extraction and subsequently to qPCR had no amplification. The amplification efficiency percentage calculated from the slope of the standard curve was 96 %. Standard curve precision evaluated via coefficients of determination was 0.99 (Fig. 5.2). The cycle threshold (Ct) values of replicate samples (standards and controls) differed by no more than one cycle. No amplification occurred in the negative-control samples and only those samples included as positive controls for the real-time PCR produced a positive result. Analysis of these results using the FreeCalc software program (Cameron 2002), suggests that the number of samples from subpopulations of *E. capensis*, *M. capensis*, *M. paradoxus* and *M. cephalus* were not adequate to conclude that the population is free from *I. hoferi*-infection within those species. The number of samples from S. *sagax* South coast subpopulation was adequate to conclude that the subpopulation is free from disease at the expected minimum prevalence of 2 % at a confidence interval of >99 %. The number of samples from subpopulations of S. *sagax* from the East and West coast was not adequate to conclude that the population is free from *I. hoferi*-infection.

Table 5.1: Sampling details for sardines (S*ardinops sagax*), anchovies (*Engraulis capensis*), hake (*Merluccius capensis* and *Merluccius paradoxus*) and mullet (*Mugil cephalus*) tested for *Ichthyophonus hoferi*, showing sample species, subpopulation, number of fish, their mean total length ± standard deviations and mean weight ± standard deviations. [E/S/W]Significant difference between those subpopulations (P <0.05).

Geographical region	Species	Number sampled	Average length (cm)	Average weight (g)	Number of positive samples
East coast	S*ardinops sagax*	30	14.997 ± 0.868[S]	35.843 ± 5.02[S]	0
	Mugil cephalus	9	18.389 ± 3.508	122.350 ± 84.208	0
	Engraulis capensis	39	12.525 ± 1.274	31.874 ± 8.604	0
South coast	S*ardinops sagax*	341	19.009 ± 2.569[EW]	71.943 ± 27.524[EW]	0
	Merluccius capensis	26	29.262 ± 11.977[W]		0
	Engraulis capensis	20	13.570 ± 1.294	33.712 ± 8.045	0
	Merluccius paradoxus	25	44.004 ± 11.814		0
West coast	S*ardinops sagax*	67	15.067 ± 1.609[S]	44.082 ± 17.794[S]	0
	Merluccius capensis	16	60.688 ± 8.977[S]		0
	Merluccius paradoxus	25	37.564 ± 10.681		0
	Mugil cephalus	150	19.552 ± 3.374	126.380 ± 54.745	0

Table 5.2: Means and standard deviations of gonadosomatic index (GSI), hepatosomatic index (HSI), organosomatic index of spleen (SSI) and condition factor (CF) in sardines (S*ardinops sagax*), anchovies (*Engraulis capensis*) and mullet (*Mugil cephalus*). $^{E/S/W}$Significant difference between those subpopulations (P <0.05). *Significant difference between genders within subpopulations (P <0.05).). Parenthesis: 95 % confidence interval.

Geographical Region	Species	Gender	HSI	SSI	GSI	CF
East coast	S*ardinops saga*x	**Total**	3.973 ± 0.725^{S}	0.166 ± 0.204	1.437 ± 1.187^{SW}	1.066 (1.041 – 1.091)^{W}
		Male	3.841 ± 0.621	0.119 ± 0.115	1.060 ± 0.924*	1.073 (1.017 – 1.128)
		Female	4.236 ± 0.884	0.213 ± 0.28	2.192 ± 1.338*	1.053 (0.999 – 1.106)
	Mugil cephalus	**Total**	0.803 ± 0.413	0.132 ± 0.070	0.553 ± 0.365	1.779 (1.724 – 1.834)
		Male	0.617 ± 0.203	0.134 ± 0.080	0.293 ± 0.186*	1.748 (1.553 – 1.942)
		Female	1.036 ± 0.233	0.129 ± 0.066	0.876 ± 0.239*	1.818 (1.683 – 1.953)
	Engraulis capensis	**Total**				1.626 (1.548 – 1.704)^{S}
		Male				1.618 (1.343 – 1.893)
		Female				1.639 (1.556 – 1.721)
South coast	S*ardinops saga*x	**Total**	2.139 ± 1.687^{EW}	0.168 ± 0.106^{W}	3.139 ± 1.171^{EW}	1.010 (0.945 – 1.074)^{W}
		Male	2.171 ± 1.583	0.172 ± 0.098	2.968 ± 0.990*	0.995 (0.968 – 1.021)
		Female	2.092 ± 1.833	0.160 ± 0.119	3.403 ± 1.369*	1.033 (0.999 – 1.066)
	Engraulis capensis	**Total**				1.354 (1.295 – 1.413)^{E}
		Male				1.422 (1.22 – 1.623)
		Female				1.309 (1.237 – 1.381)

West coast	*Sardinops sagax*	**Total**	3.706 ± 1.181[S]	0.247 ± 0.097[S]	2.272 ± 1.127[SE]	1.257 (1.223 – 1.291)[SE]
		Male	3.640 ± 1.205	0.249 ± 0.083	2.151 ± 1.115	1.269 (1.214 – 1.323)
		Female	3.787 ± 1.167	0.246 ± 0.109	2.421 ± 1.141	1.243 (1.203 -1.282)
	Mugil cephalus	**Total**	0.951 ± 0.425	0.177 ± 0.086	0.667 ± 0.436	1.690 (1.453 – 1.927)
		Male	0.910 ± 0.418	0.159 ± 0.075	0.4334 ± 0.209*	1.589 (1.515 – 1.663)*
		Female	1.019 ± 0.430	0.210 ± 0.094	1.058 ± 0.438*	1.860 (1.578 – 2.141)*

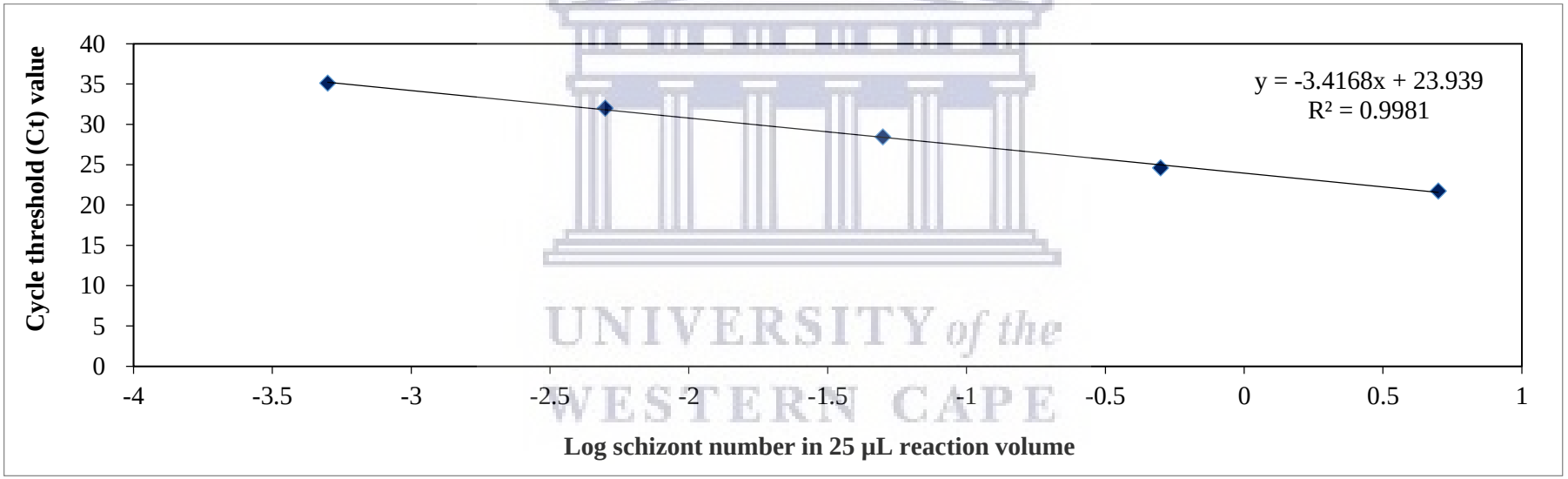

Fig. 5.2: Average cycle threshold (Ct) value obtained following qPCR analysis on serial dilutions of *I. hoferi* DNA obtained from known quantities of *I. hoferi*. Real-time qPCR was carried out using the *I. hoferi* specific primers, BW2-F and BW2-R. The amplification efficiency percentage calculated from the slope of the standard curve was 96 %. Standard curve precision evaluated via coefficients of determination was 0.99.

5.4) Discussion

Paperna, (1986) revealed that 2 out of 86 flathead mullet (*Mugil cephalus*) examined from the Kowie lagoon were heavily infected with *I. hoferi* (2 % prevalence). Assuming that if the pathogen was present in at least 2 % of fish, was uniformly distributed among shoals and schools of fish within a subpopulation of >100000 individuals at a confidence level of 95 %, and diagnostic sensitivity for microscopic examination was 95.2 % and diagnostic specificity for qPCR was 100 % then 156 samples per subpopulation would need to produce negative infection results to conclude that the population is free from disease at the expected minimum prevalence of 2 % at a 95 % confidence level. None of the sardines (S*ardinops sagax*), anchovies (*Engraulis capensis*), hake (*Merluccius capensis* and *Merluccius paradoxus*) or mullet (*Mugil cephalus*) examined tested positive overall for *I. hoferi*-infection, by microscopy and real-time qPCR. Using the FreeCalc software program, these findings suggest that if the minimum expected prevalence of infection with *I. hoferi* is assumed at 2 % in a subpopulation of the South African sardine, S. *sagax*, the South subpopulation is free from disease at the expected minimum prevalence of 2 % at a > 95 % confidence level and that these fish are susceptible to infection by *I. hoferi*. The other fish sampled from the other subpopulations were lower than the required sample size of 156, thus the results were not adequate to conclude that the subpopulations of *E. capensis*, *M. capensis*, *M. paradoxus*, *M. cephalus* and West and East coast subpopulations of S. *sagax* are free from disease. These sample sizes were too small to distinguish a population with prevalence of 2 % from a disease-free population.

In this study the wet-mount squash preparations was the method of choice for screening the subpopulations because all the different tissues and areas of the tissues could be examined, thus improving detection ability because of the uneven distribution of the parasite in host tissues (Kocan *et al.*, 2011) and because examination of all tissues is imperative to accurately determine the true infection prevalence (Chapter 4). The wet-mount squash preparation technique was therefore used on all available tissue targets; the liver, spleen, heart, gonad and kidney of sardines, anchovies and mullet and only livers of the hake due to the availability of samples, and used in conjunction with qPCR in order to confirm infection status and prevent false positive and negative results which could arise from low infection intensities (Chapter 4). Standardization for qPCR in the form of controls was included to allow for the interpretation of the qPCR results to be valid (Walker and Subasinghe, 2000). These controls included the DNA from positive extraction reference

samples to indicate that the extraction method and qPCR had been successful. The controls also allowed the recognition of qPCR inhibition in the sample if there was any change in the Cq values between assay runs controls. Appropriate control reactions to indicate false positive results were also performed even though there were no positive results. By knowing whether contamination is from a diagnostic target or from a positive control permits a more focused approach in eliminating the source of contamination (Walker and Subasinghe, 2000). Negative control reactions included a negative reference control in the DNA extraction step to distinguish whether contamination from a positive sample to another sample would have occurred and included a sample without template (non-template control) in the qPCR amplification step.

There are difficulties in determining the true prevalence of *I. hoferi*-infections in natural populations due to the non-uniform distribution of the parasite between geographic areas and within populations (Mcvicar, 1999). While attempts were made to collect as many samples from the different ecoregions, sample size and areas were dependant on sample bycatch locations, thus the low numbers of samples collected and examined was not representative of respective source populations. Since transmission of *I. hoferi* is thought to occur through the ingestion of the thick-walled schizonts with infected food particles, the conclusion that the feeding of infected unprocessed marine fish to susceptible cultured fish populations in both marine and freshwater farms is a reason for *I. hoferi* occurring in cultured populations (Mcvicar, 1999). We attempted to test for the occurrence of *I. hoferi* in aquaculture and aquaria fed pelagic species to identify routes of transmission into the exhibit at the Two Oceans Aquarium, however we failed to detect any positive infected samples in our low sample sizes. In conclusion from our preliminary study *I. hoferi* was not detected prevalent in the local populations. However due to the small sample sizes within some of the subpopulations of the species, caution must be exercised. An expanded surveillance assessment is recommended to provide a better estimate of infection prevalence to better determine the probability of *I. hoferi* to be introduced into local captive fish populations.

CHAPTER 6

Final Discussion

The effective identification of diseases of aquatic animals, to estimate parasite prevalence for epidemiological studies or for the control and treatment of disease, requires diagnostic assays to be sensitive, specific, reproducible and to perform consistently (Thrusfield, 2005; Altinok and Kurt, 2003; Banoo *et al.*, 2010; Hunt, 2011; Peeler and Taylor, 2011). For commercial fisheries and aquaculture industries these diagnostic assays are critical to identify early infections, high risk areas and potential risks to captive fish population as infections with *I. hoferi* are detrimental to fish and can negatively impact commercial fisheries and aquaculture industries (Rahimian, 1998; Franco-Sierra and Alvarez-Pellitero, 1999; Mcvicar, 1999; Kocan *et al.*, 2009). The improved control of rearing conditions in aquaculture has resulted in the increase of stocking densities of target fish, which can increase stress amongst the fish and cause faster transmission of disease and result in increased mortalities (Reno, 1998; Altinok and Kurt, 2003; Bondad-Reantaso *et al.*, 2005; Murray and Peeler, 2005). The natural transmission of *I. hoferi* occurs among susceptible hosts via predation or scavenging (Mcvicar, 1999) and in cultured fish the occurrence of *I. hoferi* is traced to the feeding of un-processed tissues from infected fish (Hansen and Pethon, 1985; Kocan *et al.*, 1999; Mcvicar, 1999). Using diagnostic techniques that are sensitive and can allow for specific identification and rapid diagnosis of *I. hoferi* infection are critical for effective management, improving control and preventative strategies. The identification of high risk affected areas and the prompt removal of the infected individuals can reduce the spread of the disease within an aquaculture farm or exhibit in aquaria during disease outbreaks.

As there are a variety of methods currently available to detect *I. hoferi* in infected fish hosts, we evaluated the comparability of wet-mount squash preparations (Paperna, 1986), histological examination (Kocan *et al.*, 2011), *in vitro* culture (Mcvicar, 1999; Hershberger *et al.*, 2002) and real-time quantitative PCR (qPCR) (this dissertation) for detecting *I. hoferi* in *Rhabdosargus globiceps* fish samples. Histopathology such as microscopic visualization of wet-mount squash preparations of tissues and histological evaluations of thinly sliced, stained tissues provides useful information for the diagnosis of the disease (Mcvicar, 1999), such as revealing the organisms within infected tissues and the host response to disease, and allow for the rapid screening of samples. It can reveal the presence of thick-walled

spherical schizonts usually surrounded by host granulomatous tissues, with germination tubes typically observed after the infected host has been dead for a period of time (Mcvicar, 1999). Our observations from the wet-mount squash preparations and histology samples confirmed the classic pictures described for ichthyophoniasis in a variety of hosts described in various reports (Paperna, 1986; Sitja-Bobadilla and Alvarez-Pellitero, 1990; Franco-Sierra and Alvarez-Pellitero, 1999; Mcvicar, 1999; Jones and Dawe, 2002; Schmidt-posthaus and Wahli, 2002; Zubchenko and Karaseva, 2002). Although this method is useful in confirming the identity of the parasite and determining the host cellular response (Paperna, 1986; Rahimian, 1998; Mcvicar, 1999; Kocan *et al.*, 2004), histopathology can underestimate *I. hoferi* infection prevalence (Kocan *et al.*, 1999, 2011) and can produce false positive results (this dissertation). *In vitro* culture is an alternative to histopathology and has the advantages that it allows a live *I. hoferi* cell to reproduce during incubation, which allows for the multiplication of the microorganism thus increasing the probability of detection in low intensity infections, and allows the examiner to observe all the stages of parasite development. When observing the morphological characteristics of *I. hoferi* and assessing the growth of laboratory grown cultures of *I. hoferi* in our study, we found a predictable sequence of development similar to that observed by Okamoto *et al.* (1985), Spanggaard *et al.* (1995) and Kocan *et al.* (1999). We examined large resting spores that began to germinate and produce hyphae with rounding up in the tips of the hyphae, with some dissolution of hyphae as what was similar to those experienced in Okamoto *et al.* (1985) and Spanggaard *et al.* (1995). Although *in vitro* culture allows a live *I. hoferi* schizont to reproduce during incubation, *in vitro* culture has the limitation that it relies upon growth using specific culture conditions which can be impractical when samples are collected at remote sites and is susceptible to contamination and being overrun by bacteria and fungi. These limitations have led to the increase in supplementary molecular approaches such as PCR, which involves the amplification of a unique nucleotide sequence of the pathogen using a single pair of oligonucleotide primers.

Kocan *et al*. (2011) however describes that *in vitro* culture from infected tissues is the diagnostic standard for assessment of *Ichthyophonus* sp. infection prevalence. Whipps *et al.* (2006), Hamazaki *et al.* (2013a), (b) and Lapatra and Kocan (2013) therefore have discussed the equivalency of PCR to culture. Whipps *et al*. (2006) suggested that PCR can underestimate the prevalence of *Ichthyophonus* sp. infections as the sensitivity and specificity of PCR when evaluating heart muscle on infected Chinook salmon both dropped in samples with low pathogen loads or low infection intensity; Hamazaki *et al*. (2013)

however re-examined their data and reported that PCR is equivalent to or could be more accurate than pathogen culture for diagnosing *I. hoferi* in infected tissue; Lapatra and Kocan (2013) however challenged this statement which Hamazaki *et al.* (2013b) responded were assumptions and misinterpretations. We therefore sought to evaluate the comparability of the various methods to detect *I. hoferi*. It was noted that since PCR can produce false positive or negative results if PCR cycling conditions are not optimized correctly and/or PCR primers are not designed properly, Hiney and Smith (1998), Bott *et al.* (2010) and the MIQE guidelines highlighted a framework and criteria respectively for the validation of PCR-based detection techniques for the development of a reliable PCR assay with high analytical specificity and sensitivity. We therefore developed a new SYBR green real-time quantitative PCR assay that is highly specific to the SSU rDNA sequence of *I. hoferi*, that has been designed, optimised and validated based on the criteria proposed by Hiney and Smith (1998) and Bott *et al.* (2010) and in compliance of the MIQE guidelines, for use in *I. hoferi* identification and quantification from *in vitro* culture and *I. hoferi* infected fish tissue samples. The SSU rDNA gene region was selected to design species-specific PCR primers for *I. hoferi* as these genes are highly abundant within eukaryotic cells and contain sufficient variable regions to distinguish between heterologous species (Hillis and Dixon, 1991; Bott *et al.*, 2010). The qPCR primers were designed to specifically target a conserved region of the *I. hoferi* small subunit rDNA gene within a mixed genomic DNA background, including DNA of closely related non-target organisms and liver tissue of *R. globiceps*. As there was only amplification of *I. hoferi*, and no cross amplification occurred when screened against closely related species and environmental isolates it was ideal for targeted diagnosis and surveillance. Furthermore since the physical and chemical properties of a tissue matrix can have an effect on the performance of the overall dynamics of the PCR assay as suggested by Hiney and Smith (1998), the qPCR assay developed in this study performance was also evaluated within the host tissue matrix, which we tested using the liver tissue of *R. globiceps*. We demonstrated a 9 % reduction in the overall sensitivity of the assay when compared to dilution series made up with a sterile matrix of PBS. This finding agrees with Hiney and Smith (1998) and supports the importance of including this step when developing a diagnostic assay. Since it had been left out in previous PCR development studies (Whipps *et al.*, 2006; White *et al.*, 2013), we therefore suspect that the lower detection limits in these previous studies may have been over estimated. Furthermore as the quality of extracted DNA can also adversely affect PCR results either by co-purification of contaminants which can inhibit the PCR, or cause false negative results by the loss of DNA in the extraction method (Cunningham, 2002; Altinok

and Kurt, 2003), we tested a heat-lysis extraction reported by Greeff *et al.* (2012) and compared the results to the QIAGEN DNeasy blood and tissue kit extraction (Cat No./ID: 69504) that has been used in previous PCR studies of *I. hoferi* (Criscione *et al.*, 2002; White *et al.*, 2013; Gregg *et al.*, 2014). The heat-lysis method has been shown to successfully extract high quality genomic DNA, from *Haliotis midae* tissues infected with *Halioticida noduliformans* (Greeff *et al.*, 2012) and from tissues of the Eastern Oyster, *Crassostrea virginica* infected with *Vibrio campbellii* (Macey *et al.*, 2008a) and the Atlantic blue crab, *Callinectes sapidus* (Macey *et al.*, 2008b), allowing for accurate identification of the specific target organisms. We demonstrated that the heat-lysis extraction method is reproducible and successful for extracting DNA from *I. hoferi* cultures and from *R. globiceps* fish infected with *I. hoferi*, and is the most efficient method for extracting DNA from *I. hoferi* compared to the QIAGEN DNeasy blood and tissue kit extraction. The method does not require any specialised or expensive equipment and can be used in any laboratory that is fitted with some basic equipment.

In this study, *R. globiceps* samples that were naturally infected with *I. hoferi* at the Two Oceans Aquarium were studied and screened using the various diagnostic techniques. The test with the highest diagnostic sensitivity and negative predictive value was the wet-mount squash preparations of all organ tissues (95.24 % and 88.89 % respectively) which was followed closely by real-time qPCR of homogenised liver tissue (90.48 % and 87.50 % respectively). The data showed using more than one diagnostic method and the examination of all tissues of *R. globiceps* is imperative to accurately determine true infection prevalence. Real-time qPCR was the test that had the highest combination of diagnostic sensitivity and diagnostic specificity (and NPV and PPV) in detecting *I. hoferi* infection (followed closely by *in vitro* culture), thus confirmed that the test was a good test to confirm disease occurrence. Although the wet-mount squash preparation of all of the tissues tested was the method that obtained the highest diagnostic sensitivity and the highest negative predictive value, this test also had the poorest diagnostic specificity (57.14 %) and the poorest positive predictive value (76.92 %) of all the methods tested. We also noted that to obtain the best estimate of *I. hoferi* prevalence, it is recommended that more than one tissue type is collected for analysis (Table 4.4 & Fig. 4.2). Furthermore since Mcvicar (1999) had highlighted that *I. hoferi* infection is focal rather than diffuse in hosts due to the granulomatous response containing the pathogen, and Kocan *et al.* (2011) reporting that there is an uneven distribution of the parasite in host tissues, discrepancies in parasite detection sensitivity between different diagnostic techniques are eminent. We therefore

tested whether the homogenization of tissue would improve the diagnostic sensitivity of some of the assays, namely culture and qPCR and observed a 9.53 % increase in the diagnostic sensitivity from qPCR, and a 14.28 % increase in the diagnostic sensitivity from *in vitro* culture after the liver tissues were homogenized in buffer. This showed that the diagnostic sensitivity of the culture and qPCR assay can be improved when tissues have been homogenized. We concluded that the wet-mount squash examination technique was a viable diagnostic test to detect *I. hoferi*-infections, especially when evaluating all the visceral organs of the host, and it can be used as a screening test and must be utilized in conjunction with another highly specific method in order to prevent misidentification. These can either therefore be *in vitro* culture or qPCR depending on the logistics and availability of the equipment, as the wet-mount squash examination technique lacks specificity and diagnosis can be difficult when *I. hoferi* is present in low numbers (Walker and Subasinghe, 2000). As cost was mentioned in Lapatra and Kocan (2013), with the increased interest and technological developments within the molecular field, costs are rapidly coming down, furthermore by increasing ones sample volumes costs can be lowered further.

Based on the results from the study of the evaluation of the various diagnostic techniques, we therefore utilized the wet-mount squash preparation technique to screen samples and used qPCR for confirmation attempting to test for the occurrence of *I. hoferi* in aquaculture fed pelagic species subpopulations of South Africa. We trusted that this would improve our understanding of the distribution of *I. hoferi* around South Africa and would aid in identifying risk pathways for *I. hoferi* transmission into captive populations and whether the infection of *R. globiceps* at the Two Oceans Aquarium were due to the feeding of fish with infected unprocessed feed. This was proposed because since the disease is more frequently seen in marine fish, the aquaria *R. globiceps* may have been fed using contaminated marine food. Real-time quantitative polymerase chain reaction (qPCR) was used to confirm infection in any samples that exhibited any pathology or granulomatous tissue reaction with microscopic inclusions suspect of infection with *I. hoferi* observed using microscopic examination of wet-mount squash preparations. We however failed to detect any positive infected samples in our samples, which could be due to the low sample sizes analysed. Although attempts were made to collect as many samples from the different ecoregions, an expanded surveillance assessment is recommended to provide a better estimate of infection prevalence within and between these species subpopulations. There have also been other suggestions for natural transmission of *I. hoferi* infections to occur

through the ingestion of schizonts that transform into hyphae directly from the water and as such testing the influent water may also be a possibility in identifying the cause of transmission and infection.

In conclusion the selection of a diagnostic technique or combination of techniques can influence the accuracy of ones results and be selected based on a few considerations such as the available technology at the laboratory, the resources at sample locations, the total cost of sampling, the speed required for diagnosis and the nature of the intended results (Thrusfield, 2005; Gozlan *et al.*, 2006; Kocan *et al.*, 2011; OIE, 2016). Since molecular based diagnostic approaches are increasingly being applied as alternatives to conventional pathogen detection techniques, other diagnostic tools available such as the LAMP (Loop-mediated isothermal amplification) assay and metagenomics/next generation sequencing applications cannot be ignored. Although the qPCR assay developed in this dissertation has been optimized and validated to detect *I. hoferi* infections, it is recommended that the test be validated amongst other labs to ensure reproducibility. Furthermore to provide a better estimate of *I. hoferi* prevalence in South Africa and its potential to spread to local fish populations, an expanded surveillance assessment should be endorsed.

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APPENDIX

Appendix A1: MIQE checklist for the development and evaluation of a real-time SYBR green quantitative PCR method for the detection of *I. hoferi* in *Rhabdosargus globiceps* fish tissue samples. NA= Not Applicable.

Item to check	Importance	Yes/No/N/A	Details
Experimental design			
Definition of experimental and control groups	E	Yes	Quantitative PCR was developed for amplification of a 299 bp fragment of the small subunit ribosomal DNA gene from *I. hoferi* extracted DNA from laboratory grown cultures (n=5), as well as from spiked uninfected *Rhabdosargus globiceps* tissues (n=24).
Number within each group	E	Yes	*I. hoferi* extracted DNA from laboratory grown cultures (n=5). Spiked uninfected *Rhabdosargus globiceps* tissues (n=24).
Assay carried out by the core or investigator's laboratory?	D	Yes	Assay carried out by investigator's lab.
Acknowledgment of authors' contributions	D	Yes	Explained in acknowledgments.
Sample			

Description	E	Yes	*Ichthyophonus hoferi* was isolated from the infected liver of euthanized Cape White Stumpnose, *Rhabdosargus globiceps*
Volume/mass of sample processed	D	Yes	≈ 0.5 g of infected liver tissue for culture.
Microdissection or macrodissection	E	Yes	Macrodissection.
Processing procedure	E	Yes	Cape White Stumpnose, *Rhabdosargus globiceps*, were euthanized with an overdose of 2-phenoxyethanol and liver tissue (≈ 0.5 g) was aseptically excised from the infected deceased fish and cultured according to 2.2.1. Samples were subcultured to obtain pure cultures, enumerated, prepared and pelleted for extraction.
If frozen, how and how quickly?	E	N/A	Samples were not frozen.
If fixed, with what and how quickly?	E	N/A	N/A
Sample storage conditions and duration	E	Yes	Cultures were maintained at 15 ˚C before subjecting the cultures to enumeration and subsequent preparation and centrifuging to create a pellet of pure cultures.
Nucleic Acid Extraction			

Procedure and/or instrumentation	E	Yes	Total genomic DNA was extracted from each pelleted sample of *I. hoferi* (~125 schizonts) using the heat-lysis method described by Greeff *et al.* (2012).
Name of kit and details of any modifications	E	N/A	N/A
Source of additional reagents used	D	N/A	N/A
Details of DNase or RNase treatment	E	N/A	N/A
Contamination assessment (DNA or RNA)	E	Yes	Using the ratio of 260 nm/280 nm.
Nucleic acid quantification	E	Yes	Spectrophotometric analysis with measurements at 280 nm and 260 nm.
Instrument and method	E	Yes	Jenway Genova Nano spectrophotometer.
Purity (A260/A280)	D	Yes	Purity was determined by measuring the absorbance ratio 260/280 – displayed in Fig. 3.1.
Yield	D	Yes	Displayed in Fig. 3.1.
RNA integrity: method/instrument	E	N/A	N/A

RIN/RQI or Cq of 3' and 5' transcripts	E	N/A	N/A
Electrophoresis traces	D	No	Pictures are available upon request.
Inhibition testing (Cq dilutions, spike, or other)	E	Yes	To assess non-target DNA in PCR inhibition, DNA extraction and subsequently qPCR was performed on diluted *I. hoferi* samples in the presence of a liver tissue matrix. This was compared to samples that were prepared in PCR-grade water. Overall the increase in Cq values at each corresponding dilution demonstrates a reduction in qPCR sensitivity by 3.34 cycles (Fig. 3.5).
qPCR protocol			
Complete reaction conditions	E	Yes	Real-time qPCR reactions were conducted using the Bio-Rad CFX96™ real-time PCR detection system and C1000™ thermal cycler. All qPCR reactions were carried out in a final volume of 25 µL and each reaction contained 50 ng of *I. hoferi* genomic DNA, 1 x KAPA SYBR fast qPCR master mix, 10 µM of each primer and PCR grade H_2O. Amplification consisted of an initial denaturation at 95 °C for 10 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing gradient from 55 – 65 °C for 30 seconds, and elongation at 72 °C for 45 seconds, melt curve from 50 °C for 10 seconds with an increment of 0.5 °C per

			cycle. Optimal annealing temperature for the BW2F/R primer set is displayed in Table 3.2.
Reaction volume and amount of cDNA/DNA	E	Yes	Real-time qPCR reactions were carried out in a final volume of 25 µL and each reaction contained 50 ng of *I. hoferi* genomic DNA.
Primer, (probe), Mg2, and dNTP concentrations	E	Yes	Final concentration of 10 µM of each respective primer in the reaction, 2.5 mM $MgCl_2$ and dNTP concentration provided with the KAPA SYBR FAST qPCR Master Mix (proprietary information).
Polymerase identity and concentration	E	Yes	KAPA SYBR FAST DNA Polymerase
Buffer/kit identity and manufacturer	E	Yes	KAPA SYBR FAST qPCR Master Mix
Exact chemical composition of the buffer	D	Yes	The KAPA SYBR FAST qPCR Master Mix contains a proprietary buffer.

Additives (SYBR Green I, DMSO, and so forth)	E	Yes	SYBR Green I
Manufacturer of plates/tubes and catalog number	D	Yes	Biorad individual PCR tubes 8-tube strip, Cat # TL50801 and Biorad hard-shell 96-well multiplate PCR plates low profile, clear, Cat # MLP9601.
Complete thermocycling parameters	E	Yes	For the BW2-F/R primer set initial denaturation at 95 °C for 10 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 59 °C for 30 seconds, and elongation at 72 °C for 45 seconds, melt curve from 50 °C for 10 seconds with an increment of 0.5 °C per cycle.
Reaction setup (manual/robotic)	D	Yes	Manual
Manufacturer of qPCR instrument	D	Yes	Bio-Rad CFX96™ real-time PCR detection system and C1000™ thermal cycler.
Real-time qPCR validation			
Evidence of optimization (from gradients)	D	No	The melting curve was generated by an initial denaturation step (95 °C for 20 s) followed by a gradual heating from 60 °C to 95 °C (ramp of 0.3 °C).

Specificity (gel, sequence, melt, or digest)	E	Yes	Real-time PCR amplification using the *I. hoferi* specific primers BW2-F/R produced a melting temperature of 80 ± 0.5 °C for the 299 bp product, with an optimum annealing temperature of 59 °C. Amplified DNA was also sequenced to confirm the specificity of the PCR reactions and subsequent analysis of the nucleotide sequences showed that the PCR products were identical to *I. hoferi*.
For SYBR Green I, Cq of the NTC	E	Yes	Non–template controls (NTC) generated Cq values >32.
Calibration curves with slope and y intercept	E	No	Available on request
PCR efficiency calculated from slope	E	Yes	The amplification efficiency percentage calculated from the slope of the standard curve was 101.07 %. Standard curve precision evaluated via coefficients of determination was 0.99.
CIs for PCR efficiency or SE	D	-	N/A
r2 of calibration curve	E	Yes	Coefficients of determination was 0.99.
Linear dynamic range	E	Yes	Linear dynamic range was considered taking into account the linearity of the standard curves; from 10 to 10^{-4} per 25 µL qPCR reaction. Fig. 3.4.
Cq variation at LOD	E	Yes	Cq value of 29 ± 0.5 (Fig. 3.4).

CIs throughout range	D	No	N/A
Evidence for LOD	E	-	The lowest detectable limit that could be detected with the BW2-F/R primer set in PCR-grade water following real-time PCR amplification was 0.005 schizonts in a 25µL reaction at a Cq value of 29 ± 0.5 (Fig. 3.4).
If multiplex, efficiency and LOD of each assay	E	N/A	N/A
Data analysis			
Real-time qPCR analysis program (source, version)	E	Yes	Bio-Rad CFX96 Manager 2.1
Method of Cq determination	E	Yes	Any samples that recorded a cycle threshold (Ct) value after setting the PCR cycle number to 32 and the baseline threshold value to 200, with a melt peak of 80 ± 0.5 °C on the melt curve graph were considered to be positive.
Outlier identification and disposition	E	Yes	None of the Cq values was discarded.
Results for NTCs	E	Yes	The non-template control did not produce any fragments, confirming that the reagents used for PCR were not contaminated.

Justification of number and choice of reference genes	E	Yes	Explained in text.
Description of normalization method	E	Yes	Each qPCR assay included positive controls, consisting of *I. hoferi* genomic DNA extracted from a pure culture.
Number and concordance of biological replicates	D	Yes	There were five biological replicates (Fig 3.4).
Number and stage (RT or qPCR) of technical replicates	E	Yes	There were three technical replicates (Fig 3.4).
Repeatability (intraassay variation)	E	Yes	Fig 3.4
Reproducibility (interassay variation, CV)	D	No	Not displayed
Power analysis	D	N/A	N/A
Statistical methods for results significance	E	Yes	Student´s t Test
Software (source, version)	E	Yes	Microsoft Excel
Cq or raw data submission with RDML	D	No	
Real-time qPCR target information			

Gene symbol	E	Yes	Small subunit (SSU) rDNA
Sequence accession number	E	Yes	See Table 3.1 for the sequence accession numbers used in sequence alignment of members of the genus *Ichthyophonus* and other related species.
Location of amplicon	D	Yes	Described in text 3.2.3.
Amplicon length	E	Yes	Table 3.2: 299bp.
In silico specificity screen (BLAST, and so on)	E	Yes	Gene specificities were tested by BLAST analysis (NCBI) showing 100 % homology. Species used to test for cross-reactivity of the *Ichthyophonus hoferi* primers designed in this study are displayed in Table 3.3.
Pseudogenes, retropseudogenes, or other homologs?	D	N/A	N/A
Sequence alignment	D	Yes	Displayed in Fig. 3.2.
Secondary structure analysis of amplicon	D	N/A	N/A
Location of each primer by exon or intron (if applicable)	E	Yes	Explained in 3.2.3 and displayed in Fig. 3.2
What splice variants are targeted?	E	N/A	No splice variants were targeted because there is no information about them in the analyzed organism.

Real-time qPCR oligonucleotides			
Primer sequences	E	Yes	Table 3.2.
RTPrimerDB identification number	D	N/A	N/A
Probe sequences	D	N/A	N/A
Location and identity of any modifications	E	Yes	No modifications were done.
Manufacturer of oligonucleotides	D	Yes	Integrated DNA Technologies.
Purification method	D	Yes	Desalted