OCCURRENCE, GENETIC RELATEDNESS AND EFFECTS OF *Diplostomum*
(DIGENEA: DIPLOSTOMIDAE) SPECIES IN *Oreochromis niloticus* L. AND
DISTRIBUTION AMONG VECTOR SNAILS IN KIBOS AREA

BY

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ABSTRACT

Diplostomum species metacercariae, are trematodes that pose economic threat to aquaculture globally. Despite their diversity, species identification is difficult owing to their striking morphological similarities. Poor knowledge of diplostomid diversity in fish species coupled with inadequate data from larval parasites in snails represent an obstacle in creation of link sequences to formal description of *Diplostomum* species. In addition, effect of the eye fluke *Diplostomum* on fish growth is not clear because most studies have been conducted in the natural systems where biotic and abiotic factors are uncontrolled. This study aimed to determine the occurrence of *Diplostomum* parasites in cultured *Oreochromis niloticus* L., establish the genetic relatedness to other reported *Diplostomum* parasites, determine the effect on growth and finally to determine prevalence of the infection in intermediate snail vectors at three fish farms in Kibos area. A cross sectional study design was adopted in three fish farms namely Lake Basin Fry Production Centre and two privately owned fish farms (Dr. Mzungu's and Auji fish farms) in Kibos area. Three fish ponds were selected per fish farm using a computer random number generator technique to increase chances of establishing diversity of *Diplostomum* parasites among the fish. Sixty-four fish were sampled in each pond. The sampling of fish was conducted every three weeks for a period of three months (December 2011 – February 2012). *Diplostomum* parasites were detected in fish eyes using microscopy to ascertain parasitic indices, genetic discrimination of *Diplostomum* species was assessed using sequences of the internal transcribed spacer region (ITS1–5.8S–ITS2) and 18S ribosomal genes in 23 diplostomoids, while effect of the parasite on fish was determined using the length – weight relationship formula. A minimum of 100 snails were sampled from the selected ponds using a scoop net and identified based on shell morphology, and screened for *Diplostomum* infection based on cercariae morphology and behaviour. 52.3% of the fish were positive of *Diplostomum* parasites. Parasite abundance increased with fish size and later declined in larger fish > 15 cm. No significant relationship was observed between parasite abundance and fish condition factor in all the study sites ($P = 0.516$, $P = 0.565$, $P = 0.357$ respectively). Phylogenetic analyses of ITS rDNA gene dataset in the metacercariae revealed close relationship to *Diplostomum mashonense* and *D. baeri*. Molecular identification using 18S rDNA sequences revealed close relationship to *D. compactum*, *D. phoxini* and *D. spathaceum*. Distribution of vector snails indicated presence of *Lymnea*, *Biomphalaria*, *Bulinus* and *Cerratophalus* snail species, however; *Diplostomum* larval communities were reported only in *Biomphalaria* sp. at a prevalence rate of 21.7%. Presence of metacercariae in fish eyes reared in all the farms together with the high abundance of *Biomphalaria* sp. shedding cercariae in all the sampled ponds confirmed positive transmission cycle of *Diplostomum* parasites in Kibos fish farms. Genetic analyses depicted significance of unrecognized genetic diversity. Internal transcribed spacer and 18S rDNA successfully differentiated species of *Diplostomum* genera suggesting that ribosomal markers are effective genetic markers for inter-species phylogenetic analysis and should be employed in future for identification of diplostomoidea. Diplostomiasis control interventions within fish farms in Kibos area need to prioritize thorough screening of fingerlings from hatcheries before restocking activities in any farm. Secondly, snails should be controlled around pond areas by clearing vegetation within the vicinity of the ponds.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Capture fisheries has traditionally met the requirements for fish and fish products in the world. However, there has been a steady decline of fish catch due to overfishing and changes in environmental conditions (FAO, 2012). Aquaculture which is broadly defined as the farming of finfish and shellfish in water systems has continued to grow and provides the most feasible means of supplementing the shortfall in the capture fish supply. Although aquaculture is the fastest growing food production sector in the world, fish diseases has not been prioritized as one of the major limiting factors in aquaculture systems. Aquaculture production however, is vulnerable to adverse impacts of diseases that result in partial or sometimes total loss of production. In 2010, aquaculture in China suffered production losses of 1.7 million tonnes caused by diseases (FAO, 2012). In 2011, disease outbreaks virtually wiped out marine shrimp farming production in Mozambique (FAO, 2012). The impact of diseases to aquaculture in Kenya has not been published. Though, fish farming serves as the best alternative to ensure food security in Kenya, a major challenge exists: that of diseases (Fioravanti *et al.*, 2009). Earthen ponds are found in most parts of Kenya, the bottom is usually made of mud, the edges consist of grass, and the water depth is commonly shallow. These factors promote an environment that attract a wide variety of biotic life inform of insects, frogs, pond snails and birds which provide a conducive environment for the completion of parasites lifecycles.

The trematode genus *Diplostomum*, (family: diplostomidae) represents a large group of parasites of economic importance worldwide due to their pathogenic metacercariae which parasitize the eyes of fish, in both natural and aquaculture systems (Chappell *et al.*, 1994;

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Chappell, 1995). *Diplostomum* species parasites have a complex life-cycle. They mature in the small intestine of piscivorous birds and pass through snails as first intermediate host and fish as second intermediate host (Chappell *et al.*, 1994). In fish, the parasites inhabit the lens, retina and aqueous humour of fish eyes as well as the brain, spinal cord and nasal spaces thereby resulting into substantial losses of wild and farmed fish.

Diplostomum parasites have been encountered in fish farms in the northern hemisphere (Europe and North America) and as such received wide theoretical and empirical attention due to their pathogenic consequences to fingerlings (Stables and Chappell, 1986; Field and Irwin, 1994; Marcogliese *et al.*, 2001; Sangster *et al.*, 2004). The eye fluke infection has been associated with decreased growth of fish in rearing conditions (Buchmann and Uldal, 1994; Paaver *et al.*, 2004) and increased mortality in fish as a result of reduced vision caused by cataracts which impairs the fish's feeding efficiency (Voutilainen *et al.*, 2008) and makes the fish more vulnerable to avian predation (Seppälä *et al.*, 2005). However, in Africa, our understanding on the distribution of *Diplostomum* infection in fish farming areas remains incomplete. This is related to low sampling efforts in tropical countries due to lack of expertise in the field of fish parasitology. In addition, identification of these parasites is problematic due to (i) the presence of morphologically similar species; (ii) the phenotypic plasticity of the adults and metacercariae; (iii) the simple larval morphology; and (iv) the difficulties in linking life-cycle stages (Kostadinova, 2008 and references therein). These problems represent a major impediment for the assessment of the distribution and the actual role of diplostomids in fish populations.

Taxonomic studies on the diplostomid digeneans from the African continent are scarce and limited to few *Diplostomum* species descriptions published in the 1930s–1960s, and virtually

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little is known of their natural history (e.g., Beverley-Burton, 1963; King and Van As, 1997). Secondly, most of the published African diplostomid studies have focused on the catfish *Clarias gariepinus* (Mwita and Nkwengulila, 2008, 2011; Chibwana and Nkwengulila, 2010, Chibwana *et al.*, 2013). Therefore, basing taxonomic identification of African *Diplostomum* species to a few ancient descriptions is misleading. Furthermore, relying on results from one fish species (*Clarias gariepinus*) makes it unreliable to extrapolate results to other fish species because of differences in feeding and resting behaviours among fish species.

Diplostomum parasites have been reported in *Oreochromis niloticus* in Kenya (Matolla, 2009; Kembenya *et al.*, 2012) and recently from another African freshwater fish host, *Synodontis nigrita* (Chibwana *et al.*, 2013) in Nigeria. This is an indication that *Diplostomum* parasites affect a number of fish species and therefore more research should be undertaken to elucidate the occurrence, role and diversity of these parasites in other fish populations. In Kenya, Nile tilapia (*Oreochromis niloticus* L. 1758) ranks the most commercialized fish in western Kenya. However, decline in current fish production estimates of pond reared Nile tilapia from 1000 tonnes/year to 65 tonnes/year in Nyanza region has been recorded (Fisheries Department, 2012). This has posed severe threats to the aquaculture component of the stimulus package on fish farming initiated by the Kenyan government to revamp the economy among riparian communities. Kibos, is one of the informal settlement areas located within Kisumu City and has a population of 250,000 people (Central Bureau of Statistics', 2010). The location has a density of 4.3 people per household (2009 Census) whose main economic activity is fishing from Lake Victoria situated 10 km away. In order to reduce overdependence of these communities to fishing, as well as migration of the expanded population to Kisumu City, the government of Kenya initiated the Economic Stimulus Programme in the area, which focused on fish farming. Establishment of the programme in

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Kibos area was also driven by the fact that Lake Basin Development Authority (LBDA) is situated in the area and therefore this initiative would reinforce activities in the LBDA centre such as increase fingerling production, create employment to local community inform of staff as well as cater for the increased demand of fingerlings to stock the fast-growing number of fishponds by fish farmers. Despite this initiative, the fish farming sector is faced by a challenge in lack of programs or standards for certification of seed quality in Kenya, which pose as a health risk in the transfer of fingerlings from hatcheries to newly constructed or existing ponds.

Kibos area is well endowed with black cotton soil suitable for aquaculture, however fish ponds constructed in the area in the year 2009 to stimulate economic development has not translated into increased fish production over the years. Farmers shy away from the initiative, while most of the constructed ponds remain abandoned due to poor yields. Evidence of decline in the average size of fish (300g to 150g) harvested in fish ponds in the area over the last 3-5 years was as well reported by Otieno, (2010); being attributed to low-level of extension services and lack of quality fingerlings (Ngugi and Manyala, 2004). These limitations have led to decline in pond fish culture production in Nyanza region with the total annual production being only around 65 tonnes (Ngugi and Manyala, 2004); compared to around 1,000 tonnes/year countrywide (FAO, 2013). Although, studies by Chappell, (1995), Machado *et al.* (2005) and Seppala *et al.* (2005) associated reduced growth of fish to presence of *Diplostomum* digenetic trematodes, lack of studies on this assertion in Kenya is yet to be confirmed.

1.2 Statement of the problem

Due to the dwindling stocks for capture fisheries in Kenya as is presently the case worldwide, there is a growing interest in the development of fish culture. Whereas the latest attempt by the government was to introduce the Fish farming Enterprise Productivity Programme (FFEPP) to boost fish and food production among riparian communities, this initiative has been threatened by low production of farmed fish in Kenya (Rothuis *et al.*, 2011). Generally, the reasons for slow aquaculture development in Kenya have been (1) lack of a tradition of fish and water husbandry (Brummett and Williams, 2000), (2) lack of information on fish farming technology (Fisheries Department, 2012) and (3) Lack of information on fish diseases leading to unknown investment return-rates by fish farmers (Ngugi and Manyala, 2004; Shitote *et al.*, 2011). Similarly, decline in the average size of fish (300g to 150g) harvested in fish farms in Kibos area over the last 3-5 years was reported by Otieno, (2010). Low yields from fin aquaculture in Nyanza region coupled with small sized fish harvested in Kibos area could be associated to a number of factors including: lack of quality seeds, poor pond management, diseases and epizootics among others. Owing to few government seed production centers, Kibos Fry production centre located in Kibos area serves as the main source of fingerlings for farmers in Rift valley and Western Kenya regions. However, there is no program or standard for certification of seed quality in Kenya, which pose as a health risk in the transfer of fingerlings from the hatchery to newly constructed or existing ponds. Most farmers in the area lack information on fish diseases that affect fish and therefore do not understand factors that contribute to fish diseases, as well as the impact of fish diseases to production.

1.3 Justification

The Food and Agriculture Organization of the United Nations (FAO, 2005) recommended that for supplementary high protein food and additional income for riparian communities residing in Sub-Saharan Africa, intensive culture of indigenous species of fish should be adopted by farmers. However, parasitic infections of fish can be a major setback in achieving maximum production per unit area of culture. In order to promote maximum production with an aim to achieve some objectives of the Millennium Development Goals on poverty and hunger, we need information on parasites affecting the natural fish populations in fish-farming areas. Such information enable us understand the health and viability of fish stocks before transferring fish to new ponds with an aim of controlling spread of diseases or transfer of low quality fish seeds that may eventually affect fish production turn over thus demining the aim of the goal. This can only be achieved by considering reliable identification of parasites affecting the fish population. Molecular techniques aid in quick and reliable identification of cryptic species in a community. In this study, ribosomal molecular markers were successfully employed to genetically relate *Diplostomum* parasite diversity affecting *Oreochromis niloticus* to other published *Diplostomum* sp. This is because most studies on *Diplostomum* parasites in Africa have been studied on the clarid fish species which exhibit a different resting and feeding behaviour compared to *Oreochromis niloticus* (Al-Harbi and Siddiqui, 2000). Data from this study provide a foundation for future comparison work regarding *Diplostomum* parasites whereas information on pond snail distribution will foster successful ecological management and control of diplostomiasis infection in aquaculture. Information on Length - weight measures (growth) of farmed fish will aid in necessary accurate prediction of production levels in the farms.

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1.4 General Objective

The general objective of the present investigation was to study the occurrence and genetic relatedness of *Diplostomum* parasites in fish, establish biotic factors influencing parasite occurrence and how these parasites affect the growth of fish in privately managed fish farms in Kibos area.

1.4.1 Specific Objectives

1. To determine prevalence, abundance and intensity of *Diplostomum* parasites infesting *Oreochromis niloticus* in fish farms in Kibos area of Kisumu County.

2. To identify phylogenetic relatedness between *Diplostomum* species from fish population in Kibos area and other reported *Diplostomum* species.

3. To survey *Diplostomum* trematodes in pond snails in Kibos area using 18S rDNA.

4. To compare the length (cm) and weight (g) measurements of parasitized and non-parasitized fish from three fish farms in Kibos area.

1.5 Research questions

1. What is the prevalence rate, abundance and intensity of *Diplostomum* parasites in *Oreochromis niloticus* in Kibos area?

2. What are the *Diplostomum* genotypes found in *Oreochromis niloticus* in fish farms within Kibos area?

3. What is the distribution and prevalence of *Diplostomum* infection in pond snail intermediate hosts within Fish Farms in Kibos area?

4. What is the effect of *Diplostomum* parasites on the growth (length and weight) of farmed *Oreochromis niloticus* in Kibos area?

CHAPTER TWO

LITERATURE REVIEW

2.1 Prevalence, Abundance and Intensity of *Diplostomum* parasites infesting *Oreochromis niloticus* L.

In the northern hemisphere, *Diplostomum* species has been extensively studied because of their pathogenicity on fingerlings in fish farms (Field and Irwin, 1994; Chappell, 1995). Metacercariae of *Diplostomum* species have been recorded in over 150 fresh and brackish water fish (Shigin, 1993; Niewiadomska, 1996) in Europe and America. In Europe, salmonids, such as rainbow trout (*Oncorhynchus mykiss*), have proved to be vulnerable to cataract forming eye fluke infections (Speed and Pauley, 1984). Chappell (1967) recorded a count of 231 *D. spathaceum* from the lenses of a six year old trout, while Wotten (1974) recovered over 550 metacercariae from a mature rainbow trout. In Mexico, diplostomiasis caused by the digenean *Diplostomum* (Austrodiplostomum) *compactum* species was recorded in 33 freshwater fish species from ten families (Salgado-Maldonado, 2004), including the African tilapia *Oreochromis mossambicus*, *O. niloticus*, and *O. urolepis hornorum*. In the state of Guerrero, *Diplostomum compactum* has been recorded in ten species of fish (Violante-González and Aguirre-Macedo, 2007; Violante-González *et al.*, 2007; Gonzalez *et al.*, 2009). Considering reports, majority of published information on *Diplostomum* sp. has been conducted in the natural waters with scanty of information on aquaculture systems. Considering data from fish farms, *Diplostomum* parasites have been encountered in farmed fish and has been associated with decreased growth of fish in rearing conditions (Buchmann and Uldal, 1994; Paaver *et al.*, 2004) and increased mortality in fish as a result of reduced vision caused by cataracts (Voutilainen *et al.*, 2008).

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The environmental conditions seen in fish farms predispose cultured fish to significant stress factors owing to water quality, quantity and its circulation, stocking densities (Biomass), re-stocking, handling among others which disrupt the dynamics of host-parasite balance. These conditions greatly favour disease transmission in the pond system which may pose significant impact on the captive fish populations. This necessitates the need to have a good knowledge of parasites affecting the natural fish populations in fish-farming areas.

Studies on *Diplostomum* parasites in Africa have been reported by Kassaye *et al.* (2009) who reported 100% prevalence of *Diplostomum* parasites in wild *Oreochromis niloticus* and *Clarias gariepinus* found in Lakes Hora, Babogaya, Ziway as well as from Wonji and Yemilo ponds in Ethiopia (Kassaye *et al.*, 2009). Recent reports from Eldoret and Chepkoilel fish farms at Moi University, Kenya, indicated prevalence of diplostomid infection in the region at 100%, 84% and 66% in Kerita dam, Kesses dam and Chepkoilel fish farm. Parasite mean intensity was recorded at 12, 9 and 14 respectively in the three sites (Matolla, 2009; Kembenya *et al.*, 2012). This was a clear indication of *Diplostomum* infection in the region. As stated earlier, prevalence of parasitism could be related to the environmental conditions seen in aquaculture systems. In addition, there is scarcity of documentation on the transmission of parasites from hatchery centers to fish farms with most of the documentations having associated wild fish to the transmission of parasitic infections to farms. Farmed fish has been reported to be vectors of diseases of concern to human health (FAO, 2011), however, studies pertaining to the role of farmed fish as sources of parasitic infections to other fish farms has not been investigated. Kibos Fry Production Centre serves as the main source of fingerlings for farmers in Uasin-Gishu and Nyanza regions. Reports by Matolla, (2009) and Kembenya *et al.* (2012) indicated prevalence of *Diplostomum* parasites in Chepkoilel fish farms, which acquired fingerlings for restocking from Kibos Fry Production

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Centre, yet the health status of the fingerlings at the Kibos fish farm, has not been investigated. Inadequacy of information on the wellbeing of fingerlings from the hatchery center is likely to be a major constraint to fish farming development in Western Kenya.

2.2 Genetic relatedness of *Diplostomum* species in fish population using ribosomal markers

Gallazo *et al.* (2002) pointed out that the identification of species within *Diplostomum* is difficult at all stages in the parasite life cycle due to a number of reasons including the phenotypic plasticity of the organisms themselves, the paucity of morphological features in certain life-cycle stages, age and host induced variation, artefacts produced during fixation, and the extensive overlap in morphological characteristics that occur among species. Although adults appear to be the least difficult to identify to the species level, identification of metacercariae which are much more frequently encountered, is more difficult (Niewiadomska, 1996; Niewiadomska and Laskowski, 2002). Keys and monographs produced to identify the metacercariae (e.g., Shigin, 1986) have not been satisfactory and disagreement exists over identifications that have been based on metacercarial morphology (Niewiadomska, 1996). The classical method of elucidating metacercariae is to carry out experimental infection of animals in the laboratory (Ostrowski de Nu´n˜ ez, 1989). However, such research is logistically demanding, and it is usually feasible to study only a single parasite species and in most cases the identity of digenean larval stages can, at best, only be resolved to the generic level (Moszczynska *et al.*, 2009). In this context, molecular markers offer powerful and much-needed tools that have the potential to distinguish between morphologically similar species at any stage in their life cycle.

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DNA-based approaches provide an independent method of distinguishing between species when morphological criteria are ambiguous or are subject to variation (McManus and Bowles, 1996).

Ribosomal DNA (rDNA) is one of the most useful markers in taxonomic studies because of its availability in high copy numbers and also contains variable regions flanked by more conserved regions (Chilton, 2004). These regions vary from highly conserved (18S, 5.8S and 28S) to highly variable (transcribed and non-transcribed or intergenic spacer) regions (Fig. 1).
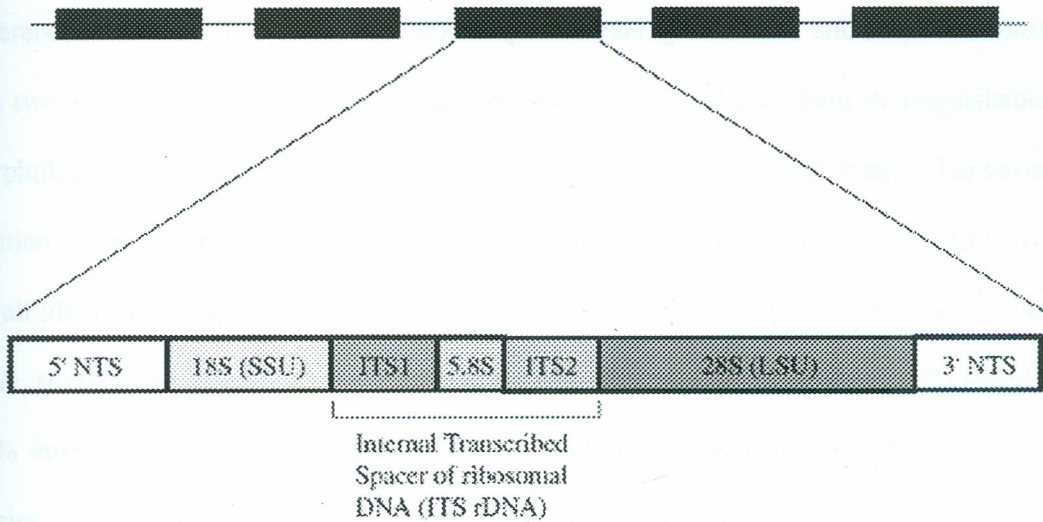

Figure 1: Diagrammatic representation of ribosomal DNA within the nucleolar organiser region of the eukaryotic genome (tandem repeats (black boxes), the transcription unit, the non-transcribed spacers and the two internal transcribed spacers) (not to scale). (Adopted from Nolan and Cribb, 2005)

The 18S rRNA, or small subunit ribosomal gene, is among the slowest evolving sequences found in living organisms and is used to infer deep phylogenetic relationships among ancient lineages (Hillis and Davis, 1986). The 28S rRNA, or large subunit rRNA gene, is larger than 18S and shows a faster rate of evolution through its different domains than does the 18S gene. However, the 28S rRNA gene does possess regions with levels of gene conservation similar to those of 18S (Hillis and Dixon, 1991). Both regions have been used to detect

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species boundaries within digenean families (Leon-Regagnon and Paredes, 2002) however, this is not common.

The internal transcribed spacer (ITS) includes two spacers; ITS1 and ITS2, separated by the 5.8S rRNA gene. Previous studies have characterized *Diplostomum* in the northern hemisphere (Europe and North America) using the ITS sequences of rDNA. Five European species and three North American species of *Diplostomum* were distinguished based on divergence in ITS sequences ranging from 1.6% to 4.6% (Galazzo *et al.*, 2002; Niewiadomska and Laskowski, 2002). Niewiadomska and Laskowski (2002) found no differences between ITS1 sequences from *Diplostomum spathaceum* and *D. parviventosum*. The two species were not synonymised because they considered them distinguishable by morphological differences between adult, cercarial and metacercarial stages. However, in addition to the two sequences for each species lodged by these authors on Genbank (which are all identical as reported) there are a further four otherwise unpublished ITS1 sequences for *D. spathaceum* as reviewed by Nolan and Cribb (2005). Galazzo *et al.* (2002) found 0.0–0.8% intraspecific variation in the ITS1 and ITS2 sequences from three North American species of *Diplostomum*. Five *Diplostomum baeri* sequences were identical; four *D. huronense* sequences were identical whereas a fifth had a single transition. Six of nine specimens identified as *D. indistinctum* were identical. The other three differed at three sites in the ITS1, while one differed at a further five sites in ITS1 and a single site in ITS2. The three specimens exhibiting intraspecific variation had been identified only tentatively (Galazzo *et al.*, 2002).

Taxonomic studies on African diplostomids are scarce and limited to few species descriptions that were published in the 1930s–1960s (e.g., Beverley-Burton, 1963; King and Van As,

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1997) and whose originality has never been established. Inappropriate naming of *Diplostomum* species in tropical countries has led to misidentification of the species and thus inaccurate estimates of species diversity. A study by Chibwana and Nkwengulila (2010) assigned the name *D. mashonense* to a parasite species recovered from the brain of clarid fish in Tanzania, however, a recent study on the same by Chibwana *et al.* (2013), elicited a controversy in the naming of the parasites thereby reallocating *D. mashonense* to *Tylodelphys mashonense* using ITS1-5.8S-ITS2 molecular markers. This controversy depicts a complicated taxonomic situation in Africa coupled with poor taxonomic resources and expertise. Furthermore, misidentification of species is a fact that cannot be neglected because, concurrent infections with the eye-infecting diplostomids appear to be frequent and widespread geographically being reported in Tanzania, South Africa and Kenya (Chibwana and Nkwengulila, 2010; Madanire-Moyo *et al.*, 2010; Kembenya *et al.*, 2012). This fact, coupled with the morphological similarity of the metacercariae makes the practical species diagnosis based on morphology very difficult. Use of rapid and accurate molecular identifications using standardized tools and barcoding approaches appear most suited (April *et al.*, 2011) for uncovering diplostomid taxonomic diversity in different regions worldwide.

To date, a total of eight named species of *Diplostomum* using ITS rDNA sequences are now available. These include: complete sequences of the ITS1-5.8S-ITS2 gene cluster for *Diplostomum huronense* (La Rue, 1927), *Diplostomum indistinctum* (Guberlet, 1923) and *Diplostomum baeri* (Dubois, 1937) from fish and/or gulls collected in Canada (Galazzo *et al.*, 2002; Locke *et al.*, 2010a, b) and partial ITS1 sequences for *Diplostomum baeri*, *Diplostomum mergi* (Dubois, 1932), *Diplostomum paracaudum* (Iles, 1959), *Diplostomum parviventosum* (Dubois, 1932), *Diplostomum pseudospathaceum* (Niewiadomska, 1984) and *Diplostomum spathaceum* (Rudolphi, 1819) from larval stages collected in Poland

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(Niewiadomska and Laskowski, 2002). Furthermore, ITS1-5.8S-ITS2 sequences for nine additional presumed species (unidentified isolates labelled as *Diplostomum* spp. 1–9) have been generated recently from fish metacercariae in Canada (Locke *et al.*, 2010a, b; Rellstab *et al.*, 2011). These studies only shed light on previous studies conducted in the St. Lawrence River in Canada which is geographically limited to the northern hemisphere (Europe and North America).

2.3 Survey of *Diplostomum* trematodes in pond snails using 18S rDNA

The life cycles of many parasites are poorly known (Locke *et al.*, 2010b). Among the digeneans, it is difficult to determine the host species used during different phases of a parasite's life cycle. This is because the alpha taxonomy of digeneans is based on the morphology of the adult, and this information generally cannot be linked to larval stages bearing little resemblance to adults. This problem is acute for cercariae in the Diplostomoidea, in which even generic level identification is often difficult.

The taxonomy of the genus *Diplostomum* is in a controversial state due to several limiting factors as stipulated by Kostadinova, (2008). The classical method of elucidating trematode life cycles is the experimental infection of animals in the laboratory (Ostrowski de Nuñez, 1989). However, such research is logistically demanding, and it is usually feasible to study only a single parasite species and a small number of host species. In addition, it is often necessary to use non-natural hosts in experimental studies, which reduces their relevance to parasite – host associations in nature.

This challenge has led to controversial naming of *Diplostomum* species as well as contradictions in the number of species identified among the hosts in the life cycle. A recent checklist data in Europe representing *Diplostomum* species data collected in Czech and

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Slovak Republics provided a ratio of 9:3:2 species in birds, fish and snails, respectively (Georgieva *et al.*, 2012). This is an indication of scarcity of data on *Diplostomum* infection in snails compared to numerous records available on *Diplostomum* sp. infections in fish and birds. This is mainly due to identification challenges of *Diplostomum* sp. exhibited in the metacercariae as well as cercariae. So far studies on *Diplostomum* sp. cercariae are rare as reported by Georgieva *et al.* (2012) whilst challenges faced in aquaculture as a consequence of the parasites. The major constraint is lack of taxonomic resources and expertise in this field as well as identification challenges of the parasite. Temporary and spatial distribution studies on snail vectors have been limited to schistosomatidae due to the significant socio-economic importance of the digenean family. Reports on schistosomatidae have established that *Biomphalaria* and *Lymnea* species of snails play an important role in the transmission of schistosomiasis within the informal settlements of Kisumu city (Opisa *et al.*, 2011). Nkwengulila and Kigadye (2005) likewise reported cercariae resembling those of the Diplostomidae from *Biomphalaria pfeifferi* snails at Mindu dam in Tanzania. Kibos area, is among the informal settlements of Kisumu City, however, the area was not included in a pilot study conducted by Opisa *et al.* (2011) owing to financial constraints. Considering that Kisumu city display temperatures of between $22^0C - 32^0C$ which are optimum for snail development and reproduction, Kibos area which is located within Kisumu City share a similar microclimate environment as the studied informal settlements. Thus, lack of studies on the role of different snails in water shed environments in Kibos area predispose the community to major problems of health and fish farming development.

2.4 Effect of *Diplostomum* parasites on Length - Weight relationship of farmed *Oreochromis niloticus* L.

Diplostomum parasites have been encountered in farmed fish and as such received wide theoretical and empirical attention due to their pathogenic consequences to aquaculture (Sangster *et al.*, 2004). The eye fluke infection has been associated with decreased growth of fish in rearing conditions (Buchmann and Uldal, 1994; Paaver *et al.*, 2004) and increased mortality in fish as a result of reduced vision caused by cataracts which impair the fish's feeding efficiency (Voutilainen *et al.*, 2008) and makes the fish more vulnerable to avian predation (Seppälä *et al.*, 2005). The loss of vision, as a result of metacercariae in the eyes was observed to reduce the alimentary efficiency in *Leuciscus leuciscus* and *Gasterosteus Aculeatus* (Crowden and Broom, 1980; Owen *et al.*, 1993). However, assessment of the impact of the parasite in aquaculture has rarely been investigated. In Kenya, recent studies on *Diplostomum* parasites affecting cultured *Oreochromis niloticus* (Matolla, 2009; Kembenya *et al.*, 2012) have been published, but little is known about the extent of economic loss caused by these parasites in fish farming. Evidence of decline in the average size of fish (100g-180g) from 300 grams has been reported previously in Kibos area over the last 3-5 years (Otieno, 2010). In addition, current small sizes of fish available in Kibos fish market clearly suggest that fish farming in the area is faced by major challenges. A report by Shitote *et al.* (2011) examined the challenges facing fish farming development in western Kenya and established that farmers do not know / understand the diseases affecting fish and it is, therefore, difficult for them to identify whether diseases have a negative impact on their fishing endeavours. Geographically, Kibos area is located 200 Km North East of Eldoret town where prevalence of *Diplostomum* was earlier reported by Matolla (2009) and Kembenya *et al.* (2012). Eldoret town and Kibos area share a similar microclimate environment and therefore the environmental conditions are similar to favour transmission of

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parasites between the two regions. Currently, no studies have been carried out in Eldoret and Kibos area to establish temporary and spatial distribution of *Diplostomum* infection in order to understand the effect of the parasite on fish production.

CHAPTER THREE

# MATERIALS AND METHODS

3.1 Study area

Kibos area is located at Kajulu village of Kisumu East district near Kisumu City. Kibos area lies between latitudes - 0.07001′ N/ 0° 4'0.0012"S and longitudes 34'81092′E / 34° 49' 0.0012"E (Fig. 2). The area is largely endowed with black cotton soil which is very sticky with pH values ranging between 6.55 and 6.85, base saturation percentages of 95.3 and cation exchange capacity estimated at 72.5 Cmol/kg (Bowman and Seim, 1995). These characteristics enhance aquaculture through low soil permeability, relatively high availability of nutrients, relatively low amounts of acidity, and high base saturation percentages, which result in minimal lime requirements. Period of sampling for this study (December 2011- February 2012) was preferred based on previous literature by Kembenya *et al*. (2012) in Kenya that reported diplostomid transmission patterns to be higher during dry seasons compared to wet seasons. Farms were selected for the study because they acquire fingerlings from Lake Basin Fry Production Centre which serves as the main supplier of fingerlings in western Kenya. Generally the farms selected were distantly situated (> three km apart) to ensure spatial distribution of parasites affecting fish.

3.2 Study design

This study adopted a cross-sectional survey in sampling of fish and snails in three fish farms namely; Lake Basin Fry Production Centre and two privately owned fish farms (Dr. Mzungu's fish farm and Auji fish farm) located in Kibos area. Cross sectional survey design was chosen because it aims to describe the relationship between a disease and other factors of interest as they exist in a specified population at a particular time.

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Each farm consisted of more than six fish ponds for grow-out and nursery ponds. Three fish ponds representing the grow-out and nursery ponds were randomly selected for this study using a computer random number generator technique. This technique ensured that fish of target population had equal and independent chance of being included in the sample.

KIBOS AREA STUDY SITE
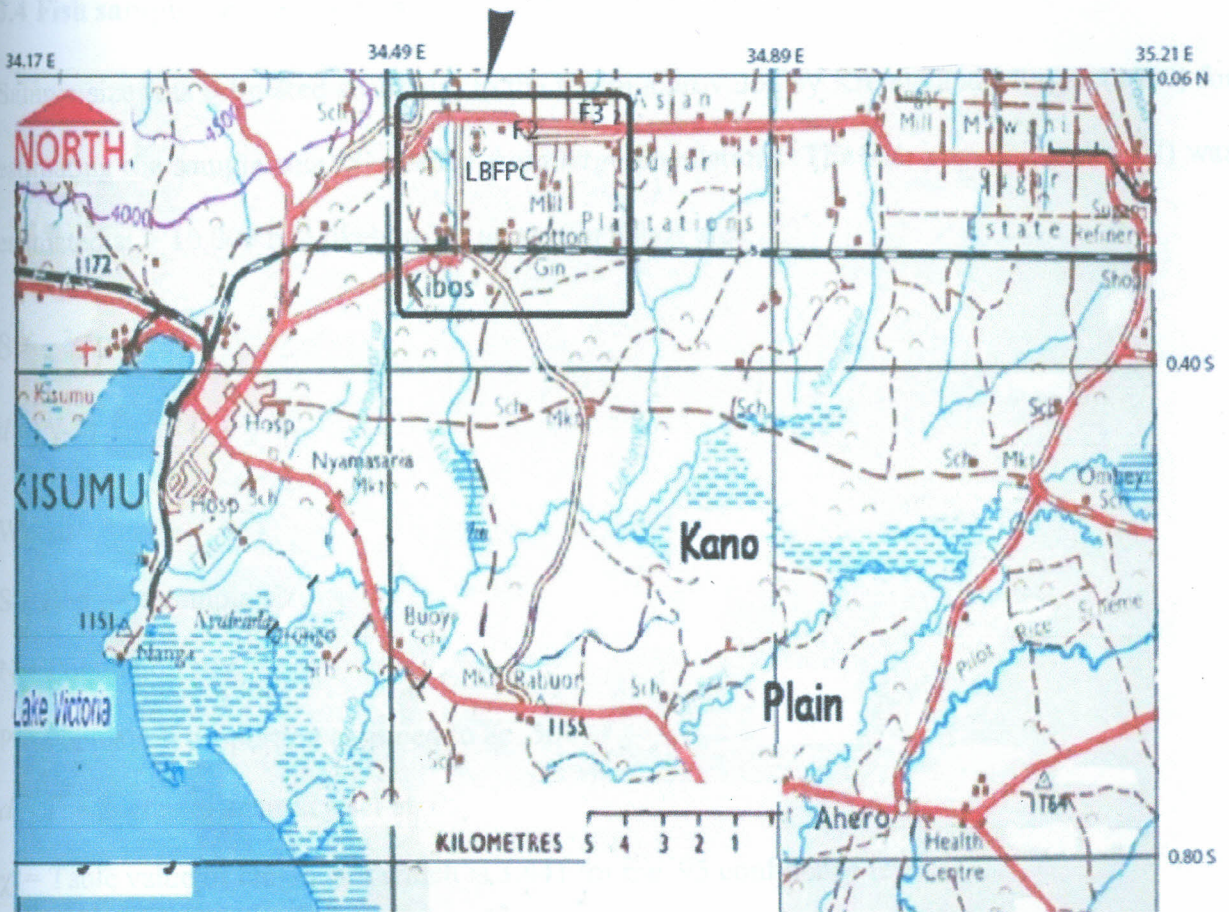
LEGEND
LBFPC - Lake Basin fry Production Centre
F2   - Auji Fish farm
F3   - Dr. Mzungu's Fish Farm

Figure 2 : Map of Kano plains showing Kisumu Municipality and the study area site (marked in box).

3.3 Study specimen

Nile tilapia, *Oreochromis niloticus* L.1758 (Cichlidae) fish was used in this study. It is the main farmed fish species in Kisumu County and one of the major parasitized fish species by *Diplostomum* parasites because of its phyto - detritus feeding habits (Schiemer, 1996).

3.4 Fish sample size calculation

Sample size was estimated according to the formula provided by Krejcie and Morgan (1970) for estimating the sample size (S) needed from large populations. The fish population size (N) was estimated at > 10,000 fish likely to be present in Kibos area.

$$S = \frac{\chi^2 NP(1-P)}{d^2(N-1) + \chi^2 P(1-P)}$$

Where:

S = Required sample size

N = Population size

P = Population proportion assumed to be .50

d = The degree of accuracy set at .05

χ^2 = Table value of chi-square which is 3.841 for the .95 confidence level.

A P value of 0.50 was assumed because, as suggested by Krejcie and Morgan (1970), if there is no previous estimate available of the proportion of fish in the target population assumed to have the characteristics of interest (*Diplostomum* infection), 50% should be used. A precision of 5% was chosen (d value 0.05) as a good compromise between an appropriate precision and the resources available for this preliminary study. With these remarks, the resulting S was estimated at **384**.

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3.5 Fish sampling procedure and transportation

Sampling was done after every three weeks for a period of three months (December 2011 – February 2012). Sixty four (64) Nile tilapia fish were randomly sampled from three fish ponds representing nursery and grow-out ponds in each fish farm. These ponds were chosen using a simple random computerized technique to eliminate biasness and increase chances of establishing diversity of *Diplostomum* parasites among the fish. Sampling of fish was conducted between 0900 h and 1100 h. The sampling time coincided with feeding time of fish in all the farms. This was to ensure that all the fish were active and therefore eliminate biasness during fishing. A seine net of 1.5m diameter and 6mm mesh was used for sampling. At the end of the study, a total of 576 fish was collected (64× 3×3). Sampled fish were then transported in iced cool boxes at 8^0C to department of Zoology laboratory, Maseno University, for analysis.

3.6 Sampling of snail population and transportation

A minimum of 100 snails were randomly collected from the selected ponds using a scoop net. Snail sample size calculation was estimated according to Huspeni *et al.* (2005). Sampling was performed between 0830 h and 1030 h. This was following the hypotheses that most snails during morning hours are dormant and begin shedding of cercariae at 1100 hours coinciding with the presence of the next host (Combes *et al.*, 1994). At each collection time, snails from each site were appropriately labelled and transported in separate perforated plastic containers to the department of Zoology laboratory, Maseno University for analysis.

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3.7 Laboratory procedures

3.7.1 Examination of fish specimens for *Diplostomum* parasites

Fish eyes were dissected and then examined for metacercariae with a stereoscopic microscope using procedures as described by Yamaguti (1971) and Gibson (1996). The metacercariae extracted from each eye were counted as separate lots, and placed in a petri dish containing saline solution before storing in 95% ethanol. Isolated metacercariae were stored in vials at 4^0C.

3.7.2 Examination of fish for length – weight relationship

The specimens were mopped on filter paper to remove excess water from their body surfaces. Total and standard lengths were then measured using a meter ruler and recorded in centimetres. The total length (TL) was measured as the distance from snout to the tip of the caudal fin while the standard length (SL) was measured as the distance from the snout to the caudal peduncle. The body weight was taken using a table top weighing balance to the nearest 0.1g. Fish samples were recorded according to their collection sites as F1, F2 and F3 (for Lake Basin Fry Production Centre – F1, Dr. Mzungu's Fish Farm – F2, and Auji Fish Farm – F3).

3.7.3 Confirmation of snail infection

By 1100 h, snails were rinsed and placed individually in 24-well culture plates (Corning Glass Works, Corning, NY, USA) containing 1 ml of filtered Dechlorinated water for a period of 24 hours. The snails were exposed to a 15-h light/9-h dark lighting regimen according to Steinauer *et al.* (2008). After 24 hours, plate wells were examined with a dissecting microscope for presence of cercariae shed by the snails. Identification of cercariae was made according to their morphology and behaviour, as strigeoid cercariae form a distinctive right-angle resting position

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and the furcae spread apart at an angle of 180^0 (Niewiadomska, 1986). Isolated cercariae were picked and stored in vials containing 95% ethanol and stored at 4^0C.

3.7.3.1 Physicochemical characteristics of the pond water

We chose physicochemical parameters of the water suggested to influence snail distributions and *Diplostomum* development in fish. In this regard, we recorded pH and temperature. Water pH and temperature were measured directly in the field using Hanna HI 9828 Hand held Multiparameter meter.

3.7.4 Genetic characterization of *Diplostomum* species

3.7.4.1 Extraction of DNA

Extraction of DNA was performed following the method of Truett *et al.* (2000) (Appendix 1).

3.7.4.2 PCR amplification of 18S rDNA gene sequence

DNA amplification of the 18S rDNA sequence was performed in 25 µl volumes via the polymerase chain reaction (PCR) in MJ Gradient thermocycler (Gene amp. PCR system 9700, Applied Biosystems U.S.A). Each PCR reaction consisted of:

Reagent	Initial Concentration	Final Concentration	25 µl reaction Volume
Standard *Taq* Reaction Buffer	10X	1 X	2.5 µl
Mg Cl$_2$	25 mM	2.5µM	2.5 µl
dNTP	10 mM	200 µM	0.5 µl
Forward primer	10 µM	5 µM	1.25 µl
Reverse primer	10 µM	5µM	1.25 µl
Taq DNA Polymerase	1 U/µL	2.5 units/50 µl PCR	0.25 µl
Template DNA	5ng/µL	1.3ng/µL	6.5µl
Nuclease-free water			10.25 µl

The mixture was then vortexed slightly and placed into a PCR machine for amplification.

Primer sequences

Primer	Oligonucleotide sequence	Approx. Product size
18S9F 5′	TGATCCTGCCAGTAGCATATGCTTG - 3′	
18S300R_1 5′	TCAGGCTCCCTCTCCGG - 3′	400nt
18S637R_2 5′	TACGCTATTGGAGCTGGAGTTACCG -3′	600nt

F – Forward primer; R_1 – Reverse primer 1; R_2 – Reverse primer 2 (According to Moszczynska *et al.*, 2009)

The prepared mixtures were amplified according to the following conditions:

Reaction	Temperature	Time	cycles
Initial denaturation	94^0C	2min	
Initial annealing	50^0C	45sec	pre-PCR
Initial extension	72^0C	1:50sec	
Denaturation	94^0C	30sec	
Annealing	50^0C	30sec	35cycles
Extension 7	72^0C	1min	
Final extension	72^0C	10min	
Hold	4^0C	∞	

Agarose gel electrophoresis for 18S rDNA gene sequence

Amplicons were loaded onto casted 1% agarose gel alongside a 100bp DNA marker and resolved

at a constant voltage of 80 volts for one hour prior to Ultra Violet light visualization.

3.7.4.3 PCR amplification of ITS rDNA gene sequence

PCR amplification was carried out in a final volume of 25µl. The following primer sequences were used.

Primer sequences

Primer	Oligonucleotide sequence	Approx. Product size
D1 (F)	AGGAATCCTGGTAAGTGCAAG	1100nt
D2 (R)	CGT TAC TGA GGG AAT CCT GGT	

In 1.5 ml microcentrifuge tubes, the following PCR master mix according to Gallazo *et al.* (2002) with minor modification during optimization was prepared and aliquoted into respective reaction tubes as shown below.

ITS rDNA PCR amplification master mix

Reagent	Initial Concentration	Final Concentration	25 µl reaction Volume
Standard *Taq* Reaction Buffer	10X	1 X	2.5 µl
Mg Cl$_2$	25 mM	1.25µM	1.25 µl
dNTP	10 mM	0.05µM	0.125 µl
Forward primer	10 µM	0.1µM	0.25 µl
Reverse primer	10 µM	0.1µM	0.25 µl
Taq DNA Polymerase	1 U/µL	1.25 units/50 µl PCR	0.125 µl
Template DNA	5ng/µL	0.6ng/µL	3.0 µl
Nuclease-free water			17.5µl

22.0 µl of PCR master mix was aliquoted into each 1.5 µl PCR tube after vortexing. 3.0µl of sample DNA was added into each tube to bring the total reaction volume to 25µl. The mixture was then vortexed slightly and placed into MJ Gradient thermo cycler (Gene amp. PCR system

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9700, Applied Biosystems U.S.A.). The prepared mixtures were amplified according to the following conditions:

Reaction	Temperature	Time	cycles
Initial denaturation	94^0C	2min	
Initial annealing	50^0C	45sec	pre-PCR
Initial extension	72^0C	1:50sec	
Denaturation	94^0C	1min	
Annealing	56^0C	1min	30cycles
Extension	72^0C	2min	
Final extension	72^0C	5min	
Hold	4^0C	∞	

Agarose gel electrophoresis for ITS rDNA gene sequence

The product was run through electrophoresis using a 1% agarose gel containing ethidium bromide (0.5 µg/mL) alongside 0.5 µg/µl; Fermentas: GeneRuler$_{TM}$ 1kbp DNA Ladder and visualized under Ultra Violet light.

3.7.5 DNA purification

PCR products were then purified using the Gene – Jet PCR purification kit (Fermentas, No. K 0701) following the manufacturer's protocol (See purification protocol, Appendix 2).

3.8 DNA sequencing

DNA was sequenced using an ABI 377 Prism Automated DNA Sequencer. Sequencing for 18S rDNA gene was performed using the forward PCR primers whereas the ITS rDNA gene was sequenced using sequencing primers according to Gallazo *et al.* (2002). The automated sequence data were analyzed using the SEQUENCHER v.3.0 software (Gene Codes Corporation, Inc.).

Chromatograms were visually inspected and consensus sequences were aligned manually prior to further analysis.

3.9 Molecular phylogenetic analysis of *Diplostomum* species

Contiguous sequences of the small subunit region and internal transcribed spacer regions of ribosomal DNA from each specimen were created from forward and reverse chromatograms and edited using DNA Baser version 2.7 (Appendix 3). Multiple alignments of the contigs was conducted using Muscle 3.8.31 multiple alignment software. Nucleotide sequence data of ITS rDNA and 18S rDNA sequences from all clusters were submitted to Basic Local Alignment Search Tool (BLAST,www.ncbi.nlm.nih.gov/blast) for similarity searches in Gen Bank with the aim of matching our representative sequences with those published in other studies of diplostomoids (Appendix 4). *Diplostomum mashonense* MS14 18S ribosomal RNA gene (see appendix iv) was used as a reference sample and our isolates depicted a 100% similarity to *Diplostomum mashonense*. Potential species were distinguished by clustering in Neighbour-Joining (NJ) phenograms using MEGA version 4.0.2 (Tamura *et al.*, 2007). The reliability of internal branches in the Neighbour-Joining trees was assessed using bootstrap analysis with 1,000 replicates. The resulting networks were rooted with the out-group taxa.

3.7 Data Analysis

Analysis of parasite occurrence among three fish farms

Prevalence (%) of *Diplostomum* parasites was estimated as the ratio between the number of infected fish and the number of examined fish expressed in percentages. The mean intensity (M.I) was determined as the ratio between the total number of parasites in a sample and the number of infected fish in a sample. The mean abundance (M.A) was determined as the ratio

between the total number of parasites in a sample and the total number of fish examined (infected + uninfected).

One-way ANOVA was used to test for differences in abundance of parasites among the farms. To determine the possible correlation between the parasite number and host standard length, Pearson's linear correlation "r," was used. Kruskall-Wallis H test was used to test for differences in parasite number among the host length classes. Comparison for prevalence of infection between the snail populations among the farms was performed using Fisher's exact test. Associations between snail abundance and physicochemical variables were determined using spearman correlations (rs). Results from various statistical tests were considered significant at p ≤ 0.05 using SPSS v. 17.00 (USA) software packages.

Analysis of Fulton's condition factor

The length measurements were converted into length frequencies with constant class intervals of 5 cm. The mean standard lengths and weights of the classes were used for data analysis, the format accepted by FISAT (Gayando and Pauly, 1997). The Fulton condition factor (K) of the experimental fish was compiled from values of the growth exponent according to Froese (2006):

$$K = \frac{100\ W}{L^b}$$

Where: **K** = Fulton's Condition factor; **W**= Mean weight of all specimens in a given length class (g); **L**= Mean length of the respective length class (cm) and b = regression coefficient. K values obtained were compared with the standard K = 1.0 by Student's t-test. The independent-samples t-test was used to test for differences in the K values between parasitized and un-parasitized

hosts. To determine the relationships between condition factor and the number of parasites, Pearson product moment correlation coefficient was used.

Analysis of the length – weight data

Relationship between weight (W) and length (L) of fish was expressed by equation: $W = aL^b$ (Froese, 2006). Where: W = Mean weight of all specimens in a given length class (g); L= Mean length of the respective length class (cm); **a** = exponent describing the rate of change of weight with length (= the intercept of the regression line on the Y axis); **b** = The slope of the regression line (also referred to as the allometric coefficient). The "a" and "b" values were obtained from a linear regression of the length and weight of fish. The degree of adjustment of the model studied was assessed by the Correlation Coefficient (r). Student's t-test was applied to verify whether the declivity of regression (constant "b") presented a significant difference of 3.0. In all cases a statistic significance of 5% was adopted.

CHAPTER FOUR

RESULTS

4.1.0 Prevalence, Abundance and Intensity of *Diplostomum* parasites from three Fish Farms in Kibos area

Metacercariae of *Diplostomum* sp. were found actively moving in the vitreous humor and lens of the sampled fish. Prevalence, mean abundance and mean intensity of parasitic indices were investigated. Prevalence among fish from Lake Basin Fry production centre, Auji fish farm and Dr. Mzungu's fish farm was 47.4%, 43.2 % and 66.1% respectively (Table 1). *O. niloticus* from Lake Basin Fry production centre had a mean intensity of 12 parasites per host fish with an abundance range of between 5 and 8 parasites. Auji fish ponds had a mean intensity of 11 parasites per fish with an abundance range of between 4 and 8 metacercariae. Dr. Mzungu's fish ponds had a mean intensity of 9 parasites per fish and abundance range of between 5 and 7 parasites (Table 1).

Table 1: Summary of Prevalence, Mean Abundance and Mean Intensity of *Diplostomum* parasites from three Fish Farms in Kibos area

Farm	No. of Fish Examined	No. of Fish infected	Prevalence (%)	No. of Parasites recovered	Mean Abundance	Mean Intensity
LBFPC	192	91	47.4	1101	5.7	12.1
Auji Fish Farm	192	83	43.23	934	4.9	11.3
Dr. Mzungu's Fish Farm	192	127	66.1	1228	6.4	9.7
Mean Total	192	100.3	52.3	1242	6.5	12.4

Prevalence is the proportion of infected hosts among all hosts examined at a particular time.
Mean abundance is the mean number of parasites found in all hosts.
Mean intensity is the mean number of parasites found in infected hosts.

4.1.1 Prevalence of Diplostomiasis infection in Kibos area

A total of 576 fish were collected in three fish farms in Kibos area. General prevalence of infection was 52.3% with 301 fish infected. Lake Basin Fry Production Centre had a prevalence of 47.42%, Auji Fish Farm had a prevalence of 43.23% and Dr. Mzungu's Fish Farm had a prevalence of 66.1% (Table 1)

4.1.2 Abundance of *Diplostomum* parasites among the Fish Farms

Kruskal-Wallis test which is a nonparametric test equivalent to the one-way ANOVA, and an extension of the Mann-Whitney U test was adopted. The test revealed a statistically significant difference in parasite abundance among the three farms during the first sampling [H(2) = 40.480, p = 0.000], with a mean rank for farm I, 91.66 for Farm II, 46.24 and 91.91 for Farm III. Similarly during the second sampling period, the parasite abundance among the farms were significantly different with H (2) = 7.232, p = 0.027, with a mean rank for farm I, 70.46 for Farm II, 90.10 and 70.44 for Farm III. Third sampling period did not indicate any significant difference in parasite abundance among the farms with [H(2) = 0.575, p = 0.750], with a mean rank for farm I, 75.51 for Farm II, 74.78 and 80.71 for Farm III.

4.1.3 Intensity of *Diplostomum* parasites within different classes of standard lengths

Regression analysis indicated mean parasite intensity varied among the fish length groups. The highest number of parasites was observed between the class size of 10.1 – 15.0 cm (Fig. 3). Contrary to the parasite increase, a drastic decrease in the parasite numbers was observed among fish $\geq$ 15 cm in all the farms.
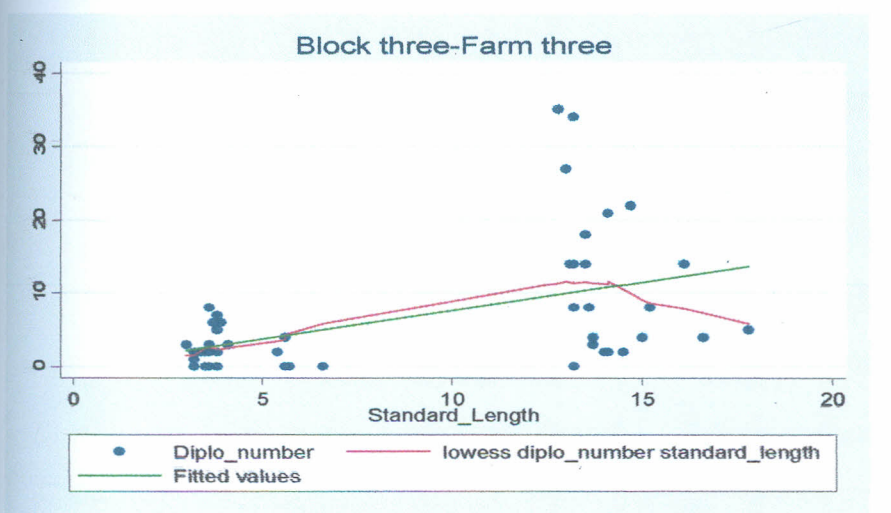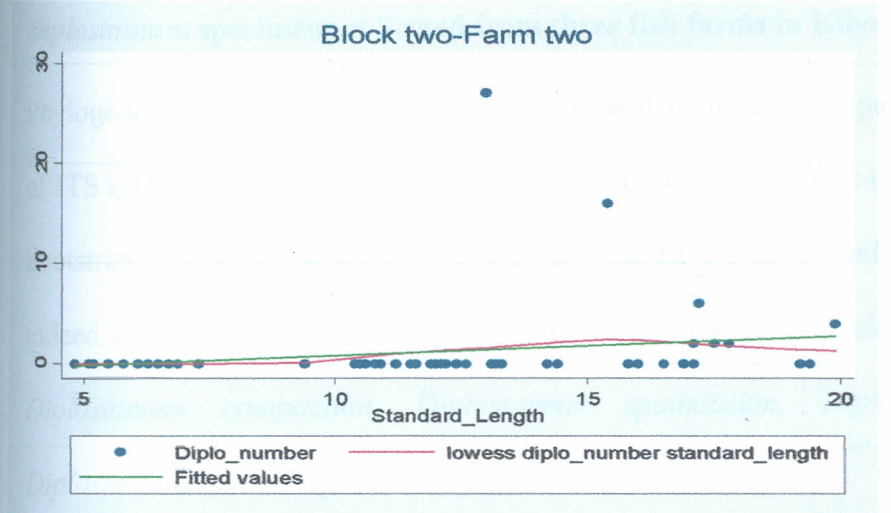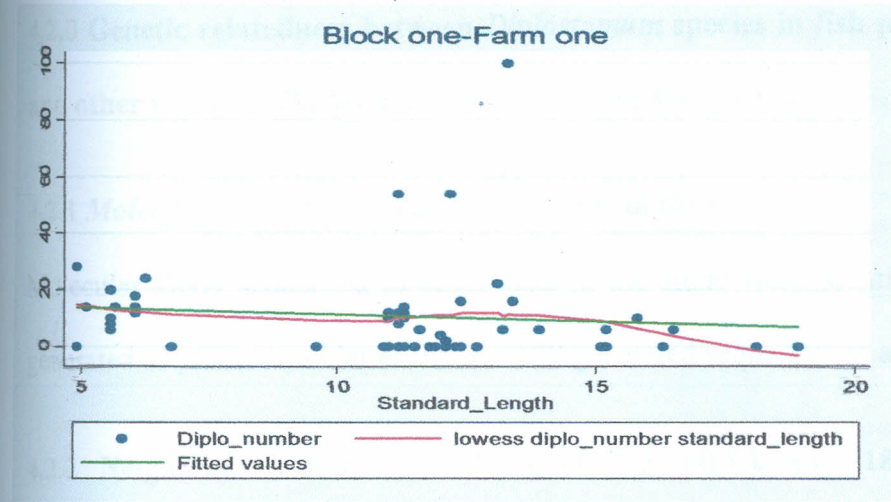
Figure 3: Mean intensity of *Diplostomum* parasites as observed between class lengths (cm) of *Oreochromis niloticus*

4.2.0 Genetic relatedness between *Diplostomum* species in fish population from Kibos area and other reported *Diplostomum* species in the Gene bank

4.2.1 *Molecular DNA sequences of specimens in the study*

Molecular DNA sequences of specimens in the study were identified with letter 'D' for ITS generated sequences and letter 'M' for 18S generated sequences as attached in appendix 5.

4.2.2 Neighbour - Joining analyses of ITS rDNA and 18S rDNA sequences from *Diplostomum* specimens collected from three fish farms in Kibos area

Phylogenetic analyses were conducted based on the alignment of partial and complete sequences of ITS rDNA and 18S rDNA using the NJ method. The resultant tree as shown below presented bootstrap consensus values of >50% for almost all branches confirming that the samples were indeed members of the *Diplostomum* genus and were closely related to *Diplostomum phoxini*, *Diplostomum compactum*, *Diplostomum spathaceum*, *Diplostomum mashonense* and *Diplostomum baeri* (Fig. 4 and Fig. 5).
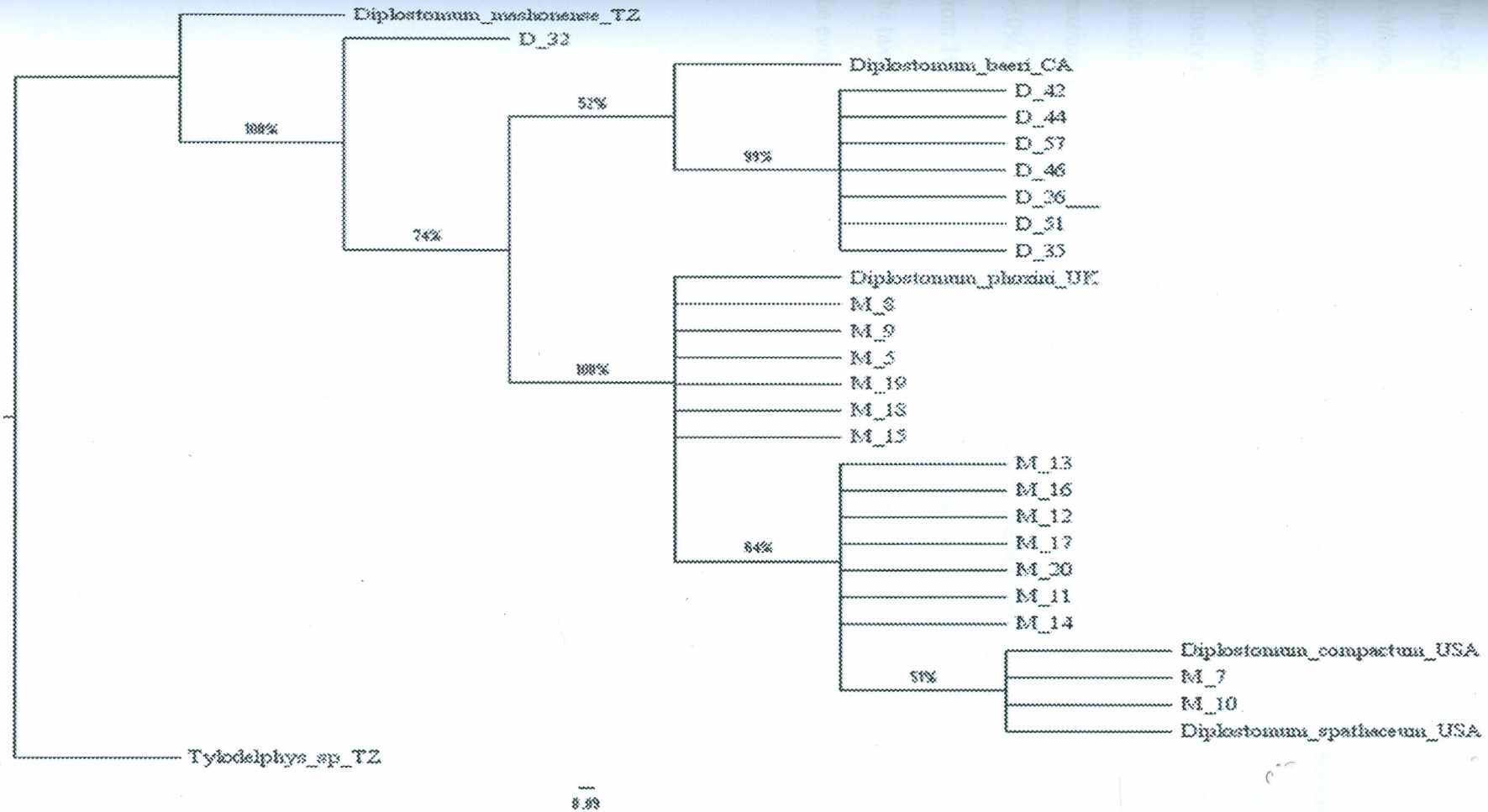
Figure 4: Neighbour-Joining tree of sequences constructed in this study in comparison with representatives in the *Diplostomum* sp. as inferred from ITS rDNA (Specimen labelled D's) and 18S rDNA (Specimen labelled M's) sequences. Numbers at the nodes represent bootstrap values. *Tylodelphys spp.* was set as an out group.

The NJ analyses for 18S rDNA sequences alone revealed presence of single species of *Ichthyocotylurus, Strigidae* and *Bolbophorus,* two species of *Apharyngostrigea* and *Posthodiplostomum,* one species of *Alaria* and at least three species of Diplostomids (*Diplostomum phoxini, Diplostomum compactum* and *Diplostomum spathaceum*) that were closely related to the sample specimens (Fig. 5). The NJ analyses for ITS rDNA (Fig. 4) revealed genetic relationship of the sample specimens to two *Diplostomum* species (*Diplostomum mashonense* and *Diplostomum baeri*). The resultant tree presented bootstrap consensus values of >50% for almost all branches. The bootstrap (Felsenstein, 1988) consensus tree was inferred from 100 replicates (Fig. 4) and 1000 replicates (Fig. 5) and taken to represent the relationship of the taxa analyzed. The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.
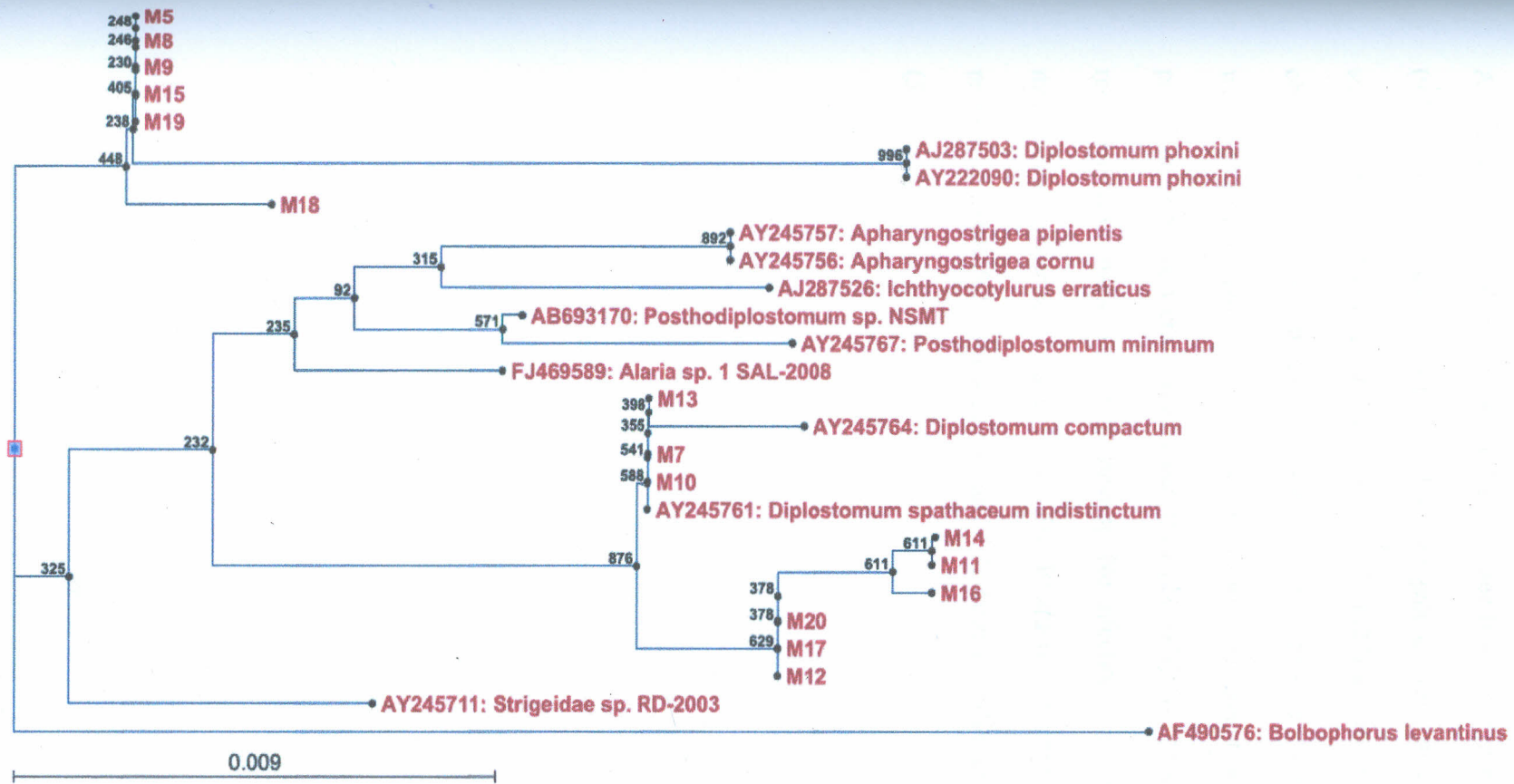
Figure 5: Neighbour joining tree depicting genetic relationship between sample specimens labelled "M" and other reported *Diplostomum* sp. as inferred from 18S rDNA sequences. Numbers at the nodes represent bootstrap values. *Bolbophorus* sp. was set as out group.

4.3 Survey of trematodes in pond snail intermediate hosts in three fish farms in Kibos area

A total of 1359 snails were collected from three fish farms in Kibos area. Out of these, 124 (9.12%) were infected with trematodes. The general prevalence of natural infection in host snails were, 0.64% (1) in *Bulinus* sp., 12.58% (123) in *Biomphalaria* sp. and no infection observed in *Lymnaea* sp. and *Cerratophalus* sp. (Table 2). Nonetheless, no individual snail was infected with more than one type of digenetic cercariae (multiple infections). The most prevalent cercariae were strigeoid cercariae (21.69%) (which contain genus *Diplostomum*) recovered from snails of *Biomphalaria* sp. No infection was observed among the snails for mammalian cercariae. Lake Basin Fry Production Centre had the highest infection prevalence (10.42%) compared to Auji fish farm (5.56%) and Dr. Mzungu's fish farms (5.71%).

Table 2: Summary of the distribution and prevalence of trematodes in pond snails collected from three fish farms in Kibos area

Site	Type of snail species	Number of snails collected	Number of snails infected with:			
			Mammalian cercariae	Xiphidiocercariae	Amphistomes	Strigeoid cercariae
LBFPC	*Biomphalaria* spp	288	0	0	0	30 (10.42%)
	Lymnea spp	180	0	0	0	0
	Cerratophalus	0	0	0	0	0
	Bulinus spp	6	0	0	0	0
Auji Fish Farm	*Biomphalaria* spp	270	0	18(6.67%)	24(8.89%)	15 (5.56%)
	Bulinus spp	156	0	0	1 (0.64%)	0
	Lymnea spp	12	0	0	0	0
Dr. Mzungu's Fish Farm	*Biomphalaria* spp	420	0	0	12 (2.86%)	24 (5.71%)
	Lymnea spp	3	0	0	0	0
	Bulinus spp	24	0	0	0	0

4.3.1 Survey of *Diplostomum* trematodes in pond snails using 18S rDNA molecular marker

PCR micrographs of cercariae by use of 18S rDNA gene sequence

Analysis of PCR products of cercariae from snails by electrophoresis using 1% agarose gel showed successful DNA amplification. PCR products of expected size were observed at 400 and 600 bp from the infected samples as shown in figures 6 and 7. Loaded wells that did not show any band were considered negative for *Diplostomum* infection.
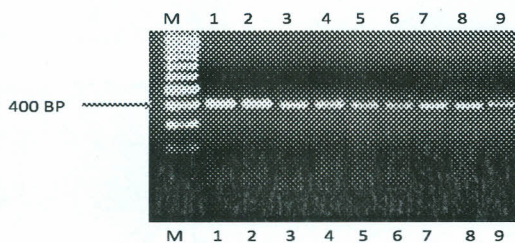
Figure 6: PCR gel band showing distinctive gene products of 400bp.
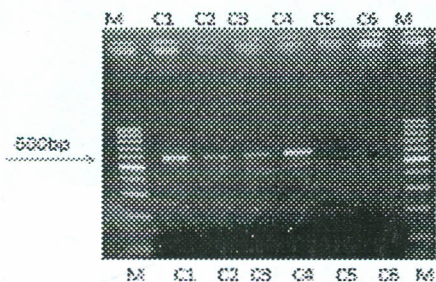
Figure 7: PCR gel band showing distinctive gene products of 600bp.

4.3.2 Environmental factors

The most common vegetation identified in close proximity to the fish farms was grass, trees, papyrus and sugarcane plantation (Plates 1 - 3). Qualitative data indicated that the vegetation cover was associated with snail abundance in the farms.

Plate 1: Ducks in water, Egrets and Comorants in a pond in Auji Fish Farm
Plate 2: Sacred Ibis in Lake Basin Fry Production Centre (LBFPC)
Plate 3: Comorants in Dr. Mzungu's Fish Farm

4.3.3 Physicochemical parameters of water

There was a monthly variation in water temperature in all the sites. The mean values of water temperature ranged between 26 - 30°C. This significantly influenced the overall snail abundance ($F_{2, 900}$ = 21.23, p < 0.01). There was a positive association between water temperature and overall snail 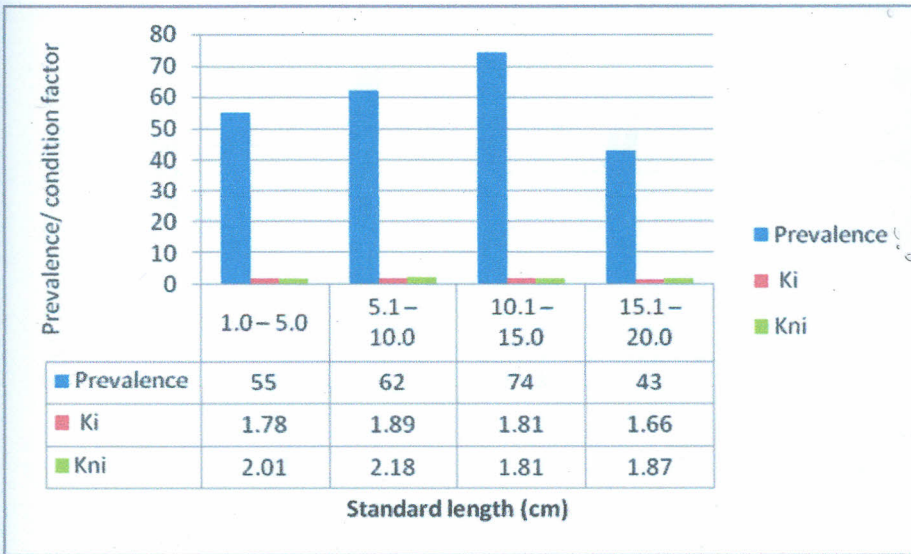abundance (r = 0.8, p = 0.01). The mean pH of water from LBFPC was 6.62 ± 1.1 (range = 5.2 - 6.2), Auji Fish Farm was 5.34 ± 1.2 (range = 5.1 - 5.38) and Dr. Mzungu's Fish Farm was 5.15 ± 0.31 (range = 5.1 – 5.3). pH was positively associated with snail abundance from all the sites (r = 0.733, p<0.001).

4.4 Condition factor of *Oreochromis niloticus* sampled in three fish farms in Kibos area

4.4.1 *Comparison of Condition factor between parasitized fish and non- parasitized fish*

Larger sized fish (> 15 cm) depicted lower K values compared to smaller sized fish (≤ 10.0 cm) as shown on figs 8, 9 and 10. T-test revealed no significant difference in the condition factor values (K) for parasitized fish (M = 1.787 ± 0.04793) and non- parasitized fish [(M = 1.970 ± 0.08437); t (4) =1.886, p = 0.1324] in Lake Basin Fry Production centre. Similarly, results obtained from Auji fish farm and Dr. Mzungu's fish farm did not reveal any significant differences in the condition factor values between parasitized and non-parasitized fish [t (5) = 0.09920, p = 0.9248 ; t (7) =0.1428, p = 0.8905] respectively. Hence, there was no significant difference in the fish condition factor (K) between parasitized and non-parasitized fish in all the study sites (p = 0.253).

Standard length (cm)	1.0 – 5.0	5.1 – 10.0	10.1 – 15.0	15.1 – 20.0
Prevalence	55	62	74	43
Ki	1.78	1.89	1.81	1.66
Kni	2.01	2.18	1.81	1.87

K*i* – Fulton condition factor of infected fish; K*ni* - Fulton condition factor of un-infected fish

Figure 8 : Comparison of prevalence and K - factor of cultured *O. niloticus* in relation to fish class size in Lake Basin Fry Production Centre
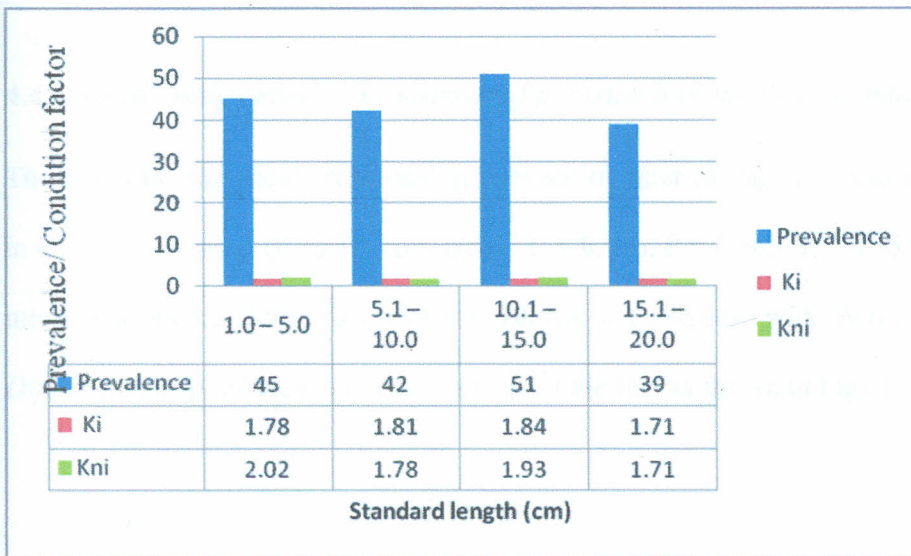
Standard length (cm)	1.0 – 5.0	5.1 – 10.0	10.1 – 15.0	15.1 – 20.0
Prevalence	45	42	51	39
Ki	1.78	1.81	1.84	1.71
Kni	2.02	1.78	1.93	1.71

K*i* – Fulton condition factor of infected fish; K*ni* - Fulton condition factor of un-infected fish

Figure 9. : Comparison of prevalence and K - factor of cultured *O. niloticus* in relation to fish class size in Auji fish farm

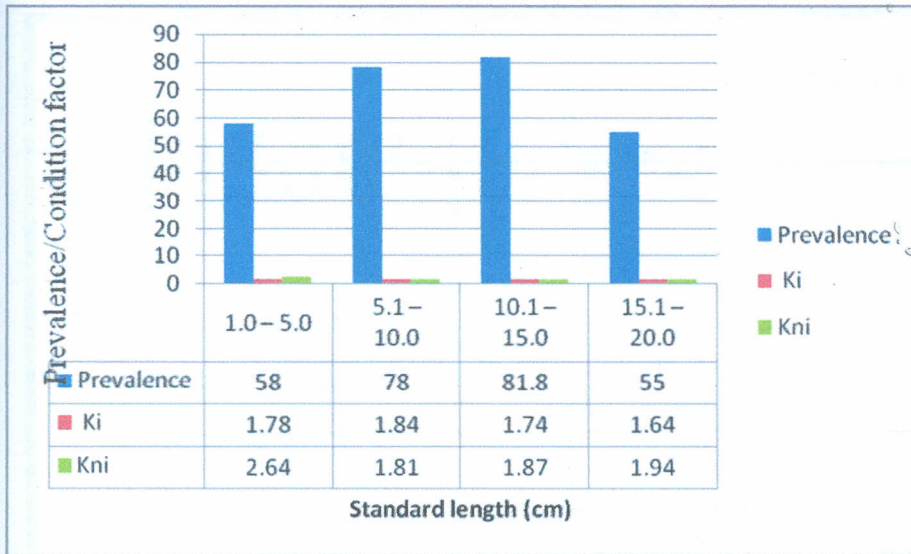| Standard length (cm)	1.0 – 5.0	5.1 – 10.0	10.1 – 15.0	15.1 – 20.0
Prevalence	58	78	81.8	55
Ki	1.78	1.84	1.74	1.64
Kni	2.64	1.81	1.87	1.94

Ki – Fulton condition factor of infected fish; Kni - Fulton condition factor of un-infected fish

Figure 10. : Comparison of prevalence and K - factor of cultured *O. niloticus* in relation to fish class size in Dr. Mzungu's fish farm

4.4.2 *Relationship between the number of parasites and the fish's condition factor*

There was no significant relationship between number of parasites and fish condition factor in all the study sites. (Pearson correlation; $P = 0.516$, $P = 0.565$, $P = 0.357$ respectively). This meant that the condition factor of the fish did not decrease with increase in the number of *Diplostomum* sp. metacercariae in the eyes of the fish as shown in Figs 11, 12 and 13.
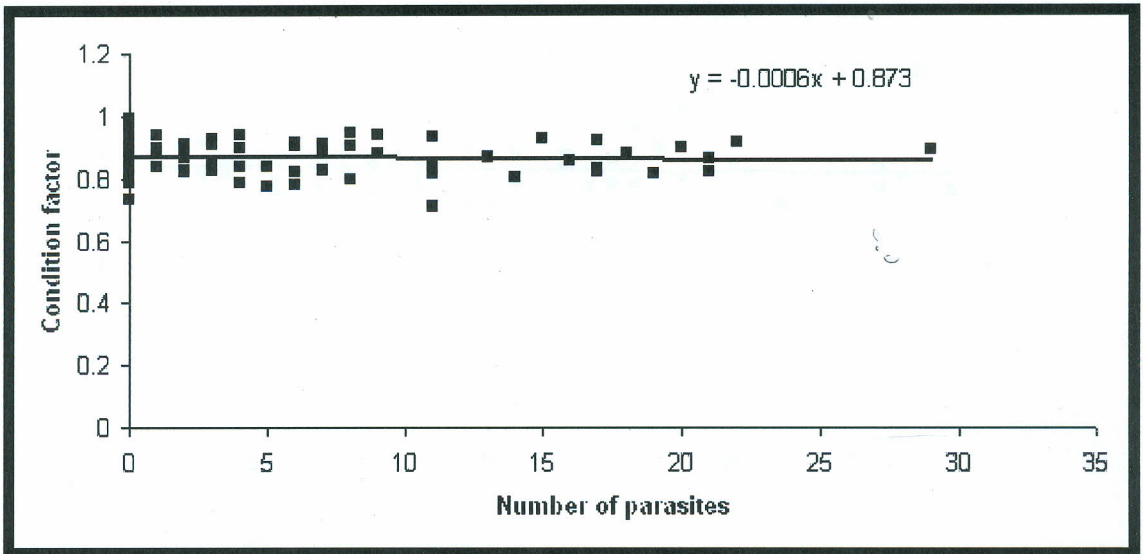
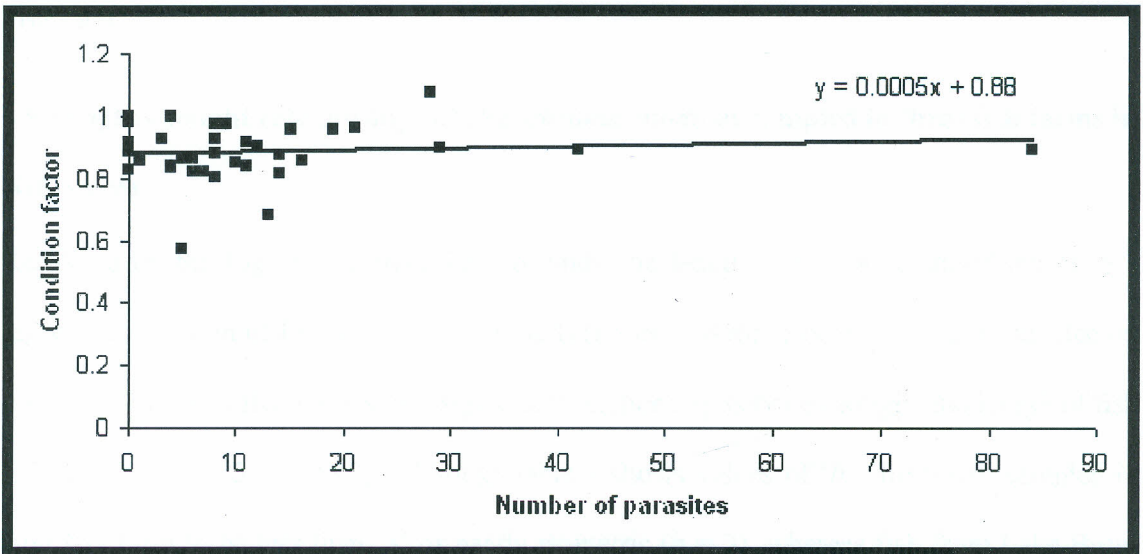**Figure 11**: Relationship between the number of parasites and the condition factor in LBFPC.

Figure 12: Relationship between the number of parasites and the condition factor in Auji fish farm.
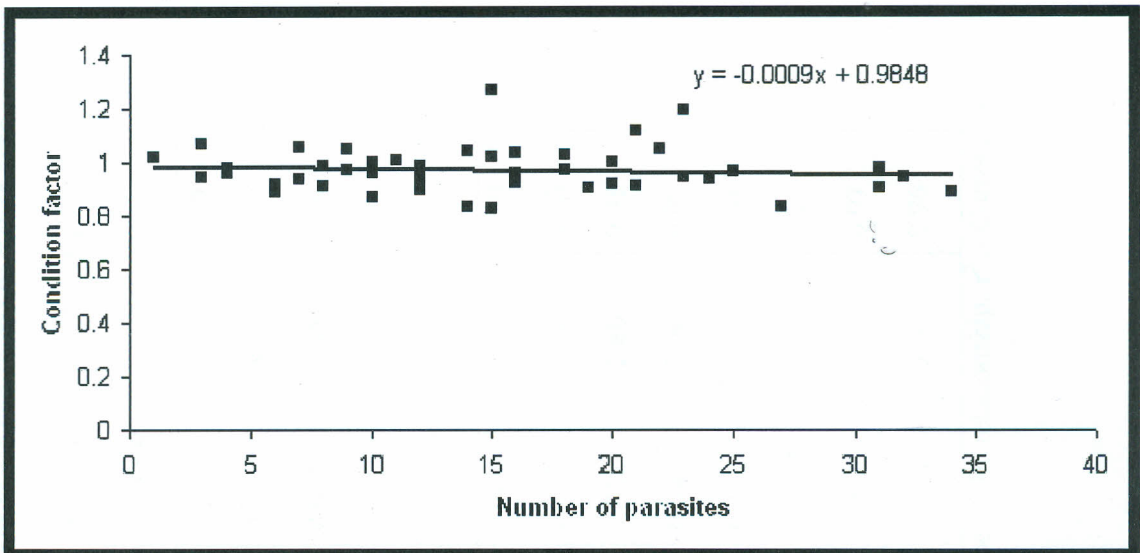
Figure 13: Relationship between the number of parasites and the condition factor in Dr. Mzungu's Fish Farm.

4.5 Length - weight relationships of *Oreochromis niloticus* sampled in three fish farms in Kibos area

Results from the log transformed data to study the length – weight relationships gave a regression equation of Log w = log a + b log L (Froese, 2006). Student's t -test conducted on **b** values confirmed that there was a significant relationship between weight and length of fish with p values <0.05 (Table 3). Although table 3 shows values of '*b*' from fish sampled in Auji fish farm to be less than '3' or nearly isometric (*b* = 3), whereas fish from Lake Basin Fry Production Centre and Dr. Mzungu's fish farm had values of '*b*' greater than '3', the median values of '*b*' from the data was 3.14. The high degree of positive correlation between standard length and total weight of grouped fish individuals was indicated by high values of correlation coefficient (r^2) ranging between 0.90 to 0.99. This implies that the exponential model applied provided reliable predictions of the weight when applied.

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Table 3: Length – weight relationship for parasitized and non-parasitized *Oreochromis niloticus* in three fish farms in Kibos area

Farm	Sample size	SL group (cm)	Mean Weight (g)		'a'		T – test	'b'	r^2
			Inf.	Not inf.	Inf.	Not inf.	*P* values		
LBFC	47	1.0 – 5.0	2.8	6.6	0.007	0.309	0.054*	3.0	0.98
	59	5.1 - 10.0	6.5	11.2	0.006	0.353	0.005*	3.18	0.99
	70	10.1 – 15.0	62.4	65	0.159	0.23	0.043*	3.08	0.99
	16	15.1 – 20.0	127.2	159.1	0.0059	0.782	0.0001*	3.44	0.96
Auji fish farm	20	1.0 – 5.0	4	7.6	0.006	0.577	0.965	2.66	0.90
	59	5.1 - 10.0	27.4	35.8	0.005	0.508	0.8026	2.92	0.96
	81	10.1 – 15.0	75.95	66.4	0.208	0.364	0.022*	2.86	0.91
	32	15.1 – 20.0	179.8	163	0.007	0.520	0.045*	2.96	0.95
Dr. Mzungu's fish farm	41	1.0 – 5.0	0.6	2.1	0.061	0.334	0.442*	3.0	0.91
	60	5.1 - 10.0	12.3	62.4	0.001	1.908	0.0781	3.14	0.99
	80	10.1 – 15.0	84.3	72.5	0.141	1.43	0.012*	3.17	0.97
	11	15.1 – 20.0	164.6	151.5	0.013	1.909	0.003*	3.0	0.96

Inf. – Parasitized fish; **Not inf.** – Un-parasitized fish; **'a'** – Intercept of the relationship; **'b'** – the slope of the relationship; r^2 – Correlation of coefficient; **SL** – Standard length (cm); * - Significant ($p \leq 0.05$)

CHAPTER FIVE

DISCUSSION

5.1 Prevalence, Abundance and Intensity of *Diplostomum* parasites from three Fish Farms in Kibos area

Results from the present study showed that prevalence of *Diplostomum* infection in three fish farms in Kibos area was 52.3%. Prevalence of the infection varied among the farms being higher in Dr. Mzungu's fish farm (66.1%) and moderately low in Lake Basin Fry Production Centre (LBFPC) and Auji fish farms (47.4% and 43.2% respectively) (Table 1). LBFPC which exhibited a prevalence of 47.4% served as the main source of fingerlings for the neighbouring fish farms in Kibos area. Infection of fish in LBFPC suggested that some of the infected fish in Auji Fish Farm and Dr. Mzungu's Fish Farm had at one time been kept in water taken from the hatchery centre and therefore the centre acted as the most likely source of the infection. *Diplostomum* parasites have a complex life cycle involving three hosts which promote continual transmission cycle of the parasites. According to Voutilainen *et al.* (2010), *Diplostomum* parasites have a long life span in fish hosts because they majorly migrate and settle in immuno-compromised sites of fish such as the eye and brain where they live for their entire life time. Microscopic examination in this study confirmed metacercariae of *Diplostomum* sp. actively moving in the vitreous humor and lens of sampled fish. Therefore transfer of fish stocks from LBFPC to other farms serve as one of the major sources of *Diplostomum* infection.

Earthen fish ponds contribute immensely to the spread of diseases in fish farms (Karvonen *et al.*, 2005). Open earthen ponds are common in Kibos area and are preferred by most fish farmers due to their affordability in construction and increased profitability as reported by (Jacobi, 2013).

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However open fish ponds accumulate nutrients, mud, clay and silt at the bottom of the pond which is stirred up from the pond bottom or comes into the pond with rainwater. This accumulation facilitates development of different biotic life such as insects and snails. Occurrence of *Diplostomum* infection in the farms was directly associated to the presence of a high abundance of *Biomphalaria* sp. snails shedding cercariae of *Diplostomum* parasites (Table 2). *Biomphalaria* snails live on water plants and mud that is rich in decaying organic matter (Karvonen *et al.*, 2005). *Biomphalaria* snails in this study were collected at the bottom of the ponds as well as near the shores of the ponds. These snails commonly occur in water that is moderately polluted with organic matter, such as faeces and urine (Karvonen *et al.*, 2005). Faecal matter along the shores of the ponds in this study was mainly contributed by birds that frequently visited the pond areas as shown in the plates 1-3. Presence of snails and birds in the environment showed that colonisation of parasites with a complex life cycle was strongly favoured. Similarly, a study conducted by Fioravanti *et al.* (2009) in Kenyan earth pond-based farms reported 1 – 4 metacercariae in farmed *Oreochromis niloticus* with a prevalence of 40.7%. Results from these findings concur with findings by Fioravanti *et al.* (2009) that suggested earthen ponds provided conducive environment that play a significant role in proliferation of parasites. In addition, the fact that these were open ponds, there is a high possibility that snails found their way into the ponds as a result of surface run offs from the vegetation cover found in close proximity to the farms. According to Bertman (1980), the highest prevalence of trematode infection in snail populations took place in small, shallow and overgrown still waters containing many aquatic organisms rather than in flowing waters like rivers and streams. As shown from the qualitative data in this study (Plates 1-3), vegetation such as papyrus, sugarcane plantations and grass dominated the nearby areas of the fish ponds. This is suggestive that the vegetation acted as

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reservoir for snails and other aquatic organisms. As suggested by Karvonen *et al.* (2005), plants serve as substrates for feeding and oviposition of snails as well as providing protection from high water velocities and predators such as fish and birds. The vegetation also seemed to offer a conducive environment for eggs emerging from motile vertebrate hosts (i.e birds) to hatch and meet with miracidium emerging from the snails. Therefore, findings from this study concur with the suggestion by Hechinger and Lafferty (2005) that snails serve as important intermediate hosts in the completion of *Diplostomum* life cycle.

Physico-chemical parameters of water served a significant role in snail population densities and transmission rates. According to Levitz *et al.* (2012), temperature and pH play a significant role in snail and parasite development. This study demonstrated that these physicochemical parameters of water appeared to be the key determinant of increased trematode prevalence among the snails. The pH of water in all the farms (range 5.1 – 6.6) was lower than the recommended range of pH for cultured fish (range 6.8 – 8.7), while the association with snail abundance was ($r = 0.733$, $p < 0.001$) positive. Although tilapia can survive at pH ranging from 5 to 10, they do best in a pH range of 6 to 9. Reduced pH in the water was likely to be related to sugarcane plantation found within the vicinity of the farms. Most of the research done in sugarcane plantations, have pointed reduction of soil pH to the use of nitrogenous fertilizers which reduce soil pH during ammonification and nitrification processes (Oliver, 2004). Equally, water temperature appeared to be a key determinant of snail abundance. The positive association between snail abundance and water temperature observed in our study ($r = 0.3$, $p = 0.01$) suggested optimal reproduction in the snail. This is in agreement with observations that

demonstrated *Biomphalaria pfeifferi* grew and survived better at 25°C than at 19°C (Sturrock, 1966).

The present study was conducted between December and February, these months were dry and hot with recorded temperature range of the pond waters being 26^0C-32^0C. Although there was a high prevalence of *Diplostomum* infection in fish, prevalence of trematodes in the snails seemed low with the highest prevalence (10.42%) observed in snails collected from LBFPC. The overall high temperatures recorded (26 - 32^0C) might have contributed to temperature stress and ultraviolet radiation that affected trematode survival and ability of miracidia to infect snails. This is typical of infection by digenean larvae (Esch and Fernandez, 1993) as high prevalence of trematode parasitism in snail population render a remarkable proportion of snails infertile due to the host-castrating effect of trematodes. As a result, the host density is reduced (Puurtinen *et al.* 2004). Snail prevalence of less than 10% of *Diplostomum* infections have similarly been reported in lymnaeid snails in both Finland (Va¨yrynen *et al.*, 2000; Faltynkova *et al.*, 2007) and other European countries (Faltynkova, 2005; Faltynkova and Haas, 2006). This study therefore indicated that very low proportion of snails tend to shed cercariae that translate to high levels of diplostomiasis infection in the farms. This is because, infection prevalence between 1% and 10% in snails is considered sufficient to generate 100% infection rates in fish (Stables and Chappell, 1986). In contrast to findings from this study, Nkwengulila and Kigadye (2005) reported higher infection prevalence levels of digenean trematodes in snails during the dry season compared to other seasons. Other workers (Vareyrynen *et al.*, 2000; Karvonen *et al.*, 2006) have reported seasonal variation of prevalence of digeneans in snails attributing high prevalence during dry seasons to be contributed by reduced water volume in conjunction with increased density of snail

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hosts and intensified use of the habitat by definitive hosts. This study was conducted only during the dry season with no comparisons to the other seasons.

5.1.1 Relationship of fish size with *Diplostomum* infection levels

The findings on correlation between fish size and parasitic intensities in this study differ from observations by Aloo *et al.* (2004), who suggested that large sized fish harbour more parasites, this may be related to the different environments taken into consideration: they examined wild marine fish, while we examined fish from controlled (ponds) freshwater environment, where the influence of several abiotic and biotic factors could change the dynamic of parasitic infections. Gradual decline in the infection levels among large sized fish (≥15.0cm) discovered in this study can be related to the feeding and resting behavioural differences between juveniles and adults in culture ponds. González *et al.* (2009) reported that in early life stages, *O. niloticus* take refuge among aquatic vegetation along the pond edges. Here, they tend to ingest mainly aquatic insects, but are also in constant proximity to snails which greatly increase their probability of becoming infected. In contrast, larger fish spend most of their time consuming the dry feed provided daily in the deeper areas of the ponds, which lack aquatic vegetation and therefore provide no habitat for snails. In addition, decline in infection parameters in older fish (≥ 15cm) might be due to low establishment rate of the parasites caused by gradual development of immunity which result in low recruitment rate (Voutilainen *et al.*, 2010). During the present study, no dead or moribund metacercariae were recorded. These results are consistent with works reported by Kennedy and Burrough (1977), Machado *et al.* (2005), González *et al.* (2009) and Turgut and Ozgul (2012).

5.2.0 Genetic relatedness of *Diplostomum* species in fish population from Kibos area and other reported *Diplostomum* species using ribosomal markers

The principal findings of this study indicated that multiple species infections of *Diplostomum* were common in the fish community. These findings were revealed using ITS and 18S ribosomal DNA. Considering the two molecular markers, ITS rDNA phylogeny generated robust and plausible topology compared to 18S rDNA. This may be explained by the high degree of divergence exhibited by ITS as a result of the high variability on the 5' end as a consequence of tandemly repeated elements located at this site, causing a greater number of possible alignments compared to SSU sequences (Hillis and Dixon, 1991). This is in agreement with the findings of Littlewood *et al.* (2008), who demonstrated that SSU datasets do not have enough resolving power to produce robust trees.

Five species of lens-infecting *Diplostomum* were found to be closely related to the specimens analysed in this study based on the distance matrix method that considered the phenotypic similarities of the species. The use of outgroup taxa (*Tylodelphys sp.*) that was closely related to *Diplostomum* and the monophyly of the cryptic species revealed herein, suggested that each species complex originated from a common ancestor. According to this study, ITS sequence data of specimen D32 was closely related to *Diplostomum mashonense* (Beverley-Burton, 1963). Similarity of specimen D32 to *D. mashonense* was associated with the resultant tree that presented a bootstrap consensus value of 100% for the branch (Fig. 4). This is in agreement with the observation by Chibwana and Nkwengulila (2010) who pointed out striking similarity between *D. mashonense* (Beverley-Burton, 1963) and *Tylodelphys* spp. 1 and 2, and later discriminated *D. mashonense* (FJ 470402) from *Tylodephys* spp. using morphometric variability

analysis. Similarity between the two *Diplostomum* species (*D. mashonense* & D32) suggest a strong association between *Diplostomum* sp. studied in Tanzania and *Diplostomum* in Kisumu, Kenya. Thus, similarity in parasite distribution in the different fish host species might be as a consequence of co-evolutionary interactions associated with geographical divergence of the species.

Phylogenetic analysis of ITS rDNA sequence data from adult forms of *Diplostomum* by Gallazo *et al.* (2002) lends support to 1 sequence from this study which demonstrated highly similar consensus sequences to *D. baeri* (JQ 665460) classified as American species (Fig. 4). Specimens closely related to *D. baeri* included D42, D52, D35, D57, D44, D26, and D46 which were equally assessed by ITS rDNA and strongly supported by a high bootstrap value (> 99%). 18S rDNA sequence data was closely related to *D. compactum*, *D. spathaceum* and *D. phoxini* classified as American or European species.

Specimen M7 and M10 were closely related to *D. compactum* and *D. spathaceum*, whereas specimens M5 – M20 were closely related to *D. phoxini*. Similarity of the specimens to reported *Diplostomum sp.* was associated with the resultant tree that presented bootstrap consensus values of >50% for almost all branches (Fig. 4 and 5). The present study therefore provides a preliminary confirmation of diplostomoid species residing in both continents with a possibility of recent divergence or hybridization.

Spatial distribution of *Diplostomum* sp. at the three fish farms in Kibos area demonstrated five *Diplostomum* communities among the fish population. This is an indication of increased gene flow among the parasites making it impossible for only one parasite to adapt to the environment.

Although our study was not designed to investigate how definitive hosts influence gene flow in parasites, we give speculations based on the biological basis of the parasite life cycle. Host mobility has been proposed to be the main determinant of gene flow in parasites since they are commonly dependent on their host for dispersal (Blouin *et al.*, 1995). Prugnolle *et al.* (2005), suggested that parasites with complex life cycles such as *Diplostomum* include multiple host species within their lifecycle and therefore gene flow is expected to be determined by the host with the highest dispersal rate. In this study, it was likely that high mobility in the bird species commonly seen around the farms (the great Egret, Cormorants and Pied-billed Grebe) was sufficient to cause high levels of gene flow among the spatially isolated *Diplostomum* parasites in the different farms when taking into account that each definitive host can harbour dozens or hundreds of adult parasites.

5. 3 Survey of *Diplostomum* trematodes in pond snails using 18S rDNA molecular marker

Genetic characterization of cercariae in snails to identify parasite species is currently the focus of many studies because accurate genotypic analysis has important implications in science for studying population biology, evolution, epidemiology, and genetic structure of these parasites thereby enhancing the efficient control of the diseases they cause.

The presence of *Diplostomum* cercariae in *Biomphalaria* snails from the study ponds was demonstrated by microscopy and confirmed by PCR. Genotypic characterization of cercariae was performed based on the amplification of 18S rDNA. The parasite DNA fragments of expected sizes (bands with corresponding sizes of about 400 and 600 bp) were obtained (Fig.6.0 and Fig. 7.0 respectively). This indicated presence of *Diplostomum* trematodes in *Biomphalaria* snails and that 18S rDNA genes employed was reliable in identification of unknown trematodes to genus *Diplostomum*.

5.4 Effect of *Diplostomum* parasites on Length - Weight relationship of farmed *Oreochromis niloticus* L.

5.4.1 Fulton's condition factor (K)

According to reports by Chappell (1995), infection with metacercariae such as *Diplostomum compactum* and *Diplostomum spathacaeum* in the eyes of fish, can cause severe damage in hosts including massive fish kills. Disruption of vision by metacercariae may also reduce feeding efficiency, as was observed in dace (*Leuciscus leuciscus*) and threespine sticklebacks (*Gasterosteus aculeatus*) (Crowden and Broom, 1980; Owen *et al.*, 1993). However, using the data reported herein, no effect of *Diplostomum* sp. on growth or condition, as measured by condition factor was detected although the number of *Diplostomum* sp. per individual exceeded 12. This can be attributed to the fact that there were few numbers of metacercariae in the lens to affect the fish condition factor. Marcogliese *et al.* (2001) and Kembenya *et al.* (2012) similarly observed no significant effect of *Diplostomum* parasitism on the condition of fish studied despite the high number of parasite intensity observed ranging between 1 – 248 parasites per host.

Using the data reported herein, most of the isolated metacercariae were found actively moving in the vitreous humor while few were located in the lens. Seppa¨la¨ *et al.* (2006) showed that high numbers of metacercariae in the lens impair vision and anti-predator behaviour in fish. Therefore, lack of significant effect of the parasites on condition factor (K) could be attributed to the fact that there were few numbers of metacercariae in the lens to impair vision. Secondly, it is possible that infected fish in pond reared culture system received other cues from conspecifics in

a group (such as active movement towards the surface when food was being introduced) which initiated and facilitated their feeding activity.

Variation in condition factor by size (standard length) showed that larger sized fish (>15 cm) had lower K values compared to smaller sized fish (≤10cm). This could be attributed to the resources transferred to the gonads in the latter stages of the life history of a fish for purposes of reproduction or construction of gonads. These results coincide with Saliu (2001), Lizama and Ambrosia (2002) and Anene (2005) who observed relatively lower values of condition factors for large sizes of fish, while relatively higher values of condition factors for rather small sized fish in *Brycinus nurse*, characid and tilapia fish species respectively. Reported K values for *O. niloticus* in this study (range 1.7 – 2.1) could not be compared to values of 2.25, obtained for *Brycinus nurse* fish species (Saliu, 2001), 2.67 and 2.3 in Parana river and man-made lakes (Lizama and Ambrosia, 2002 ; Anene, 2005). Lower K values of fish in this study could be explained by the fact that pond fish culture favour persistence of a wide variety of parasitic diseases since high stocking densities predispose fish to stress which lowers their immune response (Costas *et al.*, 2008).

5.4.2 Length - weight relationships

The transformed length fitted over weight in the current study, gave linear growth indicating the three dimensional growth structures of the assayed fish species. The median value of coefficient '*b*' for the overall samples was 3.14 ±0.05 pointing out an overall positive allometric growth. This is a clear indication that the weight of these fish increased faster than the cube of their total lengths. However, fish from Auji fish farm demonstrated a negative allometric growth (b<3,

p<0.05) while fish from subsequent fish farms reflected positive allometric growth (b>3, p<0.05). Several factors can be attributed to this, such as abundant food supply and other favourable conditions as suggested by Veeramani *et al.* (2010). Even though the change of *b* values depend primarily on the shape and fatness of the species, various factors may be responsible for the differences in parameters of the length/weight relationships. According to Taskavak and Bilecenoglu (2001) and Özaydin *et al.* (2007), the parameter '*b*', unlike the parameter '*a*', may vary seasonally, and even daily, and between habitats. Thus, the length/ weight relationship in fish is affected by a number of factors including gonad maturity, sex, diet, stomach fullness and health as well as season and habitat. Considering the present study, only one aspect of fish health was taken into consideration. Therefore, information gained in the present survey can only be used by fish biologists to derive weight estimates for tilapia fishes that are measured but not weighed. These results are analogous with the suggestion of Carlander (1969) that the exponent *b* should normally fall between 2.5 and 3.5.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1. Prevalence of *Diplostomum* parasites infecting fish in three fish farms in Kibos area was 52.3% with mean parasitic indices of 12.4 parasites per fish representing mean intensity and 6.5 parasites per Fish Farm representing mean abundance. *Diplostomum* abundance in all the farms was closely related to the abundance of host snails.

2. Five species of *Diplostomum* were genetically related to sample specimens in this study as shown: D 32 - *D. mashonense* (FJ 470403); D 42, D 51, D 35, D 57, D 44, D 26, D 46 - *D. baeri* (JQ 665460); M5, M8,M9, M15, M18, M19 - (*D. phoxini*); M13 - (*D. compactum*); M7, M10 - (*D. spathaceum*). These findings provide a preliminary confirmation of diplostomoid species residing in Africa, America and European continents with a possibility of recent divergence or hybridization.

3. *Biomphalaria* sp. snails collected around fish ponds were confirmed to harbour *Diplostomum* trematodes responsible for diplostomiasis infection. Presence of these snails around the ponds was positively associated with pH and temperature of the water.

4. High degree of positive correlation (r^2) ranging between 0.90 to 0.99 between the length and weight measurements of fish implied that the exponential model applied provided reliable predictions of the weight when only the length of fish was known.

5. Internal transcribed spacer (ITS1-5.8S-ITS2) of the rRNA gene and 18S rDNA successfully differentiated species of *Diplostomum* genera suggesting that ribosomal markers are effective genetic markers for inter-species phylogenetic analysis.

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Recommendations

6.2.1 Recommendation according to the present work

1. Prevalence of *Diplostomum* parasites at the rate 47.4% in Kibos Fry Production Centre leads to the conclusion that the source of fingerlings is an important determinant of the distribution and occurrence of parasites. Therefore, it is recommended that thorough screening and sanitary control of fingerlings from hatcheries is necessary before restocking activities in any farm.

2. Internal transcribed spacer (ITS) and 18S rDNA successfully provided phylogenetic relationship of *Diplostomum* genera and therefore should be employed for species detection and identification.

3. The high abundance of *Biomphalaria* sp. and confirmation of cercarial shedding in snails around fish farms in Kibos area calls for mollusciding or manual removal of snails around the ponds. Secondly, local sanitation around pond areas should be improved by clearing vegetation such as papyrus reeds and grass.

4. Metacercarial counts were low and did not cause any abnormalities in the length – weight measures of fish, however, increased numbers of the parasites in fish eyes cause blindness and indirectly hinders growth. Therefore control measures should be prioritized in fish farms.

6.2.2 Recommendation for future studies

1. In order to understand *Diplostomum* spatial distribution in Kibos area, seasonal and annual data will be necessary for accurate determination of prevalence and other parasitic indices in fish farms. This is because, this study was limited to the dry season and therefore comparison of data during different seasons will be of benefit to future studies.

2. This study did not give formal description names to the sample specimens due to lack of sequence links to the adult stages and cercarial stages; this stresses the need for further taxonomic research on this group.

3. Highly specific and sensitive methods such as RFLP should be adopted in future for detection of diplostomidae species in snails. This detection technique is based on simple PCR, and no sequencing is necessary making it economically advantageous compared to other techniques that require sequencing to be performed.

4. The current study did not consider diet, gonadal development and stomach fullness of the sampled fish. These factors may influence the length-weight relationships of fish and therefore, seasonal or annual data are necessary to verify this hypothesis.

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