Study of *Diplostomum* (Digenea: Diplostomoidea) in South Africa: Diversity and effect of metacercariae on fish behaviour

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Abstract

A large and widely distributed group of parasites within the genus *Diplostomum* (Digenea: Diplostomoidea) utilises a complex life cycle with life stages that parasitise freshwater snails, fish (intermediate hosts) and piscivorous birds (definitive hosts). Metacercariae of *Diplostomum* infecting the eyes (lens, vitreous humour, and retina) and brains of fish, have a well-known reputation for their pathogenicity in aquaculture fish farms. In taxonomy, the genus *Diplostomum* have been a controversial topic for many years because identification of most of the nominal species currently known have been based solely on morphological characteristics of the life stages. To date, almost 80 nominal species of *Diplostomum* have been reported worldwide; with the majority of the species recorded from the Palearctic region. However, most of the morphology-based identifications of species within this genus require critical revision due to difficulties in identifying larval stages based on their simple morphology and disagreements among parasitologists of the validity of some of the reported species. The application of molecular methods based on multiple genetic markers has increased available knowledge on the species diversity within *Diplostomum* in the last decade, making accurate identification of cryptic species possible (by primary use of mitochondrial markers). So far, based on the development of the molecular approach, eight species and 38 unidentified species-level genetic lineages have been reported globally. In Africa, only eight species of *Diplostomum* were described based on morphology and only one species from Nigeria has been identified based on molecular evidence. One of the major challenges in Africa is the lack of baseline data for the diversity and distribution of *Diplostomum* parasitising freshwater fishes and is mainly due to a lack of knowledge, expertise, sampling effort and funding in the field of parasitology. Numerous experimental studies exploring the effect of metacercariae on fish behaviour, predominantly done in Europe, found that metacercariae of *Diplostomum* have an effect on the escape response, feeding- and swimming behaviour as well as habitat selection of their intermediate hosts; thus facilitating transmission to the definitive hosts. In contrast, no published data on the influence of metacercariae of *Diplostomum* on fish behaviour in Africa exists. Thus, the aims of the present study were: (i) to determine the diversity of *Diplostomum* in South African fishes by applying molecular and traditional morphological methods, and (ii) to determine the effect of *Diplostomum* infections on fish behaviour using the Plain squeaker *Synodontis zambezensis* Peters, 1852 as model species. To achieve this aim, a total of 160 fishes belonging to 17 species were collected and the eyes and brains were examined for the presence of *Diplostomum* and analysed along with specimens from the Water Research Group (WRG) collection that were collected during previous sampling expeditions in the Phongolo (2016, 2017, 2018), Riet (2017), Usuthu (2017) and Mooi Rivers (2019). Metacercariae were recovered from the eye lenses of 38 fishes belonging to five species

of the families Anguillidae, Cichilidae and Mochokidae, with an overall low prevalence of infection (18%). Representative metacercariae were subjected to morphological analysis and molecular sequencing including partial mitochondrial *cox*1 and ribosomal 28S rDNA genes as well as the ribosomal ITS1-5.8S-ITS2 region. The presence of three species of *Diplostomum* was discovered. The three species matched those previously reported from Nigeria, Iraq and China, therefore those from *Tilapia sparrmanii* Smith, 1840 and *S. zambezensis* were identified as *Diplostomum* sp.; those from *Anguilla labiata* (Peters, 1852), *Oreochromis mossambicus* (Peters, 1852) and *S. zambezensis* were named *Diplostomum* sp. 14; and those from *Pseudocrenilabrus philander* (Weber, 1897) were named *Diplostomum* sp. 16. Ten *S. zambezensis* previously collected from the Ndumo Game Reserve (NGR) (2017) and 22 *S. zambezensis* (NGR, 2018) were used in the laboratory and field-based quantitative behavioural experiments. Analyses of video recordings and statistical data applying unpaired Welsch's t-tests and One-Way ANOVA revealed a significant difference in behaviour between infected and uninfected fish during acclimation and attacks based on the time spent in top and bottom zones, frequency of zone alternations, minimum and maximum acceleration and mobility state (immobile to highly mobile). During attack trials only, which was not found during the acclimation period, a significant difference was found in distance moved and swimming speed between infected and uninfected fish. This study is the first dedicated assessment of *Diplostomum* applying both molecular and morphological approaches in freshwater fishes in South Africa. The first morphological and molecular evidence provided for *Diplostomum* sp., *Diplostomum* sp. 14 and *Diplostomum* sp. 16 as well as statistical evidence of significant effects of metacercariae of *Diplostomum* on the behaviour of *S. zambezensis*, contributes to the elucidation of the life cycle of *Diplostomum*, expands our knowledge on the geographical distribution of species within this genus and provides baseline data for future behavioural studies of fish infected with diplostomids in Africa.

Keywords: Trematoda, Metacercariae, Freshwater fish, Morphology, DNA, Noldus EthoVision, Swimming behaviour

List of Abbreviations

A

AIC	Akaike Information Criterion
ANOVA	Analysis of variance

B

BDNR	Boskop Dam Nature Reserve
BI	Bayesian Inference
BLAST	Basic Local Alignment Search Tool

C

*cox*1	cytochrome *c* oxidase subunit I

I

ITS	Internal Transcribed Spacer region

K

KZN	KwaZulu-Natal Province

M

MCMC	Markov Chain Monte Carlo
ML	Maximum Likelihood
MNP	Mokala National Park

N

NABF	National Aquatic Bioassay Facility
*nad*1–6	NADH- nicotinamide adenine dinucleotide dehydrogenase subunit 1–6
NGR	Ndumo Game Reserve
NMB	National Museum, Bloemfontein
nt	Nucleotides
NWU	North-West University

P

PCR	Polymerase Chain Reaction
P	Prevalence

S

SEM	Standard Error of the Mean

W

WRG	Water Research Group

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction

The class **Trematoda** Rudolphi, 1808 is a large, entirely parasitic group of flatworms within the phylum **Platyhelminthes** that comprises two subclasses, the **Aspidogastrea** Faust & Tang, 1936 and the **Digenea** Carus, 1863. The subclass Digenea consists out of two orders, the **Diplostomida** Olson, Cribb, Tkach, Bray & Littlewood, 2003 and **Plagiorchiida** La Rue, 1957, 25 superfamilies, 148 families and almost 20 000 nominal species and is the largest group of parasitic Platyhelminthes (Bray *et al*., 2008). Digeneans are almost exclusively endoparasites that can be found in all classes of vertebrates. Their life cycle, in contrast to the aspidogastreans, is usually complex and includes both free-living and parasitic stages. A typical digenean life cycle involves three hosts: a mollusc first intermediate host, invertebrate or vertebrate second intermediate host and vertebrate definitive host.

The effect that digenean trematodes have on their hosts, both intermediate and definitive is always negative and often has significant consequences on host biology (Kudlai *et al*., 2017). Out of numerous families within the Digenea, several contain representatives that are of high economic or medical significance. The family Diplostomidae Poirier, 1886 is one of them. Several large genera within the family comprise species that are considered as important pathogens of their intermediate hosts (Shigin, 1986; Fried & Abruzzi, 2010; Otranto & Eberhard, 2011; Blasco-Costa & Locke, 2017). According to the most recent revision of the Diplostomidae by Niewiadomska (2002), the family comprises four subfamilies, Diplostominae Poirier, 1886 (14 genera), Crassiphialinae Sudarikov, 1960 (15 genera), Alariinae Hall & Wigdor, 1918 (11 genera) and Codonocephalinae Sudarikov, 1959 (1 genus) (Niewiadomska, 2002). Most diplostomid trematodes parasitises three hosts in their life cycle which includes freshwater snails, fish (occasionally amphibians) and piscivorous birds, mammals, or reptiles (Kennedy & Burrough, 1977; Niewiadomska, 2002). The metacercariae of the Diplostomidae – larval stages that parasitise the second intermediate host – can be found in a variety of organs, either encysted on the body surface, muscles, mesenteries and skin or unencysted in the tissue of the eye lenses, vitreous humours, retina, central nervous system and brain (Gibson, 1996; Migiro *et al*., 2012, Otachi *et al*., 2015; Stoyanov *et al*., 2017). High densities of metacercariae at sites of infection may cause haemorrhaging in the muscles and capillaries, obstructed blood vessels, cranial distortion and formation of eye cataracts that ultimately results in reduced host survival or mortality in the cases of juvenile fish (Szidat & Nani, 1951; Shigin, 1986; Chappell, 1995; Georgieva *et al*., 2013; Rosser *et al*., 2016). Of the 41 genera from the Diplostomidae currently known worldwide, seven genera have been recorded in Africa (Khalil & Polling, 1997; Kudlai *et al*., 2018; Hoogendoorn *et al*., 2019). A total of 21 diplostomid species have been reported from freshwater fishes in Africa (Williams, 1967; Prudhoe & Hussey, 1977; Mashego & Saayman, 1989; Khalil &

Polling, 1997; Barson & Avenant-Oldewage, 2006; Zhokhov *et al*., 2010; Chibwana & Nkwengulila, 2010; Chibwana *et al*., 2013; Moema *et al*., 2013; Jansen Van Rensburg *et al*., 2013; Zhokhov, 2014; Otachi *et al*., 2015; Hoogendoorn *et al*., 2019). Of these, molecular data have only provided for six species: *Diplostomum* sp. (Chibwana *et al*., 2013), *Tylodelphys mashonensis* (Chibwana *et al*., 2013; Moema *et al*., 2013), *Tylodelphys* sp. 1 and *Tylodelphys* sp. 2 (Chibwana & Nkwengulila, 2010; Chibwana *et al*., 2013), *Tylodelphys* sp. 2 (Otachi *et al*., 2015), and *Tylodelphys* sp. (Moema *et al*., 2013). Moreover, only seven species, *Diplostomum* sp. type I and *Diplostomum* sp. type II (Prudhoe & Hussey, 1977), *Diplostomum* type 3 (Madanire-Moyo *et al*., 2010), *Neodiplostomum* sp. (Prudhoe & Hussey, 1977; Van As & Basson, 1984), *Ornithodiplostomum* sp. (Barson & Avenant-Oldewage, 2006), *Tylodelphys mashonensis* Beverley-Burton, 1963 (Mashego & Saayman, 1989; Moema *et al*., 2013) and *Tylodelphys* sp. (Moema *et al*., 2013) and few metacercariae assigned to different diplostomid morphotype groups have been described or reported in freshwater fishes in South Africa (Prudhoe & Hussey, 1977; Van As & Basson, 1984; Khalil & Polling, 1997; Barson & Avenant-Oldewage, 2006; Grobbelaar *et al*., 2014, 2015). Recently, four additional species namely, *Bolbophorus* sp. 3, *Posthodiplostomum* sp. 9, *Uvulifer* sp. 4, Diplostomidae gen. sp. were reported from *Tilapia sparrmanii* Smith, 1840 by Hoogendoorn *et al*. (2019) (see Appendix A). Despite extensive studies focusing on the type-genus *Diplostomum* von Nordmann, 1832 in the past; this genus remains in a controversial state due to a lack of dedicated studies and sufficient morphological or molecular evidence and expertise; especially in South Africa (Prudhoe & Hussey, 1977; Mashego & Saayman, 1989; Khalil & Polling, 1997; Kudlai *et al*., 2018).

The genus *Diplostomum* comprises of the most species-rich group of parasites within the Diplostomidae reported from all continents, with the majority of the species described from the Palearctic region (Shigin, 1986, 1993; Niewiadomska, 2010; Blasco-Costa & Locke, 2017). Metacercariae of *Diplostomum* located in the eye lenses or eye vitreous humour (seldom in brains) of their fish hosts, have a well-known reputation as pathogens and cause mortalities in cases of high infections in both wild and farmed fish populations (Shigin, 1986; Georgieva *et al*., 2013; Blasco-Costa *et al*., 2014). In recent years, metacercariae of *Diplostomum* became the focus of numerous ecological, behavioural and evolutionary studies (predominantly in Europe and North America), due to their ecological and economic importance in fish farming and the development of molecular tools that made accurate and reliable species identification of *Diplostomum* possible (Shariff *et al*., 1980; Shigin, 1986; Chappell, 1995, Seppälä *et al*., 2004, 2005a, 2005b, 2008, 2011; Voutilainen *et al*., 2008; Georgieva *et al*., 2013; Blasco-Costa *et al*., 2014; Faltýnková *et al*., 2014; Selbach *et al*., 2015; Klemme *et al*., 2016; Flink *et al*., 2017). These larval stages served as models in many research studies focussing on the evolutionary relationships that include: host-parasite co-evolution, adaptations, mechanisms of migration,

competition and parasite community assemblages (Ballabeni & Ward, 1993; Kalbe & Kurtz, 2006; Barber, 2013; Klemme *et al*., 2016; Blasco-Costa & Locke, 2017).

It is clear from the above mentioned studies, that a research gap in the knowledge of *Diplostomum* not only in South Africa, but in Africa exists as reliable data is lacking for the known species reported from these areas. To date, the identification and delineation of species remain the two key limitations in understanding the true diversity of *Diplostomum*. However, due to the recent development of molecular tools applying multi-locus genetic markers (28S, ITS1-5.8S-ITS2 and *cox*1), reliable and accurate species delineation is possible. This provides researchers to study the bigger picture such host-parasite interactions and adaptations, elucidation of life cycles and ultimately linking the transmission of parasites with geographical distributions.

Historical notes on the genus *Diplostomum*

During parasitological examinations of freshwater fish collected in the vicinity of Berlin and Hamburg in 1832, von Nordmann found numerous trematodes in the eye lenses and vitreous humours. The nature or taxonomic placement of these parasites were unknown at the time as they have never been reported before. This led to the establishment of the genus *Diplostomum* (see Shigin, 1986 for details). *Diplostomum volvens* von Nordmann, 1832 and *Diplostomum clavata* von Nordmann, 1832 (= *Tylodelphys clavata* (von Nordmann, 1832) Diesing, 1850) were the first two species described in this genus. *Diplostomum volvens* was designated as the type-species (later synonymised with *Diplostomum spathaceum* (Rudolphi, 1819) Olsson, 1876) of the genus, whereas *D*. *clavata*, following several revisions, was transferred to the genus *Tylodelphys* Diesing, 1850. In von Nordmann's (1832) first descriptions of the species, he noted that trematodes found in cyprinids were predominantly located in the eye lenses and trematodes in percids and burbot *Lota lota* (Linnaeus, 1758) (Lotidae) were generally located in the eye vitreous humour. The researcher further noticed that metacercariae found in various fish hosts slightly differed morphologically, but these differences did not contribute to the taxonomic classification and all trematodes of this type were considered a single species, *D*. *volvens*. The nature of the metacercariae found by von Nordmann was unknown and the specimens were identified as sexually mature individuals, which led to numerous erroneous interpretations of the morphological features (see Shigin, 1986 for details).

More than 60 years later, the characteristics of the metacercariae described by von Nordmann were resolved by A. Ehrhardt and O. Ehrhardt (Braun, 1894). In the experiment conducted by the researchers, gulls were infected by feeding them lenses of the roach *Rutilus rutilus* (Linnaeus, 1758) (Cyprinidae) that contained metacercariae. Following the feeding experiment, the adult trematodes recovered from the gull showed morphological similarity with the species of *Hemistomum spathaceum* (= *D*. *spathaceum*) described by Rudolphi in 1819 from

gulls in Europe. Following this discovery, *D. volvens* was synonymised with *D. spathaceum* (see Shigin, 1986 for details).

From the 19th century until mid-20th century, five additional species of *Diplostomum* were described, namely: *Diplostomum lenticola* von Linstow, 1878 (= *Tetracotyle lenticola* (von Linstow, 1878) Faust, 1918), *Diplostomum petromyzifluviatilis* Diesing, 1850, and *Diplostomum phoxini* (Faust, 1918) Arvy & Buttner, 1954 in the Palaearctic; *Diplostomum huronense* (La Rue, 1927) Hughes, 1929 and *Diplostomum indistinctum* (Guberlet, 1923) in the Nearctic; and *Diplostomum murrayense* (Johnston & Cleland, 1938) Johnston & Simpson, 1939 in Australia (see Shigin, 1986 and references therein).

Data on the biology of *Diplostomum* available by the middle of the 20th century, supported conclusions that the metacercarial stages develop in the intermediate freshwater fish hosts. Interestingly, according to Shigin (1986), by the mid-20th century there were 30 species of *Diplostomum* described based on adults, but only eight species were reported from fishes. This discrepancy was explained by the assumption that the species of this genus may have different life cycle strategies involving other hosts (which seemed unlikely) or that some of the species were represented by species complexes.

Rigorous studies focusing on the biology of trematodes from the genus *Diplostomum* and systematics of their metacercariae initiated by Shigin and colleagues revealed that metacercariae previously identified by many researchers as *D. spathaceum*, in fact represented several valid species (Shigin, 1965a, b, 1968a, b, 1969; Razmashkin, 1969 etc.). Shigin and colleagues developed new methodology for studying metacercariae and expanded morphological criteria for their identification (Sudarikov & Shigin, 1965) that made it possible to differentiate between species based on the morphology of the metacercarial stages. The newly obtained data allowed the revision of our knowledge on diplostomid pathogens in fishes and significantly increased interest in future studies of the genus.

Subsequent studies on the genus *Diplostomum* were devoted to (i) elucidation of their life cycles, (ii) description of the morphology of all life cycle stages (adults, cercariae and metacercariae), and (iii) investigation of the pathogenic effects of the metacercarial stages on fish populations.

Life cycle of species of *Diplostomum*

As mentioned above, the first study investigating the life cycle of *Diplostomum* involving an experimental approach was conducted by A. Ehrhardt and O. Ehrhardt (Braun, 1894). This study demonstrated that trematodes found in the eyes of fishes were, in fact, metacercarial stages of species of *Diplostomum* and that members of this genus have a complex life cycle with fish-eating birds serving as definitive hosts. Another important study contributing to the elucidation of the life cycles of *Diplostomum* was that of Szidat (1924). This author reported gastropod molluscs from

the family Lymnaeidae as the first intermediate host. Based on the evidence provided by the two studies mentioned, it became clear that species of *Diplostomum* utilise a three-host life cycle that involves gastropod molluscs and fish as intermediate hosts, and birds as definitive hosts (Fig. 1-1, images modified from Pérez-Del-Olmo *et al*., 2014; Dignall, 2020; Dougalis, 2018; Robb, 2020; miracidia and sporocysts available from https://projects.ncsu.edu/project/bio402_315/platyhelminthes/Platyhelminthes%203%202012.html).

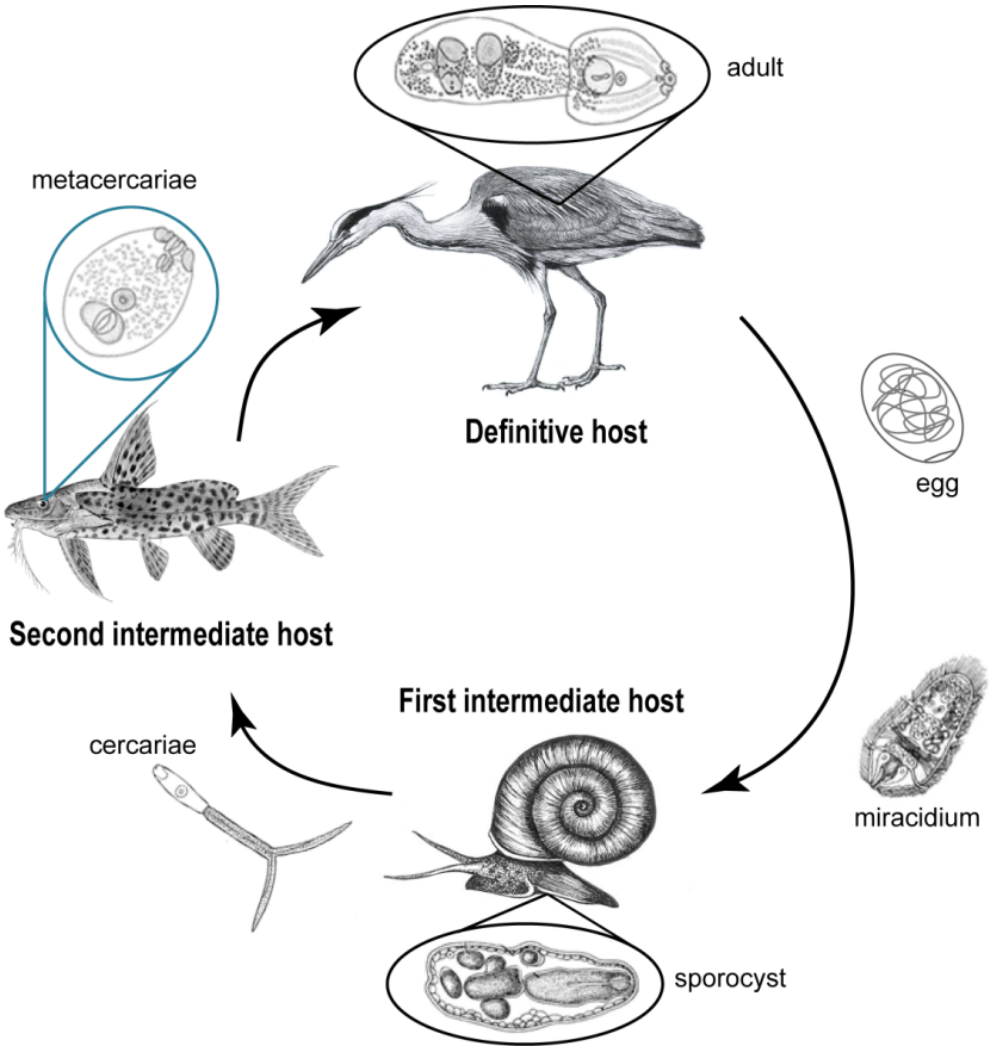


Figure 1-1: Life cycle of *Diplostomum* spp. (modified from Pérez-Del-Olmo *et al*., 2014; Dignall, 2020; Dougalis, 2018; Robb, 2020; miracidia and sporocysts available from https://projects.ncsu.edu/project/bio402_315/platyhelminthes/Platyhelminthes%203%202012.html).

In addition to the parasitic life stages: parthenitae (in molluscs), metacercariae (in fishes) and adult trematodes (in birds); the life cycle of these trematodes also includes free-living stages such as the eggs and two larval stages – miracidia and cercaria that occur in the water.

Adult worms of *Diplostomum* sexually reproduce in the intestines of piscivorous birds and reach maturity relatively quickly (within 5–7 days), but rarely live in their hosts for longer than one

month (Shigin, 1986). The first step of transmission occurs when adults shed eggs that pass in the host faeces and are released into the water. Embryonic development is only possible in water and depends on temperature, with optimum temperatures between 20ºC and 25ºC for development. The eggs hatch after 9–14 days, releasing miracidia that reach and penetrates the first intermediate host - a lymnaeid snail (Grobbelaar *et al*., 2014; Blasco-Costa & Locke, 2017). The life span of miracidia is limited to a few hours and can, during this time, swim up to 10 meters in search of a suitable host (Shigin, 1986). Following penetration, asexual reproduction inside the snail hosts occurs in three generations: mother sporocysts, daughter sporocysts, and cercariae. The mother sporocysts (first generation) develop from the miracidia and produces daughter sporocysts (second generation). The daughter sporocysts multiply and fill the entire hepatopancreas of the snail host. The cercariae are produced in the daughter sporocysts and when fully developed they exit and migrate through the host tissue until it emerges from the snail host into the water in pursuit of finding a potential second intermediate host i.e. freshwater fish (Probert & Erasmus, 1965; Shigin, 1986; Grobbelaar *et al*., 2014). The cercariae penetrates the gills or body surface of the second intermediate host, after which the tail is discarded and development of the metacercariae inside the host takes place (Grobbelaar *et al*., 2014). Thereafter, developed metacercariae get transmitted (via ingestion of fish) to the definitive hosts (fish-eating birds), in which the adults develop and sexually reproduce in the intestine of their host.

To the best of our knowledge, life cycles of 22 species of *Diplostomum* have been fully or partially elucidated experimentally: *Diplostomum adamsi* Lester & Huizinga, 1977, *Diplostomum baeri* Dubois, 1937, *Diplostomum chromatophorum* (Brown, 1931) Shigin, 1986, *Diplostomum flexicaudum* (Cort & Brooks, 1928) Haitsma, 1931, *Diplostomum gasterostei* Williams, 1966, *Diplostomum gobiorum* Shigin, 1965, *Diplostomum helveticum* (Dubois, 1929) Shigin, 1977, *D. indistinctum*, *Diplostomum mergi* Dubois, 1932, *D. murrayense*, *Diplostomum nordmanni* Shigin & Sharipov, 1986, *Diplostomum paraspathaceum* Shigin, 1965, *Diplostomum parviventosum* Dubois, 1932, *D. petromyzifluviatilis*, *D. phoxini* (Rees, 1955, 1957), *Diplostomum pseudobaeri* Razmashkin & Andrejuk, 1978, *Diplostomum pseudospathaceum* Niewiadomska, 1984, *Diplostomum pusillum* (Dubois, 1928) Nazmi Gohar, 1932, *Diplostomum rutili* Razmashkin, 1969, *Diplostomum scudderi* (Olivier, 1941) Dubois, 1966, *D. spathaceum* , *Diplostomum variabile* (Chandler, 1932) Dubois, 1937 and *D. volvens* (see Braun, 1894; Szidat, 1934; Johnson & Angel, 1941; Hoffman & Hundley, 1957; Williams, 1966; Harris *et al*., 1967; Lester & Huizinga, 1977; Shigin, 1986; Mckeown & Irwin, 1995; Field & Irwin, 1995; Niewiadomska, 1986). The data on different life stages of *Diplostomum* have globally been reported in various hosts over the course of the study on the life cycles of species belonging to this genus. Adults of *Diplostomum* have been reported from the piscivorous birds of the family Anatidae Leach, 1820 in Europe, Ardeidae Leach, 1820 in North America and Laridae Rafinesque, 1815 in Antarctica, Europe and North

America. In contrast to only one or two families of birds reported as hosts for *Diplostomum* in the above mentioned continents, a much broader host species range from the Ardeidae, Laridae and Anhingidae Reichenbach, 1849 have been reported from Australia (see Dubois & Pearson, 1965; Dubois & Pearson, 1967; Dubois & Angel, 1972; Shigin, 1986; Feiler, 1986; Galazzo *et al*., 2002; Moszczynska *et al*., 2009; Locke *et al*., 2010a, b; Rellstab *et al*., 2011; Georgieva *et al*., 2013; Pérez-del-Olmo *et al*., 2014; Brabec *et al*., 2015; Locke *et al*., 2015). In Africa, only three bird species belonging to three families have been reported as hosts for *Diplostomum ardeae* Dubois, 1969in *Ardea goliath* (Ardeidae) Cretzschmar, 1829, *Diplostomum magnicaudum* El-Naffar, 1979 in *Gallinula chloropus chloropus* Linnaeus, 1758 (Rallidae Rafinesque, 1815) from Egypt and *Diplostomum ghanense* Ukoli, 1968 in *Anhinga rufa rufa* Daudin, 1802 (Anhingidae) from Ghana (Ukoli, 1968; El-Naffar, 1979; El-Naffar, 1980).

To date, the first intermediate hosts reported for cercariae of *Diplostomum* are lymnaeid snails (Eurasia, Africa, North America) (see Shigin, 1986; Georgieva *et al*., 2013; Behrmann-Godel, 2013; Blasco-Costa *et al*., 2014; Faltýnková *et al*., 2014; Selbach *et al*., 2015; Locke *et al*., 2015; Enabulele *et al*., 2018; Gordy & Hanington, 2019). It is worth mentioning that Gordy *et al*. (2016) found cercariae of *Diplostomum* sp. 8 from planorbid snails in Alberta, Canada. However, this might be an accidental infection as lymnaeid snails are known to serve as first intermediate hosts for *Diplostomum* species. Metacercariae of *Diplostomum* have been recorded in over 150 fish species from the families Acipenseridae Bonaparte, 1831, Atherinopsidae Fowler, 1903, Catostomidae Cope, 1871, Clupeidae Cuvier, 1817, Centrarchidae Bleeker, 1859, Cobitidae Swainson, 1838, Cottidae Bonaparte, 1831, Cyprinidae, Esocidae Cuvier, 1817, Fundulidae Günther, 1866, Gasterosteidae, Gobiidae Cuvier, 1816, Ictaluridae Gill, 1861, Lotidae Bonaparte, 1832, Poeciliidae Bonaparte, 1831, Percidae Rafinesque, 1815, Percopsidae Agassiz, 1850, Salmonidae Cuvier, 1816 and Siluridae Cuvier, 1816 from Europe and North America (Niewiadomska & Laskowski, 2002; Locke *et al*., 2010a, b; Rellstab *et al*., 2011; Behrmann-Godel, 2013; Désilets *et al*., 2013; Georgieva *et al*., 2013; Blasco-Costa *et al*., 2014; Pérez-del-Olmo *et al*., 2014; Kuhn *et al*., 2015; Locke *et al*., 2015; Rahn *et al*., 2016; Kudlai *et al*., 2017; Soldánová *et al*., 2017; Ubels *et al*., 2018). Fish hosts from the Bagridae Bleeker, 1858, Channidae Fowler, 1934, Cichlidae Bonaparte, 1835, Cyprinidae, Gobiidae, Mastacembelidae Swainson, 1839, Mugilidae Jarocki, 1822 and Percidae have been recorded as hosts for *Diplostomum* spp. in Asia (Abdullah & Mhaisen, 2007; Bashe & Abdullah, 2010; Mhaisen *et al*., 2016; Locke *et al*., 2015). However, a limited number of second intermediate hosts for metacercariae of *Diplostomum* have been recorded in Africa: Centrarchidae, Characidae Latreille, 1825, Cichlidae, Clariidae Bonaparte, 1846, Cyprinidae, Hepsetidae Hubbs, 1939, Mochokidae Jordan, 1923, Salmonidae and Schilbeidae Bleeker, 1858 (see Prudhoe & Hussey, 1977; El-Naffar, 1979; Van As & Basson, 1984; Chibwana *et al*., 2013; Grobbelaar *et al*., 2014; Zhokhov,

2014). Due to difficulties in species identifications of the metacercarial stages, numerous reports remain ambiguous (Prudhoe & Hussey, 1977; Van As & Basson, 1984; Grobbelaar *et al*., 2014).

Morphological characterisation of *Diplostomum*

At each stage of their development, species of *Diplostomum* possess unique characteristics that allow to distinguish them from the rest of the members within the Diplostomidae. Morphology of the adult and cercarial stages, in contrast to the metacercariae, is more complex and provides several distinct characters that can be used for species differentiation and identification whereas the morphology of metacercariae is rather simple.

Adults of *Diplostomum* are generally small (> 5500 µm) (see Shigin, 1986). The body of worms is dorso-ventrally flattened and distinctly bipartite. The forebody is spoon-shaped and bears the organs of attachment and the reproductive organs are concentrated in the tubular hindbody. The organs of attachment include oral and ventral suckers, pair of pseudosuckers and massive holdfast organ with median slit. The digestive system is well developed and consists of oral cavity and pharynx followed by the oesophagus and two intestinal branches that reach close to the posterior extremity. The reproductive system of *Diplostomum* spp. is hermaphroditic. The male genital organs are represented by two tandem testes (anterior testis is asymmetrical, posterior testis is symmetrical, bilobed, ventrally concave), seminal ducts, seminal vesicle, and copulatory bursa. The components of the adult female reproductive system include the pretesticular ovary, oviduct, vitellaria, ootype, Laurer's canal and uterus. The vitellaria is arranged on both sides of the body and extends forward beyond the margin of the ventral sucker and the copulatory bursa in cavity form with opening of hermaphroditic ducts at the base. The genital pore is subterminal. The excretory system comprises of flame cells, conducting vessels and the excretory bladder. Characteristics of the reproductive system are one of the most important features that are used for species identification. (Niewiadomska & Laskowski, 2002; Niewiadomska, 2002).

Cercariae of *Diplostomum* belong to the morphotype furcocercariae (meaning "forked tail"). The elongate-oval body can be yellow pigmented in the parenchyma of the whole body or have yellow pigmentation on both sides of the anterior organ, around or above the ventral sucker, tail stem, the furcae or have no pigmentation at all. The body is either equal in length or shorter than the tail stem and carries the organs of attachment including the anterior organ and ventral sucker. The digestive system starts at the mouth opening followed by the prepharynx, pharynx and oesophagus that leads to the intestinal bifurcation at mid-body length extending to some distance from the excretory vesicle. There are two pairs of penetration gland-cells filled with small granular content: one smaller anterior pair and one larger posterior pair that is usually located in close proximity to the ventral sucker with the ducts opening antero-laterally to the mouth. The excretory system consists of flame cells, conducting vessels, excretory vesicle and the caudal excretory

duct that passes through the tail stem. The reproductive system is in a developmental stage in cercariae and the primordia of reproductive organs are represented by a small, compact mass of cells anterior to the excretory vesicle. The tail stem used for movement is usually shorter or equal in size to the furcae and possesses caudal bodies all along the excretory duct.

The arrangement of the body armature on the cercariae is one of the most prominent features used when identifying species. Body armature either has pre-oral or post-oral spines or both that is arranged in a median group without a lateral group or arranged in median- and lateral groups.

There are numerous morphological characteristics of furcocercariae that are used for species identification. Key features used to distinguish among species of *Diplostomum* include: the ratio between the body length, tail stem length and furca length; the ratio between the ventral sucker width and anterior organ width; the total number of pre-oral spines in the median group and number of pre-oral spines in each lateral group; the number of post-oral rows of spines; the presence or absence of transverse spine rows on the body; the number of spine rows on the ventral sucker; the size of the penetration gland cells; the presence or absence of spines on the tail stem and furcae; and finally the position of the tailstem in resting position (<45º or <90º or straight) (see Niewiadomska & Laskowski 2002; Faltýnková *et al*., 2014; Selbach *et al*., 2015).

Metacercariae of the Diplostomidae are categorised in four morphotype groups, namely “diplostomulum”, “neascus”, “prohemistomulum” and “tetracotyle”, where metacercariae of *Diplostomum* belong to the morphotype “diplostomulum” (see Niewiadomska, 2002). Metacercariae of *Diplostomum* are classified as small or medium-sized (> 1000 µm) as they are significantly inferior in size to most of the representatives within the order Strigeida Poche, 1926 (Shigin, 1986). The body is dorso-ventrally flattened and indistinctly bipartite with a large forebody and very small hindbody. The forebody is either round, oval or elongate and bears the organs of attachment and most of the digestive organs. The organs of attachment include oral and ventral suckers, a pair of pseudosuckers and massive holdfast organ with the median slit. Pseudosuckers are the most variable organs that are used to determine identity of the metacercariae. Pseudosuckers can either be lip-shaped (or everted) at the level of the oral sucker or sunken (or pocket-shaped) at the posterior margin of the oral sucker. The digestive system is well developed and consists of the oral cavity, prepharynx and pharynx followed by the oesophagus and two intestinal branches that reach close to posterior extremity. The excretory system comprises of flame cells, conducting vessels and the excretory bladder. According to Niewiadomska (2002), the main feature for distinguishing “diplostomulum” metacercariae from other genera is the structure of the excretory system. In the case of “diplostomulum” metacercariae, this structure is simple with three longitudinal canals (two lateral with ramifications moving posteriorly and one median) connected anteriorly and posteriorly (between the pharynx and ventral sucker) with ramifications at the end with enlarged pockets containing excretory bodies. Excretory pore is

subterminal, oriented ventrally (Shigin, 1986; Niewiadomska, 2002). Other features that may be used in the identification of metacercariae of *Diplostomum* includes: the ratio of the oral sucker width and ventral sucker width; the ratio of the hindbody length and forebody length; the ratio of the hindbody width and forebody width; and number and size of excretory bodies (Shigin, 1986).

Molecular approach to the study of the genus *Diplostomum*

The development of molecular techniques was especially ground-breaking for the identification of species of *Diplostomum*. The application of modern molecular approaches using different genetic markers allowed to overcome the morphological restrictions in the identification of species of *Diplostomum* and aided in the elucidation of their life cycles. However, several studies applying molecular methods for the delimitation and identification of species did not provide detailed morphological descriptions of the material (Locke *et al*., 2010a, b, 2015) which resulted in the counter-productivity of the attempts to resolve the uncertain taxonomic status of species of *Diplostomum*.

Recently, a significant amount of research effort has been invested in developing a molecular sequence library for species within this genus (Galazzo *et al*., 2002; Moszczynska *et al*., 2009; Locke *et al*., 2010a, b, 2015; Behrmann-Godel, 2013; Georgieva *et al*., 2013; Pérez-del-Olmo *et al*., 2014; Blasco-Costa *et al*., 2014; Selbach *et al*., 2015; Kuhn *et al*., 2015; Soldánová *et al*., 2017; Kudlai *et al*., 2017; Enabulele *et al*., 2018). This data largely contributed to our understanding on species diversity, evolution, and host-parasite interactions of the members of *Diplostomum*.

The first genetic markers applied in the studies of diplostomids were rather conservative, 18S and 28S rDNA, followed by the ITS1 rDNA (Galazzo *et al*., 2002; Niewiadomska & Laskowski, 2002; Olson *et al*., 2003). This later evolved into the use of the entire ITS1-5.8S-ITS2 region of the rDNA and was finally refined by the discovery of the usefulness of the mitochondrial barcode cytochrome *c* oxidase I (*cox*1) region for accurate species identification (Galazzo *et al*., 2002; Moszczynska *et al*., 2009). To date, several markers within nuclear ribosomal DNA (18S rDNA and 28S rDNA genes, ITS1-5.8S-ITS2 region) and mitochondrial DNA (*cox*1 and *nad*3) genes have been used for delineation and identification of species of *Diplostomum*.

The pioneer studies using the molecular approach focused on sequencing of the internal transcribed spacer 1 (ITS1) region (Niewiadomska & Laskowski, 2002) has been followed in subsequent studies by Anandan (2004), Rellstab *et al*. (2011) and Cavaleiro *et al*. (2012). In 2012, Cavaleiro and colleagues were the first to attempt applying both morphological and molecular classification (partial ITS1 rDNA-region) of two morphotypes of *Diplostomum* sp. found in the *Platichthys flesus* (Linnaeus, 1758, Pleuronectidae) from Portugal (Cavaleiro *et al*., 2012). It was discovered that these morphotypes were genetically identical (Cavaleiro *et al*., 2012). Two additional species of *Diplostomum* were also reported based on ITS1 sequences: *D*.

pseudospathaceum and *D*. *mergi* from snails in Denmark (Haarder *et al*., 2013). These studies resulted in obtaining ITS1 sequences for six species: *D*. *baeri*, *D*. *mergi*, *Diplostomum paracaudum* (Iles, 1959) Shigin, 1977, *D*. *phoxini*, *D*. *pseudospathaceum* and *D*. *spathaceum* from the larval stages (cercariae and metacercariae) collected in Poland, United Kingdom and Finland and an unidentified species *Diplostomum* sp. (metacercariae) from Portugal with an interspecific divergence of 1.3 – 4.7% (considered as overall low divergence). The ITS1 sequences for *D*. *spathaceum* and *D*. *parviventosum* appeared to be identical, although the isolates were morphologically distinct and represented two species. This demonstrated low variability of the ITS1 region and thus raised the need for different markers to successfully distinguish between species of *Diplostomum*. Galazzo *et al*. (2002) amplified the entire ITS1-5.8S-ITS2 region for *Diplostomum* spp. The sequence comparison analysis showed higher interspecific divergence (1.7 – 4.4%) of the sequences amplified using the ITS1-5.8S-ITS2 region compared to those sequences amplified by the ITS1 marker and distinguished three species from North America: *D*. *huronense*, *D*. *indistinctum* and *D*. *baeri*. Even though Galazzo *et al*. (2002) could distinguish between the species of *Diplostomum*, they could not successfully identify and separate a cryptic species of *D*. *indistinctum*. However, an unexpected discovery of 23 nucleotides difference between species of *D*. *baeri* from their study and *D*. *baeri* sequenced by Niewiadomska & Laskowski (2002) from Canada and Europe indicated a definite difference in species identity. Although Galazzo *et al*. (2002) was the first to successfully distinguish between different species of *Diplostomum*, the recognition of cryptic species was still necessary for accurate species differentiation and identification in attempt to uncover the true diversity of this genus.

As previously mentioned, molecular markers of ITS1 rDNA region and partial 28S rDNA gene showed low levels of divergence and can only be used for identifications to the genus level (Moszczynska *et al*., 2009; Georgieva *et al*., 2013). This led to the development and use of the barcode region of the *cox*1 gene; a more effective solution to distinguish closely related species within the genus *Diplostomum* (Moszczynska *et al*., 2009; Georgieva *et al*., 2013; Pérez-del-Olmo *et al*., 2014). The development of diplostomid-specific primers used to generate the *cox*1 barcode region designed by Moszczynska *et al*. (2009) allowed for the construction of a large barcode library of species of *Diplostomum* with more than 1000 sequences available in GenBank (Moszczynska *et al*., 2009; Locke *et al*., 2010a, b; Georgieva *et al*., 2013; Blasco-Costa *et al*., 2014; Locke *et al*., 2015; Selbach *et al*., 2015; García-Varela *et al*., 2015; Kudlai *et al*., 2017). In a more recent study by Brabec *et al*. (2015), the implementation of novel genetic markers for the identification of *Diplostomum* spp. were investigated. These authors studied the use of seven subunits of NADH dehydrogenase (*nad*1-6 and *nad*4L) and revealed that *nad4* and *nad*5 were the most promising markers to use in molecular taxonomy due its optimal sequence variability (Brabec *et al*., 2015). Recently, the mitochondrial *nad*3 gene was used to successfully identify

three species/species lineage of *Diplostomum*: *Diplostomum mergi* complex sp. Lineage 2, *D*. *spathaceum* and *D*. *pseudospathaceum* from various fishes in Hungary and Slovakia and showed higher sequence variability compare to *cox*1 gene (Kudlai *et al*., 2017).

Currently, molecular data on all life stages of members of *Diplostomum* available in GenBank includes sequences for nine identified and 38 unidentified species and species-level genetic lineages from Europe (4 species and 15 unidentified, respectively), North America (4 and 19, respectively), Asia (1 and 3, respectively) and Africa (1 unidentified species) (see Chibwana *et al*., 2013; Locke *et al*., 2015; Kudlai *et al*., 2017; Soldánová *et al*., 2017; Gordy & Hanington, 2019). Of these, sequences for nine species generated from the adult isolates have been published, with only six being identified to species level with two from Europe: *D*. *spathaceum* and *D*. *pseudospathaceum*; and seven species are from North America: four identified species, *D*. *ardeae*, *D*. *baeri*, *D*. *huronense*, *D*. *indistinctum*; and three unidentified species, *Diplostomum* sp. 1, 3, 4 *sensu* Locke *et al*. (2010a, 2010b). A summary of the molecular data available for species and species lineages of *Diplostomum* in GenBank is provided in Table 1-1. In Africa, nine species of *Diplostomum* have been reported from freshwater fishes in Ethiopia, Egypt, Nigeria and South Africa, with molecular confirmation provided for only one unidentified species of *Diplostomum* from Nigeria (Prudhoe & Hussey, 1977; Khalil & Polling, 1997; Chibwana *et al*., 2013; Zhokhov, 2014).

Even though advanced molecular techniques have largely contributed to our understanding of the diversity of *Diplostomum*, they have limitations. Most of the molecular sequences available in GenBank were from the metacercarial stages (in fish hosts) that, as mentioned previously, lack sufficient morphological characters for accurate species identification. The high percentages of metacercariae reported in fish hosts are largely due to the availability and accessibility of the fish hosts as well as permits and ethics required for sampling. Therefore, numerous metacercarial isolates remain unidentified and require the sequences from their adult parasitising bird definitive hosts to identify the larval stages to the species-level and elucidate their life cycles.

Table 1-1: Summary of the molecular data available for *Diplostomum* spp. in GenBank.

Species name	**Species name**	**Genetic markers**				
Identification according to Georgieva *et al*., 2013; Blasco-Costa *et al*., 2014; Selbach *et al*., 2015; Kudlai *et al*., 2017	**Identification as in GenBank**	***cox*1**	***nad*3**	**ITS1-5.8S-ITS2**	**28S**	**18S**
Diplostomum ardeae Dubois, 1969	–	a	–	–	–	–
Diplostomum baeri Dubois, 1937	–	a, m	–	a, c, m	a, m	a, m
Diplostomum baeri Lineage 1	*Diplostomum baeri* *Diplostomum* sp. Lineage 3	m	– –	m	–	–
Diplostomum baeri Lineage 2	*Diplostomum baeri* *Diplostomum baeri* complex sp. 2 *Diplostomum* sp. Lineage 4	c, m	– – –	c, m	–	–
Diplostomum baeri Lineage 3	–	–	–	m	–	–
Diplostomum compactum (Lutz, 1928) Dubois, 1970	–	–	–	–	–	?
Diplostomum huronense (La Rue, 1927) Hughes, 1929	–	a, m	–	a, m	a	a
Diplostomum indistinctum (Guberlet, 1923) Hughes, 1920	–	a, c, m	–	a, m	a	a
Diplostomum mergi Dubois, 1932	–	–	–	c, m	–	c, m
Diplostomum mergi Lineage 2	*Diplostomum mergi* *Diplostomum mergi* complex sp. 2	c, m	m –	c	–	–
Diplostomum mergi Lineage 3	*Diplostomum mergi*	c, m	–	c, m	–	–
Diplostomum mergi Lineage 4	*Diplostomum mergi*	c	–	c	–	–
Diplostomum paracaudum (Iles, 1959) Shigin, 1977	–	–	–	a, c, m	m	m
Diplostomum parviventosum Dubois, 1932	*Diplostomum mergi* Lineage 1 (*cox*1)	c	–	c, m	–	–
Diplostomum phoxini (Faust, 1918) Arvy & Buttner, 1954	–	c, m	–	m	m	m
Diplostomum pseudospathaceum Niewiadomska, 1984	–	a, c, m	m	a, c, m	a	a, c, m

Table1-1 (continued)

Species name Identification according to Georgieva *et al*., 2013; Blasco-Costa *et al*., 2014; Selbach *et al*., 2015; Kudlai *et al*., 2017	Species name Identification as in GenBank	Genetic markers *cox*1	 *nad*3	 ITS1-5.8S-ITS2	 28S	 18S
Diplostomum spathaceum (Rudolphi, 1819) Olsson, 1876	*Diplostomum spathaceum* *Diplostomum paracaudum*	a, c, m	m –	a, c, m	a, c	m
Diplostomum sp. Clade Q	*Diplostomum* sp. Clade Q *Diplostomum mergi*	c, m	– –	c, m	–	c
Diplostomum sp. Lineage 2	–	c, m	–	c, m	–	–
Diplostomum sp. Lineage 5	–	m	–	m	–	–
Diplostomum sp. Lineage 6	–	c, m	–	c, m	–	–
Diplostomum sp. 1	–	a, c, m	–	a, c, m	–	a, m
Diplostomum sp. 2	–	m	–	m	–	m
Diplostomum sp. 3	–	a, c, m	–	m	–	–
Diplostomum sp. 4	–	a, c, m	–	a, c, m	–	m
Diplostomum sp. 5	–	m	–	–	–	–
Diplostomum sp. 6	–	m	–	–	–	–
Diplostomum sp. 7	–	m	–	–	–	–
Diplostomum sp. 8	–	m	–	m	–	–
Diplostomum sp. 9	–	m	–	m	–	–
Diplostomum sp. 10	–	m	–	m	–	m
Diplostomum sp. 11	–	m	–	–	–	–
Diplostomum sp. 12	–	m	–	–	–	–
Diplostomum sp. 13	–	m	–	–	–	–
Diplostomum sp. 14	–	m	–	m	–	–
Diplostomum sp. 15	–	m	–	m	–	m

Table 1-1 (continued)

Species name	**Species name**	**Genetic markers**				
Identification according to Georgieva *et al*., 2013; Blasco-Costa *et al*., 2014; Selbach *et al*., 2015; Kudlai *et al*., 2017	**Identification as in GenBank**	***cox*1**	***nad*3**	**ITS1-5.8S-ITS2**	**28S**	**18S**
Diplostomum sp. 16	–	m	–	–	–	–
Diplostomum sp. 17	–	m	–	–	–	–
Diplostomum sp. 18	–	m	–	–	–	–
Diplostomum sp. 19	–	m	–	–	–	–
Diplostomum sp. A *sensu* Kudlai *et al*. (2017)	–	m	–	–	–	–
Diplostomum sp. A *sensu* Gordy & Hanington (2019)	–	c	–	–	–	–
Diplostomum sp. B *sensu* Kudlai *et al*. (2017)	–	m	–	–	–	–
Diplostomum sp. B *sensu* Gordy & Hanington (2019)	–	c	–	–	–	–
Diplostomum sp. C *sensu* Kudlai *et al*. (2017)	–	m	–	–	–	–
Diplostomum sp. C *sensu* Gordy & Hanington (2019)	–	c	–	–	–	–
Diplostomum sp. *sensu* Chibwana *et al*. (2013)	–	m	–	m	–	–
Diplostomum sp. *sensu* Tkach *et al*. (2012)	–	–	–	–	c	–
Diplostomum sp. *sensu* van Steenkiste *et al*. (2012)	–	–	–	–	–	m
Diplostomum sp. *sensu* Cavaleiro *et al*. (2012)	–	–	–	–	–	m

Abbreviations: a, adult, m, metacercariae, c, cercariae; ?, stage not reported, -, data not available

Of the above-mentioned studies, only two, Galazzo *et al*. (2002) and Cavaleiro *et al*. (2012), provided both morphological descriptions and sequences of the identified species. Since then, more studies have followed these examples and helped build the library on the diversity for species of *Diplsotomum* (Faltýnková *et al*., 2014; Selbach *et al*., 2015; Blasco-Costa *et al*., 2014; Pérez-del-Olmo *et al*., 2015; Kudlai *et al*., 2017). With that being said, the diversity of *Diplostomum* known from Europe and North America are already in an advanced stage in comparison to studies in Africa, Antarctica, Asia, Australia, and South America. From these regions, knowledge on the diversity of *Diplostomum* is still in the developmental stage, but due to molecular tools, the establishment of a baseline for future taxonomic research, species delimitation and the elucidation of life cycles is ensured. The application of both morphological and molecular methods will, therefore, provide a clear understanding on the true global diversity of *Diplostomum*.

Species composition of *Diplostomum* and its geographical distribution

Throughout the years, numerous attempts in compiling a list of species of *Diplostomum* have led to some controversy and confusion in taxonomy within this genus. Sudarikov (1960) revised available records and provided a list of 31 nominal species of *Diplostomum* based on adult stages. Of these, metacercarial stages were reported for three species, *D*. *flexicaudum*, *D*. *murrayense* and *D*. *spathaceum*. In a later study (1971), Sudarikov added seven additional species to the above mentioned list. Around the same time, Dubois (1970b) reported 22 species of *Diplostomum* that have been described and provided descriptions for six additional species to the key: *Diplostomum amygdalum* Dubois & Pearson, 1965, *Diplostomum compactum* (Lutz, 1928) Dubois, 1970, *D*. *gasterostei*, *Diplostomum sobolevi* Shigin,1959, *Diplostomum sudarikovi* Shigin, 1960 and *Diplostomum triangulare* (Johnston, 1904) Hughes, 1929 & Dubois, 1937. Shigin (1976) provided a key of described metacercariae including 13 species of *Diplostomum* and later also added seven additional species of *Diplostomum* to the key in 1986. During revisions of this genus done by Shigin (1986), a total of 37 species of *Diplostomum* (considered as valid), their zoogeographical distribution and reports from the three hosts were compiled and remains the last study to have revised the species composition of this genus on a global scale. However, a later study with a focus on application of molecular techniques in an attempt to resolve the uncertain taxonomy of *Diplostomum* species from the Palearctic region, reported a staggering 41 nominal species of *Diplostomum* based on the review of their work and that of Shigin (1993) and Niewiadomska (2010) (see Georgieva *et al*., 2013). With this high diversity reported from only one zoogeographical region (Palearctic), a much higher diversity of *Diplostomum* species is expected when studying this genus on a global scale (compared to what Shigin reported in 1986), especially when applying molecular methods in the identification of species (cryptic species or species-level genetic lineages) within this genus (Georgieva *et al*., 2013). It is worth mentioning that most

nominal species have been reported based on morphology which in most cases (especially in larval stages) may not represent accurate identification (synonymised species), therefore it may be the case that with the application of molecular methods in species identification, a lower diversity of *Diplostomum* will be found. In many years, with focus on the use of morphological characteristics only when identifying species of *Diplostomum*, almost 160 named species have been reported of which 60 species have since been re-described and transferred to other genera (Brady, 1989). The actual number of valid species within this genus is therefore considerably less than the literature would suggest (Brady, 1989). Knowledge on the global diversity of the genus *Diplostomum* is still lacking due to sampling insufficiencies and reliable species identifications. Members of *Diplostomum* have been reported on all continents, but the majority of the described species were reported from the Palearctic and Nearctic (Shigin, 1986; Niewiadomska, 1984; Georgieva *et al*., 2013; Locke *et al*., 2015; Blasco-Costa & Locke, 2017 and references therein), however, access to some of these papers or books are not always available and language barriers of some research items published in native languages present its own challenges. To date, almost 80 nominal species of *Diplostomum* are known worldwide, with highest diversity reported from Europe and North America and an overall low diversity reported from Africa, Antarctica, Australia, Asia and South America (Dubois, 1970; Yamaguti, 1971; Shigin, 1986, 1993; Locke *et al*., 2010a, b; Georgieva *et al*., 2013; Blasco-Costa *et al*., 2014; Locke *et al*., 2015; Blasco-Costa & Locke, 2017; Kudlai *et al*., 2017).

Data on nominal species of *Diplostomum* and the zoogeographical regions from where they were reported are summarised in Appendix B. To the best of our knowledge, of the nominal species of *Diplostomum* reported globally, 49 species were from the Palearctic region (countries in Europe and Asia), 18 species from the Nearctic region (North America), eight species from the Afrotropical region (Africa), eight species from the Oriental region (India), six species from the Australian region (Australia), three species from the Neotropical region (South America) and three from the Antarctic region. Available data on adult worms reported from their definitive hosts based on morphological evidence were recorded for 63 species of the total nominal species. Four species of *Diplostomum* were described based on the metacercariae from freshwater fishes in Ethiopia (Zhokhov, 2014). Metacercariae of 15 species identified to belong to the genus *Diplostomum* were reported from freshwater fishes in India (Pandey & Agrawal, 2013). However, these records require detailed revision as they were reported from sites in the host other than the eyes and brain of the fish and morphology of specimens may resemble other genera within the Diplostomidae (Pandey & Agrawal, 2013). Of the nominal species of *Diplostomum* reported to date, supporting molecular evidence is provided for only ten named species of *Diplostomum*. Despite extensive research with a focus on the diversity of *Diplostomum* conducted in North America, Europe and Asia, no similar comprehensive studies have been done in Africa, Antarctica, Asia, Australia, and South America. The fact that so little is known on the species

composition of *Diplostomum* in Africa is largely due to the absence of dedicated studies, a lack of sampling effort or funding and lack of expertise (Chibwana *et al*., 2013, Chibwana, 2018). This expertise is essential, especially in cases of the identification of the larval stages of *Diplostomum* that can be challenging without the aid of molecular techniques. Reliable data for species of *Diplostomum* and species identification based on adults are therefore extremely important in expanding our knowledge on these trematodes in South Africa (and in Africa as a whole). Thus, the probabilities of discovering new species of *Diplostomum* in South Africa are most likely.

Pathogenicity and effect of metacercariae of *Diplostomum* on fish hosts

By coincidence, the pathogenic effect of cercariae of *Diplostomum* was first recorded by Blochmann (Blochmann, 1910). The researcher brought several snails back from an excursion and placed them in an aquarium with fish. The next morning, all the fishes in the tank were found dead. The only plausible explanation for the rapid deaths of fish could be drawn from the emergence of cercariae from the snails serving as causative agents for these infections. This discovery led to the numerous studies focused on the pathogenic effect of cercariae on fish hosts. Studies have shown that mortalities in fish may occur because of blockage of gill vessels due to the migration of the parasites that leads to ruptured blood vessels and lesions of the central nervous system. Cercariae of *Diplostomum* were reported to cause mortalities in larva or juvenile fishes even though the metacercariae were known causing cataracts in fish eyes. In 1924, diplostomiasis was first recognised as a fish disease (Plehn, 1924). The high number of incidents reporting mortalities in fish due to this disease has amplified the view on the economic importance of diplostomiasis as the development of inland fish farming increased. Knowledge on the biology and life cycles of *Diplostomum* aided in the development of methods used for prevention of fish diplostomiasis. However, limitations in the treatment of diplostomiasis persisted due to the occurrence of metacercariae in the eyes (occasionally brain) of their fish hosts (Shigin, 1986). For the development of prevention methods to occur, a clear understanding on the biology, pathogenic nature, epizootic features, and the mechanisms of regulation of the pathogen is required. The first step of prevention requires the elucidation of the life cycle of the pathogen in order to identify the most accessible link to treat. After the most accessible link in *Diplostomum* was identified, prevention for diplostomiasis were established and could be carried out in two main ways. The prevention of the transmission through: (i) the definitive host – this is done by restricting contact with fish in farms by means of nets; or (ii) by controlling snail populations present in fish farms. These methods were proposed in the first manuals on icthyopathology and were first used in the practice of fish farming and remain the two main prevention methods for diplostomiasis in fish farms (Shigin, 1986; Price & Nickum, 1995; Ndeda *et al*., 2013). However, subsequent studies have identified that an increase in water flow and the treatment of fish with praziquantel (Droncit) can also be used in combating this disease (Bylund & Sumari, 1981;

Székely & Molnar, 1991; Field & Irwin, 1994). More recent studies introduced two alternative methods of the prevention of diplostomiasis from the snail hosts by applying micro screening with small mesh sizes (32 µm) that removed 99% of cercariae in the fish farms (even 200 µm mesh sizes that removed 50% of cercariae in the fish farms) or by treating the water with sodium percarbonate (concentrations of 20 mg/L or higher) as a replacement for formalin (Buchmann & Kristensson, 2003; Larsen *et al*., 2005).

The larval stages of diplostomid trematodes are considered important pathogens that may have harmful effects on both natural and aquaculture fish populations. Juvenile fish or fingerlings are especially susceptible to metacercarial infections and in effect have a higher mortality rate due to their low immunity that is not yet fully developed (Szidat & Nani, 1951). The migration of a large number of metacercariae towards a specific site in the host i.e. eye lenses or brain of the fish host, leads to cataract formation or cranial distortion which impairs proper body functions and may result in mortality due to reduced host survival behaviour (Shigin, 1986; Chappell, 1995). An increase in metacercariae results in increased mortalities in wild and farmed fish populations, which in turn may affect the economy on a local and/or global scale (Roberts & Janovy, 2009). Thus, prevention of diplostomiasis is key to the success of the treatment. However, instead of this new found knowledge on the biology, ecology, life cycles and life history of *Diplostomum* helping to resolve questions about this genus, it in fact presented new research challenges to explore the effects of metacercarial stages on fish behaviour.

Milinski (1990) suggested two main mechanisms used by parasites that causes manipulation in the host's behaviour: direct mechanism i.e. parasites affecting the neuro-endocrine system of host by releasing hormones or neurotransmitter analogues; and indirect mechanisms i.e. parasites may alter host behaviour by changing a physiological parameter that invoke a certain response in the host. As mentioned above, one of the common effects of metacercariae on their fish hosts are the cause of cataracts which may result in blindness, lens rupture and exothalmia as tested by Shariff (1980) (Scotland). This histopathological study determined that *Diplostomum* spp. were generally located in the retina of their fish hosts [*Salmo gairdneri* = *Oncorhynchus mykiss* (Walbaum, 1792) (Salmonidae)]. Although this study proves the presence of *Diplostomum* in the retina of the hosts' eye, these diplostomid metacercariae were also reported in the brain, eye or nervous tissue and vitreous humour of its host, where they reportedly cause sensory function impairment (Holmes & Zohar, 1990). However, other effects of metacercariae on their fish hosts also include stunted growth, unusual feeding behaviour, changes in time budgets, reduced ability to successfully catch prey and a general lack of response to visual stimuli (Palmieri *et al*., 1977). One of the above mentioned effects i.e. feeding behaviour of fish (*Leuciscus leuciscus* (Linnaeus, 1758), Cyprinidae) parasitised by *D. spathaceum* were investigated in England. The main findings included a decline in efficient feeding behaviour on the prey (*Gammarus pulex* Linnaeus, 1758) as an increase in parasites in the eye of the host

were recorded (Crowden & Broom, 1980). Moreover, the loss in efficiency of feeding was reported to be compensated by an increase in time spent on feeding. This phenomenon as a result meant that heavily infected fish spent more time in the surface layers making them more likely to be caught by predators (Crowden & Broom, 1980).

A study by Voutilainen *et al*. (2008), also investigating the effects of *D*. *spathaceum* on *Salvelinus alpinus* (Linnaeus, 1758) (Salmonidae) from Finland, discovered the exact same outcomes as Crowden & Broom (1980) with different prey (zooplankton). Another behavioural study conducted in England included the study of two species of *Diplostomum*: *D*. *spathaceum* (found in the eye lens of fish) and *D*. *gasterostei* (found in the retina of fish) on three-spined sticklebacks, *Gasterosteus aculeatus* Linnaeus, 1758 (Gasterosteidae) (Owen *et al*., 1993). This study tested the level of parasite intensity in the eyes of these fish hosts required to have an effect on the vision and behaviour such as selection of prey. It was discovered that a low level of infection may affect the stickleback's field of view. However, it is important to note that this conclusion was drawn from two species of *Diplostomum* present in a single host. Both these studies showed that parasites of *Diplostomum* species impaired the ability of infected fish to shoal closely and evade predatory attacks.

Another effect of metacercariae of *D*. *phoxini* occurring in the brain (aggregated in the brain lobes) of the European minnow, *Phoxinus phoxinus* (Linnaeus, 1758) (Cyprinidae) was the changes in swimming behaviour induced by the parasite and heavily infected fish were reported to swim in an "intermittent swimming pattern", other aspects such as impaired optomotor responses, changes in shoaling behaviour were also reported for the same hosts (Ashworth & Bannerman, 1927; Rees, 1955; Lafferty & Morris, 1996; Barber & Crompton, 1997; Barber, 2000). Extensive behavioural studies started from the early 2000's. Researchers were gaining more interest in the concept of parasite manipulation on the various behavioural traits from different fish host species (Barber *et al*., 2000; Barber & Wright, 2005). Most behavioural studies in Europe (Finland in particular) focused on *O*. *mykiss* (Salmonidae) as host for *D. spathaceum* (Seppälä *et al*., 2004, 2005a, b, 2006a, b) and *D. pseudospathaceum* (Seppälä *et al*., 2012; Gopko *et al*., 2015, 2017) as this fish is relatively susceptible to infections and easy to maintain under laboratory conditions. Of these, the studies focusing on the influence of *D*. *spathaceum* on this host revealed remarkable results including: (i) infected fish had less escape response behaviour towards "artificial aerial predators" as well as "human predators using dipping nets"; (ii) changes in cryptic colouration and cryptic behaviour; (iii) catchability of infected fish increased with higher cataract coverage induced by the well-developed parasites. In contrast, metacercariae of *D*. *pseudospathaceum* on this host did not affect any of the behavioural traits such as activity, use of shelter, and escape response tested. However, after studying the effects of the maturity of *D*. *pseudospathaceum* and their influence on the depth preference and activity during and after a simulated attack, it was concluded that these parasites only manipulate traits that specifically

predispose the fish host to bird predators and not the traits determining susceptibility to non-hosts (Gopko *et al*., 2015, 2017). Another study by Seppälä *et al*. (2011) from the Baltic Sea, investigated the influence of *Diplostomum* metacercariae in numerous fish hosts namely, *Gymnocephalus cernuus* (Linnaeus, 1758*,* Percidae), *R*. *rutilus*, *L*. *leuciscus*, *Alburnus alburnus* (Linnaeus, 1758, Cyprinidae), *Osmerus eperlanus* (Linnaeus, 1758, Osmeridae), *Coregonus lavaretus* (Linnaeus, 1758, Salmonidae) and *G*. *aculeatus*. These authors found that cataracts were common in hosts, but changes in behaviour were only noted when high levels of metacercariae were present in fish. This suggested that only certain hosts, *G*. *cernuus*, *R*. *rutilus* and *L*. *leuciscus* in this case, were more strongly affected by those infections due to the higher abundance within these host and were, therefore, more likely to be predispose to predation. In summary, all studies concluded that the visual impairments induced by these parasites, have a direct effect on their susceptibility to predation, however, this is yet to be conclusively shown (Seppälä *et al*., 2006b).

In Sweden, the round goby *Neogobius melanostomas* (Pallas, 1814) (Gobiidae) were selected for behavioural studies that also investigated the influence of parasite intensity (cataract formation) on the escape responses to avian attacks (Flink *et al*., 2017). These fish did not change their natural behaviour such as shelter-use, boldness or habitat preferences, but did have less escape responses to simulated avian attacks that is in accordance to previous reports suggesting that these parasites induce behavioural changes that facilitates transmission to the final host. In a more recent study in Norway, the correlation between intensity of infection (dose) with *Diplostomum* spp. and the age and size of the Arctic charr *S*. *alpinus* were investigated (Padrós *et al*., 2018). It was discovered that metacercariae of *Diplostomum* did alter the visual acuity of the host, but to a lesser extent due to the immunity of their hosts and illustrated that the pathology caused by parasites depends on fish size, age and intensity of infection. Although many of the described modifications in behaviour may have been the result of pathology associated infections, in some cases, these modifications did have a direct link to parasite transmission.

However, of the above mentioned studies, most experimental works done in the early 20th century were based on visual observations without additional quantitative methods. The development of video tracking systems (i.e. Ethovision) in the early 1990's allowed advances in the flexibility, spatial precision and accuracy of tracking organisms and quantification of data end points (Noldus *et al*., 2001). The software has developed remarkably over the last decades and can now recognise objects by their differences in shade, making tracking of moving organisms possible by capturing screenshots of video data recordings and providing coordinates for the sample depending on the difference in contrast between object and the defined arena which can be quantified and used for statistical analysis. Ethovision video tracking software was developed for the use of general purpose video tracking, movement analysis and behaviour recognition (Noldus *et al*., 2001). The use of statistical analysis as supporting evidence in behavioural

experiments have increased significantly in recent years and enables constructing comprehensive conclusions based on both visual observations and statistical evidence (Seppälä *et al*., 2005a, b, 2006a, b, 2011, 2012; Gopko *et al*., 2015; Flink *et al*., 2017).

To summarise, the pathogenic effects of metacercariae of *Diplostomum* on fish hosts have been largely investigated in Europe and North America and revealed that infections with metacercariae that occur in the eyes and brain of fish can modify their hosts behaviour by affecting foraging efficiency, habitat selection, shoaling and anti-predator behaviour, thus facilitating the transmission to the definitive hosts and enhance the parasite's own reproductive fitness in order to complete their life cycle. Even though studies on the influences of diplostomid metacercariae on their second intermediate hosts have been largely studied in Europe, information in South Africa are virtually lacking. Therefore, no studies on the influence of metacercariae of *Diplostomum* on the behaviour of naturally infected fish hosts have been published in South Africa. In Africa, the role of parasite-induced changes in bottom dwelling fish, *Synodontis zambezensis* Peters, 1852 (Mochokidae) have not yet been investigated and current information is based on studies predominantly from Salmonidae (most commonly used host), Cyprinidae, Gasterosteidae and Gobiidae (highest parasite intensities reported from this host) from Europe. Therefore, the focus of this study was to conduct behavioural experiments to assess whether *S. zambezensis* infected with *Diplostomum* metacercariae changed their natural behaviour such as swimming in surface layers and escape responses to simulated aerial predator attacks and as result becoming more susceptible to avian attacks.

1.2 Aims and objectives

Research aims

The aims of this study were to assess the diversity of digenean trematodes from the genus *Diplostomum* parasitising freshwater fishes from selected provinces in South Africa and to determine the effect of infection with metacercariae of *Diplostomum* on the behaviour of their naturally infected fish hosts.

Research objectives

In order to achieve the aims of the study, the following objectives were formulated:

- To examine fishes from the Ndumo Game Reserve (KwaZulu-Natal), the Mokala National Park (Northern Cape) and the Boskop Dam Nature Reserve (North West Province) for the presence of metacercariae of *Diplostomum* and identify species diversity via morphological examination and phylogenetic analyses.
- To generate sequence data, partial mitochondrial (*cox*1) and nuclear ribosomal (28S) genes, and internal transcribed spacer region (ITS1-5.8S-ITS2) for metacercarial isolates.

- To provide detailed morphological descriptions of species of *Diplostomum* identified based on phylogenetic analyses.
- To conduct experimental studies in order to determine the influence of infection with metacercariae on the behaviour of their naturally infected fish hosts.
- To quantify all data end points using unpaired t-tests with Welch's correction and One-Way ANOVA to illustrate significant or non-significant differences in host behaviour between infected and uninfected fish.

1.3 Hypotheses

- A low diversity of digenean trematodes from the genus *Diplostomum* in freshwater fishes in South Africa is expected.
- A low intensity of infection with metacercariae of *Diplostomum* is expected.
- Infections with metacercariae of *Diplostomum* will influence the behaviour of their naturally infected fish host.

1.4 Outline of dissertation

Following the general introduction and literature review of our current knowledge of the genus *Diplostomum* (Chapter 1), the general methods of this study are described (Chapter 2). The result section of this dissertation is divided into two main sections, each consisting of a short introduction, materials and methods specific to that chapter, results and discussion. The first section focused on the diversity of *Diplostomum* (Chapter 3) in South African freshwater fishes and the second section focused on the effect of metacercariae on the behaviour of their naturally infected fish hosts (Chapters 4). These two sections are followed by a summative discussion (Chapter 5) that summarises all findings, proposes future research and provides final conclusions, followed by the reference list (following the format of the NWU Harvard Referencing Guide). The additional appendices complete the dissertation. A summary of each chapter is provided below.

Chapter 2 comprises of the general materials and methods used during this study including site selection, fish selection and fish sampling methods that are described in detail. In **Chapter 3**, a detailed assessment on the diversity of *Diplostomum* in freshwater fishes collected from selected provinces in South Africa based on molecular and morphological results is reported and descriptions of found metacercariae are provided together with a comparison to previous records in Africa. Sections of this chapter has been published in Hoogendoorn *et al*. (2020). In **Chapter 4**, the effect of metacercariae on the behaviour of selected fish species, *Synodontis zambezensis* is investigated and the results of statistical analyses obtained from the experiments together with main findings are described and interpreted. **Chapter 5** consists of a brief discussion of the results of each of the previous chapters in the dissertation, along with future recommendations and final conclusions.

Appendix A consists of a publication on the molecular and morphological characterisation of four species of metacercariae from the family Diplostomidae found in *Tilapia sparrmanii* (Perciformes: Cichlidae) in the North West Province, South Africa. In **Appendix B**, a table is provided with the summary of nominal species of *Diplostomum* worldwide, with their hosts and geographical distribution. **Appendix C** contains documentation of the permit for sampling in the Ndumo Game Reserve. **Appendix D** contains documentation of the permit for sampling in the North West Province. **Appendix E** consists of documentation for the ethics approval for fish sampling received from the NWU. In **Appendix F**, a table is provided with summary data for sequences for *Diplostomum* spp. retrieved from GenBank and used for the phylogenetic analyses. **Appendix G** consists of a publication reporting on discovery of the identity of three species of *Diplostomum* (Digenea: Diplostomidae) parasitising freshwater fishes in South Africa.

Chapter 2: General Materials & Methods

CHAPTER 2: GENERAL MATERIALS & METHODS

2.1 Introduction

South Africa is a semi-arid country with an average rainfall value well below the world's average per year (Botai *et al*., 2018). Flooding is caused by short, high intensity storms that occur during the rainfall seasons which results in runoff of silt, organic and inorganic matter that accumulated in the catchment, into the water bodies. During the dry seasons, many rivers and streams run dry and reservoirs show significant changes in water levels. In South Africa, there are a wide variety of aquatic ecosystems with different biota that are adapted to various changes in conditions such as pH, temperature, nutrients, oxygen availability and turbidity fluctuations as well as changes in flow patterns (DWAF, 1996).

For the purpose of this study, we focused on different water bodies such as rivers, dams, pans, and lakes for fish collection. Parasitological screenings were carried out on freshwater fishes found within these water bodies, as they serve as second intermediate hosts for the focus group of organisms of the study – metacercariae of the genus *Diplostomum* (Digenea: Diplostomoidea). Since this is the first study focusing extensively on the diversity of *Diplostomum* in South Africa, the selection of various fish species from different trophic levels for parasitological examinations is important. This is due to the fact that preferences for certain habitats and food play a vital role in the likelihood of the fish becoming infected with these parasites. Therefore, the selection of fish species during this study is discussed with a focus on their biology and ecology. A brief summary for each species is provided (Skelton, 2001). It is also important to note that the sampling effort was random with the only exception for *S. zambezensis* (selected model species) used for behavioural studies (discussed in Chapter 4).

2.2 Site selection

The present study took place in the Riet River in the Mokala National Park (MNP, 32445 ha), Northern Cape; in the Ndumo Game Reserve (NGR, 11898 ha), northern KwaZulu-Natal at four different sites (Phongolo Site 1 and Site 2; Phongolo River: Lake Nyamithi; Usuthu River: Shokwe Pan); and in the Boskop Dam Nature Reserve (BDNR, 2756 ha), North West Province at one site in the Mooi River: Boskop Dam (Fig. 2-1). The illustration was compiled in ArcGIS 10.6 (Available from https://support.esri.com/en/downloads).

The MNP is located in a semi-arid area subjective to frequent thunderstorms. The park is within a predominantly summer rainfall area that ranges between 233 mm and 558 mm per year. High rainfall occurs during November to March, reaching a peak in February. The average annual rainfall recorded was 355 mm (2007–2016). Cold winter periods with less rainfall occur in June and July where temperatures reach as low as -6.6ºC (July 2011); while summer (December and

January) reach temperatures as high as 43.2ºC (January 2016) (Bezuidenhout *et al*., 2015; ARC, 2016). Similar to the MNP, the NGR also receives predominantly summer rainfall. However, this occurrence is mainly influenced by subtropical anticyclones. The average annual rainfall is 638 mm, with heaviest rains occurring in middle to late summer (January and February) (Kyle & Marneweck, 1996). Mean temperatures recorded in the summer is 21.9ºC with maximum temperatures reaching above 40ºC. Summers are hot and winters are usually mild to warm. The BDNR is situated in a summer rainfall area with an average rainfall of 649 mm. The summer temperatures range from 22ºC to 34ºC; while winter temperatures are between 2ºC and 20ºC (Koch, 1975).

Riet River

The Riet River forms part of the Modder-Riet system and is a tributary of the Vaal River (Van Rensburg *et al*., 2011). After the confluence with its main tributary, the Modder River, the Riet River continues to flow westwards to meet the Vaal. The Riet River (300 km in length) originates in the close vicinity of the town Smithfield, Free State and has a confluence with the Vaal River upstream from the town Douglas, Northern Cape (Morris, 2002). The Riet River runs through the MNP. The MNP was established in 2007. To the best of our knowledge, to date no research on the aquatic fauna diversity in the Riet River within the park has been done. Sampling on the Riet River was conducted at one site (Fig. 2-1A): Site 1 (28°59'59.5"S, 24°28'50.0"E) (Fig. 2-2A).

Phongolo River

The Phongolo River forms part of the largest and most diverse floodplain system in South Africa (Kok, 1980; Rossouw, 1985). The Phongolo floodplain system comprises of several aquatic biota including macro-invertebrates, fish species and numerous migratory birds which use the floodplain as temporary breeding and feeding habitat (Heeg & Breen, 1982, 1994; Whittington *et al*., 2013). The ecological value of the Phongolo floodplain was first evaluated with emphasis on the adverse effects caused by the construction of the Pongolapoort Dam in 1973 on fish spawning (Phélines *et al*., 1973; Dube *et al*., 2015). According to Kimberg *et al*. (2014), 46 fish species have been recorded in the floodplain, making it the highest fish diversity region in South Africa (Heeg & Breen, 1982). Moreover, half of these fish species have their southernmost limit of distribution (Kok, 1980). The NGR is considered to be the most diverse birdlife site in South Africa with 430 bird species reported.

Sampling was conducted at two sites on the Phongolo River (Fig. 2-1B): Site 1 (26°55'46.7"S 32°19'29.7"E) (Fig. 2-2B) and Site 2 (26°52'57.81"S, 32°18'41.01"E) (Fig. 2-2C).

Phongolo River: Lake Nyamithi

Lake Nyamithi is the second largest semi-permanent floodplain pan/lake in the Phongolo floodplain system in terms of surface area. It is well known for housing the highest density of *Crocodylus niloticus* (Nile crocodile) in the NGR (Calverley & Downs, 2014a). The lake is about 4.2 km long and is 700 metres at its widest point (Calverley & Downs, 2014b). Water levels vary during summer and winter periods, reaching up to 5 metre (summer) and below 1 metre (winter) in depth (Pooley, 1982). During the periods with low water levels, the Nyamithi exhibits high salinities. Lake Nyamithi is also artificially barraged at the downstream end (Kyle & Marneweck, 1996). Sampling on Nyamithi Lake was conducted at one site (Fig. 2-1B): Site 1 (26°53'35"S, 32°17'35"E) (Fig. 2-2D).

Usuthu River: Shokwe Pan

Shokwe is classified as a riverine system with a closed drainage system, i.e. lacking a water outlet (Whittington *et al*., 2013). The Shokwe Pan receives water either during high rainfall seasons or when flooding occurs from the Usuthu River that runs through Swaziland (Whittington *et al*., 2013). Shokwe Pan forms part of the wetland floodplain system of the NGR and functions as a reservoir and nursery for fish on which the rest of the Phongolo and Rio Maputo (in Mozambique) wetland system depends on for restocking (Naguran, 2002). According to the Ramsar Convention Secretariat (2011), Shokwe Pan is of international importance based on the regularity with which it support a wide variety of water birds in large quantities and the proportions of breeding or migratory populations of species (Davies, 1994; Ramsar Convention Secretariat, 2011). Sampling in Shokwe Pan was conducted at one site (Fig. 2-1B): Site 1 (26°51'49.5"S, 32°12'54.5"E) (Fig. 2-2E).

Mooi River: Boskop Dam

Boskop Dam, constructed in 1959, is an earth-fill type dam that falls within the Mooi River sub catchment in the Mooi River catchment within the Upper Vaal Water Management Area and is located near Potchefstroom, North West Province. Boskop Dam was developed for irrigation and domestic purposes. The dam wall is 18 metres in height, 1,320 metres in length and reaches a total capacity of 21 000 000 m^3 (DWA, 2015). The dam has a lot of areas with vegetation, providing habitat and food resources for various fish species. Recreational activities (boats) for communities in the surrounding areas cause increased turbidity which leads to higher levels of total dissolved solids and nutrients in the water body and therefore higher susceptibility to eutrophication. Sampling on Boskop Dam was conducted at one site (Fig. 2-1C): Site 1 (26°33'58"S, 27°07'16"E) (Fig. 2-2F).

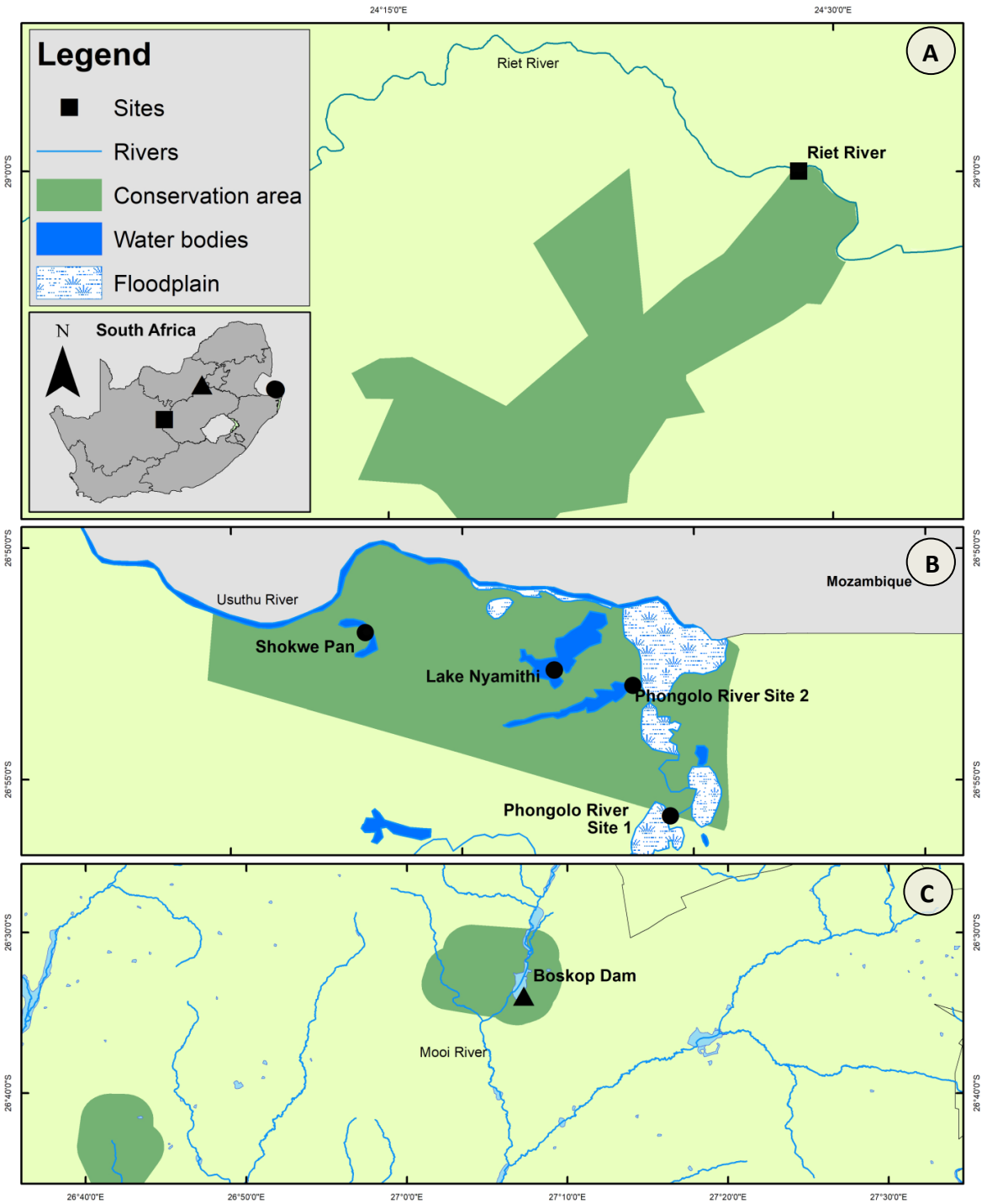


Figure 2-1: Map illustrating the sampling sites on A, Riet River in the Mokala National Park; B, Phongolo River (Site 1, Site 2 and Lake Nyamithi) and Usuthu River (Shokwe Pan) in the Ndumo Game Reserve; C, Mooi River (Boskop Dam) in the Boskop Dam Nature Reserve, South Africa.

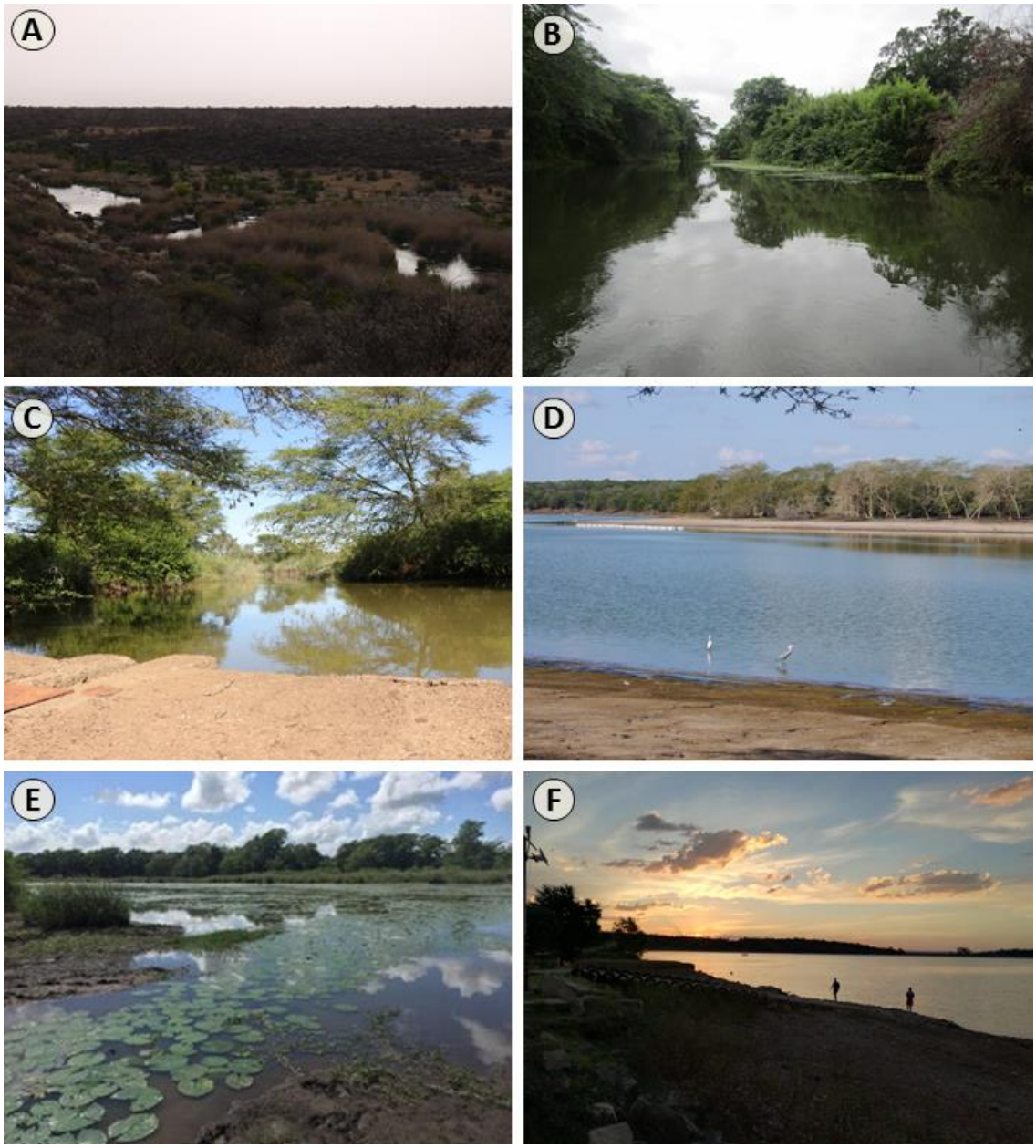


Figure 2-2: Sampling sites. A, Riet River; B, Phongolo River Site 1; C, Phongolo River Site 2; D, Phongolo River (Lake Nyamithi); E, Usuthu River (Shokwe Pan); F, Mooi River (Boskop Dam).

2.3 Selection of fish species

Currently, there are almost 50 fish species present in the Phongolo River, almost 20 fish species in the Mooi River (BDNR) and ten fish species in the Riet River (Barkhuizen, 2018). Fishes present in these systems have different feeding habits and occur in different water columns within the various water bodies. There are five ecomorphological groups in which fishes are found within a system namely, (i) group A: the open and mid water fast swimmers; (ii) group B: midwater slow swimmers; (iii) group C: surface feeders; (iv) group D: bottom dwellers; and (v) group E: dense vegetation and rocky habitat dwellers (Skelton, 2001; Ramberg *et al*., 2006). Representatives from each group, based on biology, behaviour, and habitat distribution, were selected at random

in order to identify which species may be infected with metacercariae of *Diplostomum*. More biological and ecological information on fish species selected for sampling are summarised in Table 2-1. All drawings were adapted from Skelton (2001).

Table 2-1: Summary of the data on biology of fish species examined during the study.

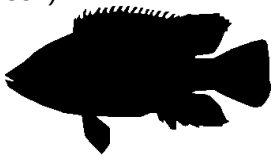
Host species	Habitat	Diet
Alestidae		
Brycinus imberi (Peters, 1852) 	**Ecomorphological group A:** Larger rivers, floodplain pans and lagoons and can be found in shallow, sheltered water of swampy bays	Aquatic and terrestrial invertebrates, various seeds, and plant material as available
Anguillidae		
Anguilla labiata (Peters, 1852) 	**Ecomorphological group B:** Adapted to marine, freshwater, brackish, demersal, and catadromous environments	Adult fish prey on fish, including trout in the streams of the eastern highlands of Zimbabwe
Centrarchidae		
Micropterus salmoides (Lacepède, 1802) 	**Ecomorphological group B:** Prefer freshwater and benthopelagic environments. Largemouth black bass can be found in lakes, ponds, swamps, backwaters, and pools of creeks, and even in small or large rivers. They prefer clear, standing, or quiet waters with over-grown backs of vegetation	Mainly piscivorous; feeds on crabs, frogs, snakes, and even small mammals
Cichilidae		
Coptodon rendalli (Boulenger, 1897) 	**Ecomorphological group B:** Freshwater, brackish and benthopelagic environments and prefer well-vegetated areas along backwaters, floodplains, and swamps	Juveniles feed on plankton where adults feed on leaves, stems, algae, vegetative detritus, insects, and crustaceans
Oreochromis mossambicus (Peters, 1852) 	**Ecomorphological group B:** Freshwater, brackish, benthopelagic and amphidromous environments; found every area except fast-flowing waters, prefers lentic waters	Feed on algae (mostly diatoms) and detritus, but larger fish may feed on invertebrates
Pseudocrenilabrus philander (Weber, 1897) 	**Ecomorphological group B:** Prefer a wide variety of habitats such as flowing waters, lakes, and isolated sinkholes; prefers vegetated areas (freshwater, benthopelagic environments)	Prey on insects, shrimps, and even small fish

Table 2-1 (continued)

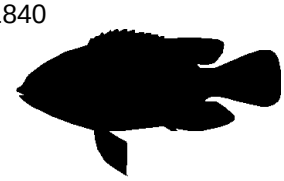
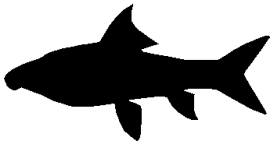
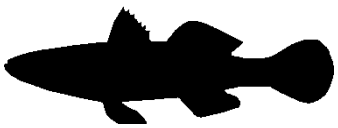
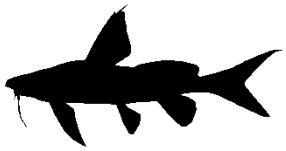
Host species	Habitat	Diet
Tilapia sparrmanii Smith, 1840 	**Ecomorphological group B:** Freshwater, benthopelagic and potamodromous environments and are tolerant to a wide habitat range	Omnivorous; feeding on available foods including algae, soft plants, small invertebrates such as insects and even small fish
Clariidae		
Clarias gariepinus (Burchell, 1822) 	**Ecomorphological group D:** Freshwater, benthopelagic and potamodromous environments and can survive in almost any habitat but favours floodplains, large sluggish rivers, lakes, and dams	Omnivorous. Preys or scavengers on any food source available including fish, birds, frogs, small mammals, reptiles, snails, crabs, shrimps, insect, other invertebrates, plant matter and may even strain fine plankton if necessary
Cyprinidae		
Cyprinus carpio Linnaeus, 1758 	**Ecomorphological group D:** Favours large water bodies with slow-flowing water and soft bottom sediments	Omnivorous, taking a wide range of plant and animal matter such as aquatic insects, crustaceans, annelids, molluscs, tree seeds, wild rice, aquatic plants, and algae; also inclined to eat the spawn of other fish and own eggs
Labeo capensis (Smith, 1841) 	**Ecomorphological group D:** Inhabits a variety of environments such as still, vegetated backwaters, prefers open flowing waters of rocky channels of large rivers and they also thrive in large impoundments	Grazers from firm surfaces of rocks and plants; mainly feed on algae and detritus from the substratum
Labeo congoro Peters, 1852 	**Ecomorphological group D:** Inhabits freshwater, benthopelagic, potamodromous environments with strong flowing rocky stretches of perennial rivers	Grazing of algae and “Aufwuchs” from rocks and firm surfaces such as the backs of hippos
Labeobarbus aeneus (Burchell, 1822) 	**Ecomorphological group D:** Prefers clear-flowing waters of large rivers with sandy or rocky substrates; also found in large dams	Omnivorous depending on the available food, with benthic invertebrates such as bivalve molluscs, vegetation, algae, and detritus forming the major food of the species
Gobiidae		
Glossogobius giuris (Hamilton-Buchanan, 1822) 	**Ecomorphological group B:** Found in marine, freshwater, brackish, benthopelagic and amphidromous environments, however, they are mainly found in freshwater and estuaries; inhabit mostly sandy or turbid streams with gravel, sand and rocky bottoms; also present in backwater habitats and floodplain pans	Diet of juveniles includes bottom-living invertebrates while larger individuals prey on fish and tadpoles

Table 2-1 (continued)

Host species	Habitat	Diet
Mochokidae		
Synodontis zambezensis Peters, 1852 	**Ecomorphological group D:** Freshwater and benthopelagic environments and pools and slow-flowing reaches of perennial and seasonal rivers; prefer riverine habitats to floodplains; bottom-dwelling organisms that shelters in holes or crevices or under logs, frequently in an upside-down position	Active at night and feeds on detritus and plant matter such as seeds as well as small invertebrates like insects and snails, and will scavenge readily
Mormyridae		
Marcusenius macrolepidotus (Peters, 1852) 	**Ecomorphological group E:** Inhabits freshwater, demersal and potamodromous environments and prefers well-vegetated (with muddy bottom) habitats in rivers and floodplains; forms shoals that move inshore after dark	Feeds on a wide range of invertebrates (with preference to midge pupae and mayfly larvae).
Petrocephalus wesselsi Kramer & van der Bank, 2000 	**Ecomorphological group E:** Fish prefer freshwater, pelagic environments and inhabits fast-flowing perennials rivers that have dense subtropical or tropical vegetation at the borders	Food resources include insect larvae and other small invertebrates
Schilbeidae		
Schilbe intermedius Rüppell, 1832 	**Ecomorphological group B:** Prefer freshwater, pelagic and potamodromous environments; these fish form shoals in mid-water or occasionally surface waters of shallow, slow-flowing open waters where emergent or submerged vegetation is available	Mostly active at night when feeding occurs; food resources include fish, insect, shrimps, molluscs, algae, bottom-dwelling planktonic organisms and plant seeds and fruits

The distribution of members of *Diplsotomum* in ecosystems depends on the presence of their intermediate (snails and fish) and definitive hosts (birds). Along with selection of fish species for the present research project, the list of bird species reported in the study areas were analysed. A total of 21 piscivorous bird species from 8 families were identified as potential definitive hosts with 15 species in MNP, 20 species in NGR and 15 species in BDNR. The detailed list of birds in each sampling area is compiled in Table 2-2 (Marnewick *et al*., 2015; Lepage & Warnier, 2014; available at https://avibase.bsc-eoc.org/checklist.jsp?region=ZA).

Table 2-2: List of the bird species - potential definitive hosts for *Diplostomum* in study areas.

Bird Host Species	Common name	MNP	NGR	BDNR
Accipitridae				
Haliaeetus vocifer (Daudin,1800)	African Fish Eagle	–	–	+
Alcedinidae				
Ceryle rudis Linnaeus, 1758	Pied Kingfisher	+	+	+
Megaceryle maxima (Pallas, 1769)	Giant Kingfisher	+	+	+
Anhingidae				
Anhinga rufa (Daudin,1802)	African Darter	+	+	+
Ardeidae				
Ardea alba Linnaeus, 1758	Great Egret	+	+	+
Ardea cinerea Linnaeus, 1758	Gray Heron	+	+	+
Ardea goliath Cretzschmar, 1829	Goliath Heron	+	+	+
Ardea intermedia (Wagler, 1829)	Yellow-billed Egret	+	+	+
Ardeola rufiventris (Sundevall, 1850)	Rufous-bellied Heron	–	+	–
Egretta garzetta (Linnaeus, 1766)	Little Egret	+	+	+
Gorsachius leuconotus (Wagler, 1827)	White-backed Night Heron	–	+	–
Nycticorax nycticorax (Linnaeus, 1758)	Black crowned Night Heron	+	+	–
Laridae				
Chlidonias hybrida (Pallas, 1811)	Whiskered Tern	+	+	+
Chlidonias leucopterus (Temminck, 1815)	White-winged Tern	+	+	+
Chroicocephalus cirrocephalus (Vieilot, 1818)	Gray-hooded Gull	+	+	+
Hydroprogne caspia (Pallas, 1770)	Caspian Tern	–	+	–
Pandionidae				
Pandion haliaetus (Linnaeus, 1758)	Osprey	+	+	+
Phalacrocoracidae				
Microcarbo africanus (Gmelin, 1789)	Reed Cormorant	+	+	+
Phalacrocorax capensis (Sparrman, 1788)	Cape Cormorant	+		
Phalacrocorax carbo (Linnaeus, 1758)	Great Cormorant	+	+	+
Strigidae				
Scotopelia peli (Bonaparte, 1850)	Pel's Fishing Owl	–	+	–

+, presence of bird species in area; –, absence of bird species in area

2.4 Collection of fish

Prior to the present study, metacercariae of *Diplostomum* were detected and sampled by Dr. Olena Kudlai during once-off surveys in the MNP in the Northern Cape (September 2016) and the NGR in KwaZulu-Natal (November 2017). Specimens of *Diplostomum* were collected from one *T*. *sparrmanii* (MNP), one *O*. *mossambicus* (Phongolo River Site 2), one *A*. *labiata* (Phongolo River Site 1) and three *S*. *zambezensis* (Phongolo River Site 1). This material was used in the

present research project. In addition to the specimens collected previously, the author with the help of colleagues from the NWU Water Research Group (WRG) collected fishes during expeditions to the NGR in KwaZulu-Natal (2018) and BDNR in the North West Province (2019). A total of 160 fish belonging to 17 species and 10 families were sampled in the Mooi, Phongolo, Usuthu and Riet Rivers (Table 2-3). Sampling was carried out under the permits OP 1582/2018 (Appendix C), NW 8065/03/2019 (Appendix D) and ethics number NWU-00160-18-S5 (Appendix E). Twenty-six fish were collected from the Riet River (28°59'60"S, 24°28'50"E), 13 fish from the Usuthu River (Shokwe Pan, 26°51'50"S, 32°12'55"E), a single fish from the Phongolo River (Lake Nyamithi, 26°53'35"S, 32°17'35"E), 93 fish from the Phongolo River [Site 1 (26°55'47"S, 32°19'30"E), Site 2 (26°52'58"S, 32°18'41"E)], and 27 fish from the Mooi River (Boskop Dam, 26°33'58"S, 27°07'16"E).

Table 2-3: Summary of fishes collected during the study.

Fish species	Mooi River: Boskop Dam	Phongolo River Site 1	Phongolo River Site 2	Phongolo River: Lake Nyamithi	Usuthu River: Shokwe Pan	Riet River	Total
Alestidae							
Brycinus imberi (Peters, 1852)	–	8	–	–	–	–	8
Anguillidae							
Anguilla labiata (Peters, 1852)	–	4	–	–	–	–	4
Centrarchidae							
Micropterus salmoides (Lacepède, 1802)	6	–	–	–	–	–	6
Cichilidae							
Coptodon rendalli (Boulenger, 1897)	–	6	–	–	–	–	6
Oreochromis mossambicus (Peters, 1852)	–	12	2	1	–	–	15
Pseudocrenilabrus philander (Weber, 1897)	10	–	–	–	–	4	14
Tilapia sparrmanii Smith, 1840	11	2	–	–	–	6	19
Clariidae							
Clarias gariepinus (Burchell, 1822)	–	–	–	–	5	5	10
Cyprinidae							
Cyprinus carpio Linnaeus, 1758	–	–	–	–	–	1	1
Labeo capensis (Smith, 1841)	–	–	–	–	–	3	3
Labeo congoro Peters, 1852	–	1	–	–	–	–	1
Labeobarbus aeneus (Burchell, 1822)	–	–	–	–	–	7	7
Gobiidae							
Glossogobius giuris (Hamilton-Buchanan, 1822)	–	1	–	–	–	–	1

Table 2-3 (continued)

Fish species	Mooi River: Boskop Dam	Phongolo River Site 1	Phongolo River Site 2	Phongolo River: Lake Nyamithi	Usuthu River: Shokwe Pan	Riet River	Total
Mochokidae							
Synodontis zambezensis Peters, 1852	–	41	–	–	–	–	41
Mormyridae							
Marcusenius macrolepidotus (Peters, 1852)	–	6	–	–	5	–	11
Petrocephalus wesselsi Kramer & van der Bank, 2000	–	1	–	–	3	–	4
Schilbeidae							
Schilbe intermedius Rüppell, 1832	–	9	–	–	–	–	9

Fishes were collected using fyke nets (NGR), electro-fishing (MNP), rod and reel (MNP; NGR), cast nets (NGR) and seine nets (BDNR) (Fig. 2-3 A-E). Fyke nets were checked twice a day (early morning and late afternoon) for the presence of fish. The collected fishes were transported to the field lab in a cool box, filled with water collected at the sampling site and aerated with battery-powered pumps. In the field lab, fish were euthanised by cranial pithing and spinal severance. Each fish was provided with a unique number and documented by photographs. The total length (TL) and standard length (SL) of fishes were measured. Thereafter fishes were identified using a freshwater field guide (Skelton, 2001). Fish taxonomy follow FishBase (Froese & Pauly, 2019).

Site selection prior to fish sampling are important in order to establish which species of fish occur within the selected system. Moreover, the biology and ecology of the fish species occurring within these systems are important in order to know which habitat they occupy and when they are active (i.e. diurnal or nocturnal). Knowledge on the behaviour of these fishes will result in the correct selection of sampling methods, ensuring successful sampling outcomes.

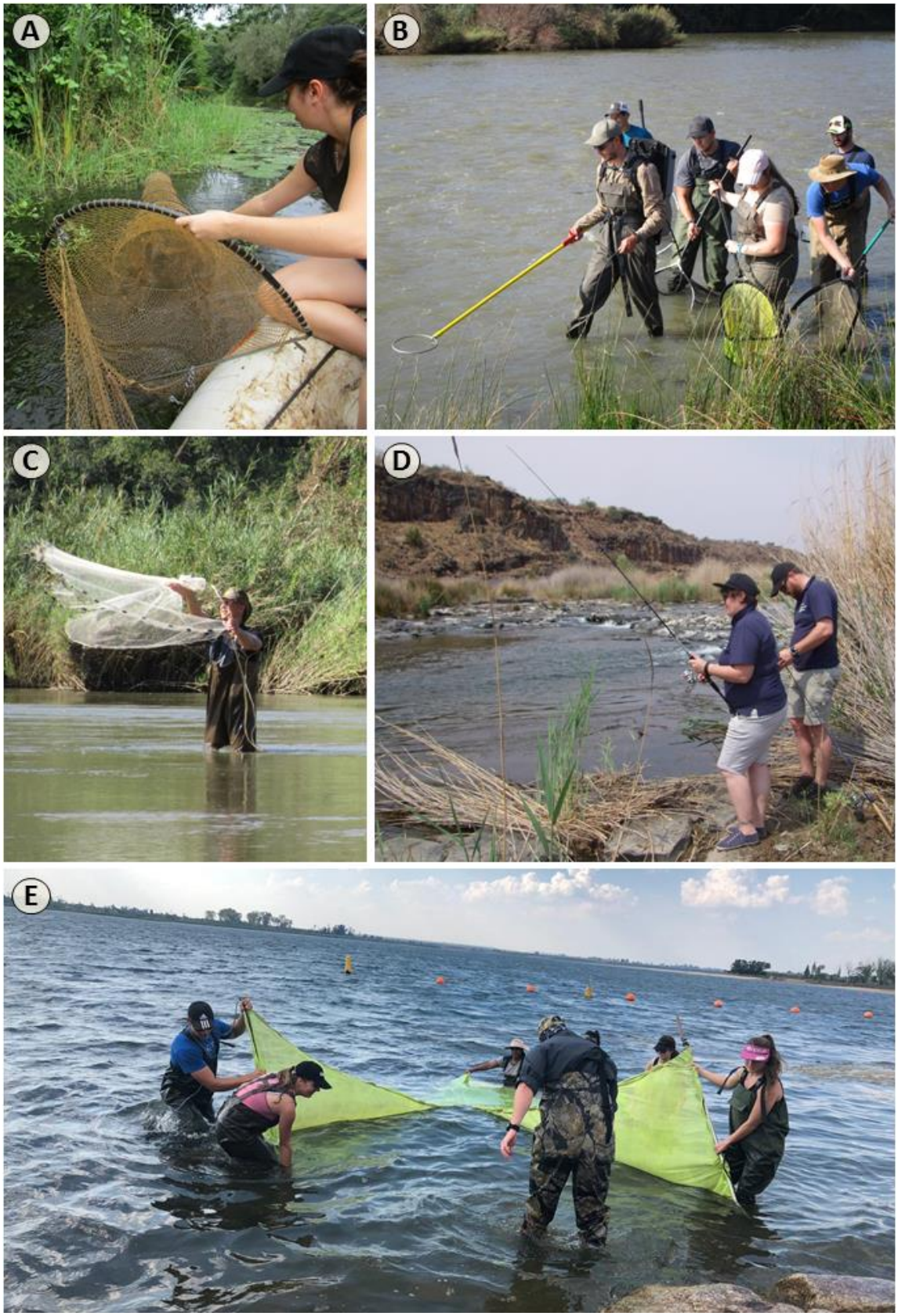
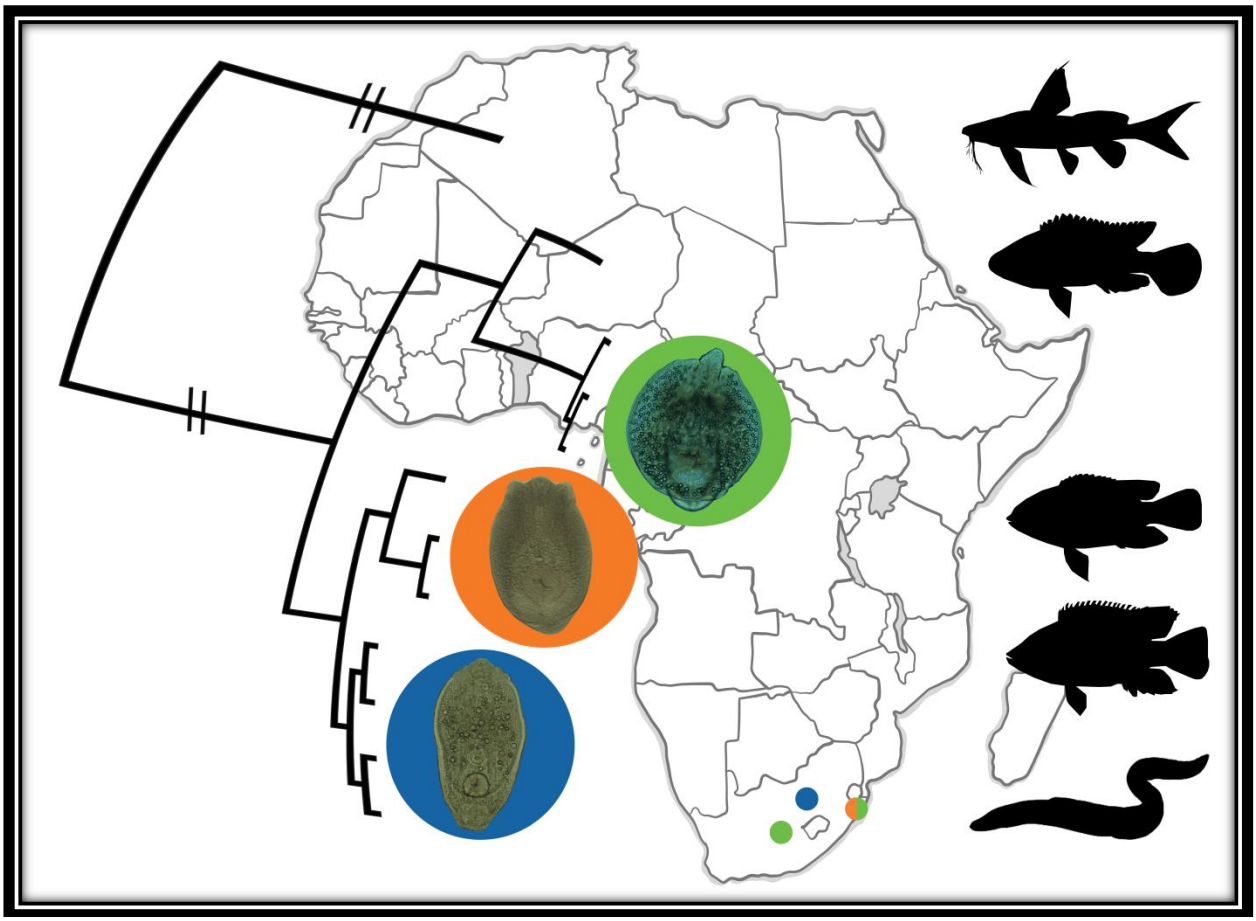


Figure 2-3: Fish collection methods. A, Fyke nets; B, Electro-fishing; C, Cast nets; D, Rod and Reel; E, Seine nets.

Chapter 3: Diversity of *Diplostomum* in South Africa



CHAPTER 3: DIVERSITY OF *DIPLOSTOMUM* IN SOUTH AFRICA

3.1 Introduction

The type-genus *Diplostomum* von Nordmann, 1832 (Digenea: Diplostomidae) represents a large group of widely distributed pathogenic parasites that, as adults, infect a wide variety of fish-eating birds (Niewiadomska, 2002). Metacercarial stages in the eye lenses of freshwater fishes are regarded as pathogens to their hosts and are the focus of numerous ecological, behavioural and evolutionary studies (Ballabeni & Ward, 1993; Owen *et al*., 1993; Seppäla *et al*., 2004; Kalbe & Kurtz, 2006; Seppäla *et al*., 2011; Benesh & Kalbe, 2016; Klemme *et al*., 2016). Owing to the extensive studies done by Shigin (1986, 1993), Niewiadomska (1986, 2002, 2010), Georgieva *et al*. (2013) and other researchers, great progress has been made in attempt to clarify the taxonomic status of *Diplostomum*. However, limitations in the development of strategies to help resolve the uncertain taxonomic status of species within this genus persist due to: (i) difficulties in clarifying the validity (due to differences in opinion between taxonomists) of species within this genus; (ii) reports or descriptions based on a single life cycle stage or only two life cycle stages for a species without providing adult descriptions; (iii) difficulties in identifying/delineating morphologically indistinguishable species present within the genus (most commonly when applying morphological analysis only on the larval stages), (iv) challenges in the elucidation of life cycles due to difficulties in linking life cycle stages; (v) misidentifications represented by previous reports based on morphological characteristics as sole method used to identify species; as well as (vi) studies only focussing on molecular identification of species of *Diplostomum* and not providing any morphological descriptions thus there is no data available for future morphological comparisons (Locke *et al*., 2010a, b; Georgieva *et al*., 2013; Locke *et al*., 2015).

In Africa, this genus is vastly unexplored and reliable data is scarce due to a lack of dedicated studies and a general lack of resources, and expertise (Chibwana *et al*., 2013). Currently, six identified and three unidentified species of *Diplostomum* have been reported from freshwater fishes in Africa of which two of unidentified species have been reported from South Africa. Two species of *Diplostomum*: *Diplostomum heterobranchii* Wedl, 1861 and *D. magnicaudum* have been reported from Egypt (El-Naffar, 1979; Khalil & Polling, 1997). *Diplostomum heterobranchii* was reported from the cranial cavity of *C. gariepinus* and *D. magnicaudum* was found encysted in the muscles of *Oreochromis niloticus* (Linnaeus, 1758). Adult worms of *D. magnicaudum* were also obtained from the small intestines of both naturally and experimentally infected hosts *Gallinula chloropus chloropus* (Linnaeus, 1758) (natural hosts) and *Columba livia* Gmelin, 1789 (experimentally infected) in Egypt (El-Naffar, 1979; Khalil & Polling, 1997).

The most recent report of *Diplostomum* species in Africa, is that of Zhokhov (2014) from Ethiopia, which includes four named species: *Diplostomum garrae* Zhokhov, 2014 reported from

the eye lens of *Garra dembecha* Getahun and Stiassny, 2007; *Diplostomum longicollis* Zhokhov, 2014 from the eye lens of *Enteromius humilis* (Boulenger, 1902), *G*. *dembecha*; *Diplostomum montanum* Zhokhov, 2014 from eye lens of *E*. *humilis*, *G*. *dembecha*, *Labeobarbus beso* (Rüppell, 1835), *L*. *gorgorensis* (Bini, 1940); and *Diplostomum tilapiae* Zhokhov, 2014 from the vitreous humour of eye of *O*. *niloticus* (Zhokhov, 2014).

In South Africa, the first report on metacercariae of *Diplostomum* from freshwater fishes was by Prudhoe and Hussey in 1977. The authors found *Diplostomum* sp. type I in thin-walled cysts within the mesenteries and *Diplostomum* sp. type II unencysted in the cranial cavity of *C*. *gariepinus* from the Transvaal area. All of the above mentioned studies based their reports on morphological examination only and the taxonomic identity of these identified species within the genus *Diplostomum* still requires critical revision.

Of the nine species of *Diplostomum* morphologically identified from freshwater fish in Africa, four species have been reported from a single host *C*. *gariepinus* (Clariidae), with the remaining species of *Diplostomum* reported from fish belonging to the families Cichlidae and Cyprinidae. Based on molecular evidence, only *Synodontis nigrita* Valenciennes, 1840 (Mochokidae) have been reported as host for an unidentified species, *Diplostomum* sp. from Nigeria (Chibwana *et al*., 2013). These reports from only seven fishes belonging to four families (Cichlidae, Clariidae, Cyprinidae and Mochokidae) are surprising especially since there are over 3200 freshwater fishes in Africa and almost 190 species that have been reported in the various freshwater ecosystems in South Africa (Snoeks *et al*., 2011). This high diversity of fish species is highly likely to mirror an even higher parasite diversity, but requires dedicated studies focusing on the parasite biodiversity occurring in other fish hosts (not only the hosts listed above) within these systems. Therefore, this study was performed to assess the diversity of *Diplostomum* parasitising freshwater fishes of three provinces in South Africa. This was done by the combined application of morphological and multi-locus molecular analyses (partial 28S rRNA and mt *cox*1 genes; and the entire ITS1-5.8S-ITS2 region) in order to get a better understanding on the true diversity of species from this genus present in South Africa. Detailed morphological descriptions and molecular sequences generated for the newly discovered species of *Diplostomum* are provided below.

3.2 Materials & Methods

Collection of fish

Fish sampling was conducted following methods described in Chapter 2: General materials and methods, Section 2.4. The vitreous humour, retina and lens of eye and brain of 160 fish belonging to 17 species and 10 families (refer Section 2.4, Table 2-3) were examined for the presence of metacercariae of the genus *Diplostomum*.

Collection of metacercariae

The brain and eyes of the fish were dissected, thereafter the brain, retina, vitreous humour and lens were placed in 0.9% saline solution and examined using a Nikon dissecting microscope for the presence of digenean parasites (Fig. 3-1). The parasites were removed from the eye lens using needles, forceps, and pipettes. All metacercariae were collected and counted.

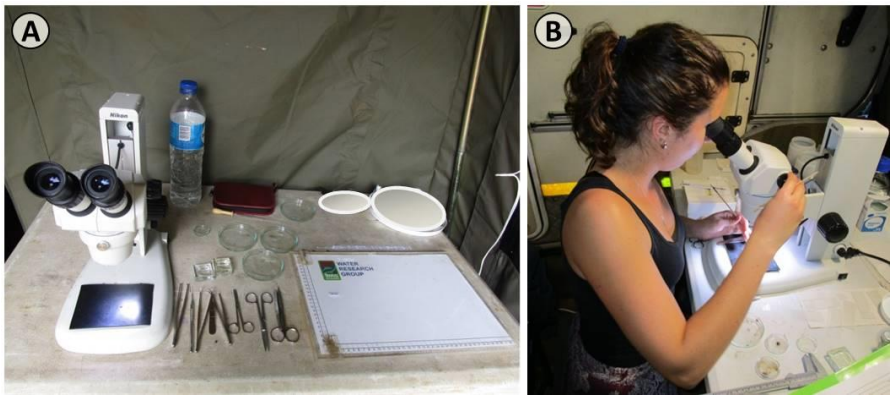


Figure 3-1: Trematode collection methods. A, Field lab station setup; B, Examination of eyes of fish for the presence of metacercariae under a dissecting microscope.

Morphological analysis

The morphology of the metacercariae selected for sequencing was initially studied using live specimens (if possible); these were then transferred to molecular grade ethanol and re-examined. During the re-examination of the fixed representative metacercariae, the specimens were rehydrated in dH_2O and a temporary wet mount was made for each specimen. Subsequently, a series of photomicrographs were taken of all selected specimens with a digital camera on a Nikon Eclipse N*i* microscope using the NIS-Elements BR Camera analysis software. All measurements were taken from the digital images with the aid of ImageJ (Available from https://imagej.nih.gov/ij/download.html). Twenty morphometric variables were measured from the digital images of live and fixed metacercariae (Fig. 3-2A, Table 3-1). In addition, the number of excretory granules was counted (Fig. 3-2B). The metrical data is presented as the range followed by the mean of the measurements taken in parentheses. All measurements are given in micrometres.

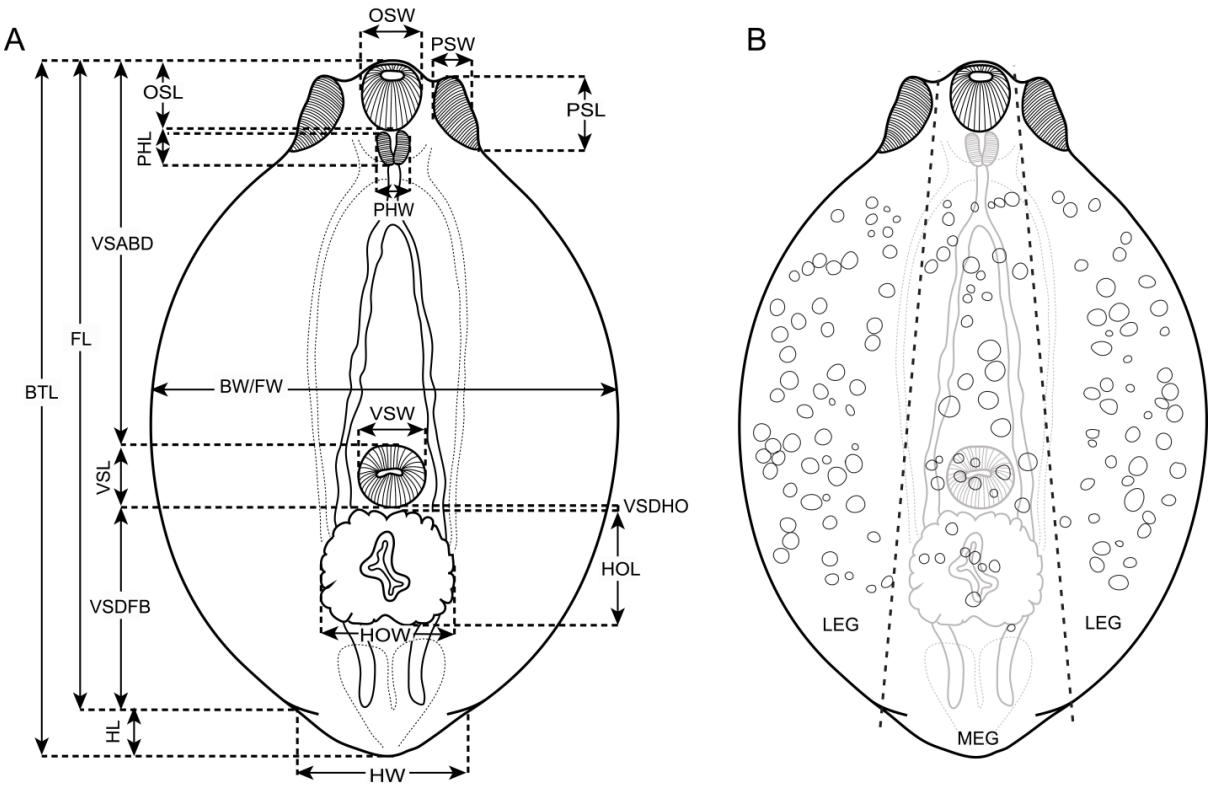


Figure 3-2: Diagram of measurements of metacercariae. A, Measurements of morphological characters. Abbreviations: BTL, Total body length; BW, Body width; FL, Forebody length; FW, Forebody width; HL, Hindbody length; HW, Hindbody width; OSL, Oral sucker length; OSW, Oral sucker width; PSL, Pseudosucker length; PSW, Pseudosucker width; PHL, Pharynx length; PHW, Pharynx width; VSL, Ventral sucker length; VSW, Ventral sucker width; HOL, Holdfast organ length; HOW, Holdfast organ width; VSDAB, Distance from ventral sucker to anterior extremity of body; VSDPFB, Distance from ventral sucker to posterior margin of forebody; VSDHO, Distance from ventral sucker to holdfast organ. B, Excretory granules. Abbreviations: LEG, Lateral excretory granules; MEG, Median excretory granules.

Table 3-1: The abbreviations of the metrical characters.

Abbreviation	Description
BTL	Total body length
BW	Body width
FL	Forebody length
FW	Forebody width
HL	Hindbody length
HW	Hindbody width
OSL	Oral sucker length
OSW	Oral sucker width
PSL	Pseudosucker length
PSW	Pseudosucker width
PPHL	Prepharynx length
PHL	Pharynx length

Table 3-1 (continued)

Abbreviation	Description
PHW	Pharynx width
VSL	Ventral sucker length
VSW	Ventral sucker width
HOL	Holdfast organ length
HOW	Holdfast organ width
VSDAB	Distance from ventral sucker to anterior extremity of body
VSDPFB	Distance from ventral sucker to posterior margin of forebody
VSDHO	Distance from ventral sucker to holdfast organ

Molecular analysis

Generation of molecular data

Total genomic DNA was extracted using the KAPA Express Extract Kit (Kapa Biosystems, Cape Town, South Africa). The DNA extractions were performed in 92µl volumes and the reaction setup included: 80µl of PCR-grade water, 10µl of 10X KAPA Express extract buffer, 2µl of 1 U/µl KAPA Express extract enzyme and >0.5g of sample tissue. Next, lysis was performed in a thermocycler at 75°C for 10 minutes, followed by enzyme inactivation at 95°C for 7 minutes. After lysis, the samples were vortexed and centrifuged at 12,000 rpm for 5 minutes. A volume of 70µl of supernatant was transferred to a 1.5 ml Graduated microcentrifuge tube and stored at -20°C.

DNA amplifications were performed following the PCR protocols (Fig. 3-3) using forward and reverse primers (Table 3-2) to amplify partial fragments of the cytochrome *c* oxidase subunit 1 (*cox*1) and 28S rRNA genes, and the entire ITS1-5.8S-ITS2 gene cluster.

PCR amplicons were visualised by agarose gel electrophoresis (1% agarose gels stained with GelRed) and sent to a commercial sequencing company (Inqaba Biotechnical Industries (Pty) Ltd. Pretoria, South Africa) for purification and sequencing. Contiguous sequences were assembled and edited using Geneious ver. 11 (Available from http://www.geneious.com) (Biomatters, Auckland, New Zealand).

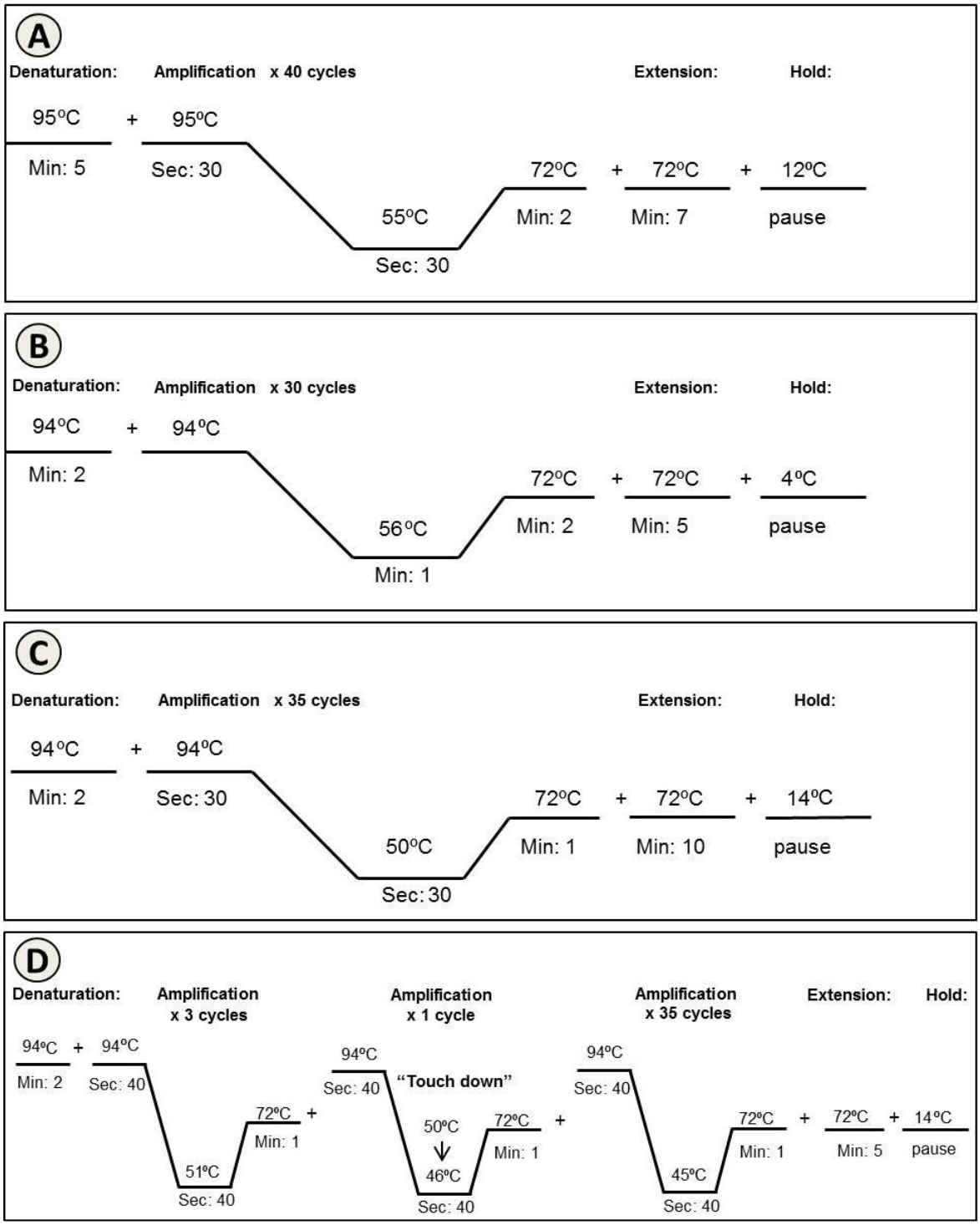


Figure 3-3: PCR thermocycle protocols used for DNA amplification of the three genetic markers. A, 28S rDNA; B, ITS1–5.8S–ITS2; C, *cox*1 (primers from Tkach *et al*., 2000; Galazzo *et al*., 2002; Tkach *et al*., 2003; Moszczynska *et al*., 2009); D, *cox*1 (primers from Van Steenkiste *et al*., 2015).

Table 3-2: Primers used for DNA amplification and sequencing.

Locus	Primer	Sequence	M[a]	Source
28S	Digl2	5'-AAGCATATCACTAAGCGG-3'	A	Tkach *et al*. (2000)
	1500R	5'-GCTATCCTGAGGGAAACTTCG-3'	A	Tkach *et al*. (2003)
	300F (internal)[b]	5'-CAAGTACCGTGAGGGAAAGTTG-3'	–	Littlewood *et al*. (2000)
	ECD2 (internal)[b]	5'-CCTTGGTCCGTGTTTCAAGACGGG-3'	–	Littlewood *et al*. (1997)
ITS1-5.8S-ITS2	D1	5'-AGGAATTCCTGGTAAGTGCAAG-3'	B	Galazzo *et al*. (2002)
	D2	5'-CGTTACTGAGGGAATCCTGGT-3'	B	Galazzo *et al*. (2002)
*cox*1	Plat-diploCOX1F	5'-CGTTTRAATTATACGGATCC-3'	C	Moszczynska *et al*. (2009)
	Plat-diploCOX1R	5'-AGCATAGTAATMGCAGCAGC-3'	C	Moszczynska *et al*. (2009)
	DICE1F	5'-ATTAACCCTCACTAAATTWCNTTR GATCATAAG-3'	D	Van Steenkiste *et al*. (2015)
	DICE11R	5'-TAATACGACTCACTATAGCWGWAC HAAATTTHCGATC-3'	D	Van Steenkiste *et al*. (2015)
	DICE14R	5'-TAATACGACTCACTATACCHACMR TAAACATATGATG-3'	D	Van Steenkiste *et al*. (2015)

[a]M, method (illustrated in Fig. 3-3)

[b]used for sequencing only

Phylogenetic analyses

The 38 newly generated sequences were compared with those ones for *Diplostomum* spp. (143 sequences) available in GenBank using the nucleotide BLAST search analysis and available sequences were downloaded from GenBank (Johnson *et al*., 2008) (see Appendix F). Phylogenetic analyses were performed using separate alignments according to the gene/ region fragment amplified.

The alignments were built with MUSCLE (Edgar, 2004) implemented in Geneious ver. 11 under default parameter values. Sequences for two species of the genus *Tylodelphys* Diesing, 1850, *Tylodelphys mashonensis* Beverly-Burton, 1963 (28S, KF189071) and *Tylodelphys clavata* (von Nordmann, 1832) (ITS, JQ665459; *cox*1, JX986909) were used as outgroups based on the results of the phylogenetic analyses of *Diplostomum* published by Georgieva *et al*. 2013. The *cox*1 dataset was aligned with reference to the amino acid translation, using the trematode mitochondrial code (translation table 21; https://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi#SG21) (Garey & Wolstenholme, 1989; Ohama *et al*., 1990).

Phylogenetic trees were constructed through Bayesian inference and Maximum likelihood analyses (Ronquist *et al*., 2012; Stamatakis, 2006). The best-fitting model (HKY+I+G for 28S dataset; GTR+I+G for ITS and *cox*1 datasets) was estimated prior to analyses using jModelTest 2.1.2 based on the Akaike information criterion (AIC) (Guindon & Gascuel, 2003; Darriba *et al*.,

2012). Bayesian Inference analysis was performed using MrBayes ver. 3.2.6 software and Markov chain Monte Carlo (MCMC) chains were run for 1,000,000 generations on CIPRES Science Gateway ver. 3.3 (Ronquist *et al*., 2012; Miller *et al*., 2010). Maximum likelihood analysis was performed using PhyML ver. 3.0 (Guindon *et al*., 2010) and run on the ATGC bioinformatics platform (http://www.atgc-montpellier.fr/). Nodal support was estimated using a bootstrap value of 100 pseudoreplicates.

Trees were visualised using FigTree ver. 1.4 software (Rambaut, 2012). Results were visualised in Tracer ver. 1.6 (Rambaut *et al*., 2014) to assess convergence and proper sampling and to identify the ‘‘burn-in’’ period. MEGA ver. 6 was used to calculate the pairwise genetic distances (p-distance) and number of nucleotide differences between sequences.

3.3 Results

General observation

Of 160 fishes collected and examined during this study, metacercariae of *Diplostomum* were found in the eye lens of 38 specimens (P = 24%) from five fish species: *A*. *labiata*, *O*. *mossambicus*, *P*. *philander*, *S*. *zambezensis* and *T*. *sparrmanii* (Table 3-3). The overall mean intensity of infection appeared high in *P*. *philander* (3−21 metacercariae per fish) collected in the Mooi River and was low (1−12 metacercariae per fish) in other fish hosts from the Phongolo, Riet and Usuthu Rivers.

Table 3-3: Total number of infections with metacercariae of *Diplostomum* in eye lenses of infected fish hosts.

Fish species	Mooi River: Boskop Dam	Phongolo River Site 1	Phongolo River Site 2	Phongolo River: Lake Nyamithi	Usuthu River: Shokwe Pan	Riet River
Anguillidae						
Anguilla labiata (Peters, 1852)	–	3	–	–	–	–
Cichilidae						
Oreochromis mossambicus (Peters, 1852)	–	–	1	–	–	–
Pseudocrenilabrus philander (Weber, 1897)	9	–	–	–	–	–
Tilapia sparrmanii Smith, 1840	–	–	–	–	–	1
Mochokidae						
Synodontis zambezensis Peters**,** 1852	–	24	–	–	–	–

Molecular identification of metacercariae

A total of 38 novel sequences were generated for 16 isolates during this study: eight sequences for the partial 28S rRNA gene, 16 sequences for the ITS1-5.8S-ITS2 region and 14 sequences for the partial *cox*1 gene (Table 3-4).

Table 3-4: Summary data for the sequences of *Diplostomum* spp. obtained during this study.

Species	Isolate	Host	Locality
Diplostomum sp.	DSZ6P	*Synodontis zambezensis*	PR S1
Diplostomum sp.	DSZ7P	*Synodontis zambezensis*	PR S1
Diplostomum sp.	DTS1R	*Tilapia sparrmanii*	RR
Diplostomum sp. 14	DAB1P	*Anguilla labiata*	PR S1
Diplostomum sp. 14	DAB2P	*Anguilla labiata*	PR S1
Diplostomum sp. 14	DOM1P	*Oreochromis mossambicus*	PR S2
Diplostomum sp. 14	DSZ1P	*Synodontis zambezensis*	PR S1
Diplostomum sp. 14	DSZ2P	*Synodontis zambezensis*	PR S1
Diplostomum sp. 14	DSZ3P	*Synodontis zambezensis*	PR S1
Diplostomum sp. 14	DSZ4P	*Synodontis zambezensis*	PR S1
Diplostomum sp. 14	DSZ5P	*Synodontis zambezensis*	PR S1
Diplostomum sp. 16	DPP1B	*Pseudocrenilabrus philander*	MR
Diplostomum sp. 16	DPP2B	*Pseudocrenilabrus philander*	MR
Diplostomum sp. 16	DPP3B	*Pseudocrenilabrus philander*	MR
Diplostomum sp. 16	DPP4B	*Pseudocrenilabrus philander*	MR
Diplostomum sp. 16	DPP5B	*Pseudocrenilabrus philander*	MR

Abbreviations: PR S1, Phongolo River Site1; RR, Riet River; PR S2, Phongolo River Site2; MR, Mooi River.

Partial 28S rDNA gene

The 28S rDNA dataset (1230 nt) comprised of six novel sequences obtained during this study and three sequences for *Diplostomum* spp. downloaded from GenBank (Appendix F). Both, Bayesian inference (BI) and maximum likelihood (ML) analyses based on the 28S rDNA alignment resulted in consensus trees with similar topologies (Fig. 3-4). The three newly-generated sequences for the isolates DAB1P, DSZ1P and DOM1P (Table 3-4) were identical and clustered with *D*. *spathaceum* (KR269765) reported from *Larus ridibundus* (L., 1766) from the Czech Republic (Brabec *et al*., 2015) with low support in both analyses. No sequence difference between the three isolates of the present study and isolate of *D*. *spathaceum* were found. The sequences of two isolates (DPP3B and DPP4B) obtained from *P*. *philander* (Table 3-4) clustered with the sequence of *D*. *pseudospathaceum* (KR269766) from *L*. *ridibundus* collected in the Czech Republic (Brabec *et al*., 2015) with low support. The genetic divergence between two identical sequences of isolates from the present study and sequence of *D*. *pseudospathaceum* was 0.3% (4 nt). Sequences of the three remaining isolates (DSZ6P, DSZ7P and DTS1R) collected from the eye lenses of *T*. *sparrmanii* and *S*. *zambezensis* (Table 3-4) clustered with *D*. *phoxini* (AY222173) from *P*. *phoxinus* in the United Kingdom (Olson *et al*., 2003). The newly generated

sequences of isolates from this study were identical to each other but differed from the sequence of *D*. *phoxini* by 1.5% (8 nt).

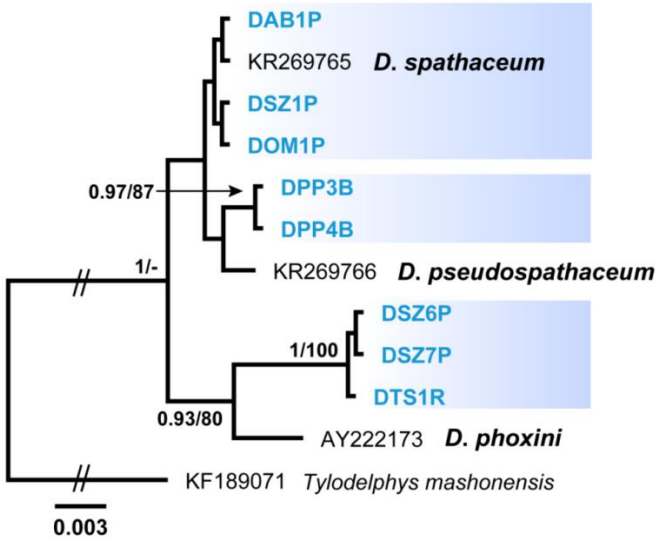


Figure 3-4: Phylogenetic tree for species of *Diplostomum* resulting from Bayesian inference (BI) analysis based on the partial 28S rRNA sequences. Nodal support from BI and ML analyses indicated as BI/ML; only values > 0.90 (BI) and > 70 (ML) are displayed. Scale-bar indicates the expected number of substitutions per site. Sequences generated in this study are in bold and indicated by blue rectangles. Codes with isolate information for newly generated sequences are provided in Table 3-4.

ITS1-5.8S-ITS2 region

Sixteen novel sequences and 42 sequences downloaded from GenBank were used in the ITS1-5.8S-ITS2 alignment (963 nt). The phylogenetic hypothesis resulted from BI and ML analyses demonstrated that the isolates sequenced in the present study clustered into three supported clades (Fig. 3-5). Sequences of the metacercarial isolates collected from *S*. *zambezensis* (DSZ6P and DSZ7P) in the Phongolo River and *T*. *sparrmanii* (DTS1R) in the Riet River (Table 3-4) clustered with the sequence of *Diplostomum* sp. recorded in the lenses of *S*. *nigrita* in Nigeria (Chibwana *et al*., 2013) in a strongly supported clade. The sequence divergence between isolates from the present study and the isolate from *S*. *nigrita* was 0.3% (2 nt). Isolates collected from *A*. *labiata* (DAB1P and DAB2P), *O*. *mossambicus* (DOM1P) and *S*. *zambezensis* (DSZ1P–DSZ5P) in the Phongolo River (Table 3-4), similarly to the results of 28S rDNA analyses, demonstrated close relationships with the isolates of *D*. *spathaceum* (KR149502; KJ726508) from *Radix auricularia* (L., 1758) in Germany (Selbach *et al*., 2015) and *G*. *aculeatus* in Iceland, respectively (Blasco-Costa *et al*., 2014). This clade also included a sequence of unidentified species *Diplostomum* sp. 14 *sensu* Locke *et al*. (2015) (KT186789) recently reported from *Tinca tinca* (L.,

1758) in China (Appendix F). The sequence divergence within the clade was 0.1% (1 nt). The clade consisting of sequences of the isolates recovered from the lenses of *P*. *philander* collected in the Mooi River was recovered as a sister to two species from North America, *D*. *huronense* and *Diplostomum* sp. 4 with no nodal support. Sequences of the isolates from *P*. *philander* were identical and did not match any sequence of *Diplostomum* spp. currently available in GenBank.

Partial *cox*1 gene

For the *cox*1 dataset, three sequences per species/ species-level genetic lineages (the longest possible) reported from different countries/continents were selected. The BI and ML analyses based on *cox*1 alignment (347 nt, 114 sequences) yielded similar phylogenetic hypotheses (Fig. 3-6), but differences from the hypotheses based on nuclear markers, 28S rDNA and ITS1-5.8S-ITS2 were apparent. Novel sequences of the isolates collected from *P*. *philander* (Table 3-4) in the Mooi River (DPP1B–DPP5B) formed a strongly supported clade with the sequences of unidentified species of *Diplostomum*, *Diplostomum* sp. 16 recently reported from *Alburnus caeruleus* Heckel, 1843 in Iraq (Locke *et al*., 2015). No sequence divergence was found between the isolates from the present study and isolate of *Diplostomum* sp. 16 which confirms their conspecificity. Sequences of the metacercarial isolates collected from the three fish species in the Phongolo River (DAB1P, DOM1P, DSZ1P and DSZ3P–DSZ5P) (Table 3-4) clustered together with the two isolates of *Diplostomum* sp. 14 collected from *Channa argus* (Cantor, 1842) and *T*. *tinca* in China (Locke *et al*., 2015) and one isolate of *Diplostomum* sp. 14 collected from *Cyprinion macrostomum* Heckel, 1843 in Iraq (Locke *et al*., 2015) in the strongly supported clade remoted from the clade of *D*. *spathaceum*. The sequence divergence within this clade ranged between 0–3.3% (0–9 nt). Sequences of the three metacercarial isolates from *S*. *zambezensis* (DSZ6P and DSZ7P) and *T*. *sparrmanii* (DTS1R) (Table 3-4), similarly to the results of the ITS1-5.8S-ITS2 analyses, clustered with the isolates of *Diplostomum* sp. from *S*. *nigrita* in Nigeria (Chibwana *et al*., 2013). The sequence divergence ranged between 0–1.1% (0–3 nt) which is considered as intraspecific. The species identification of the metacercarial isolates recovered from the lenses of freshwater fishes in South Africa during this study was based on the results of the *cox*1 gene analyses.

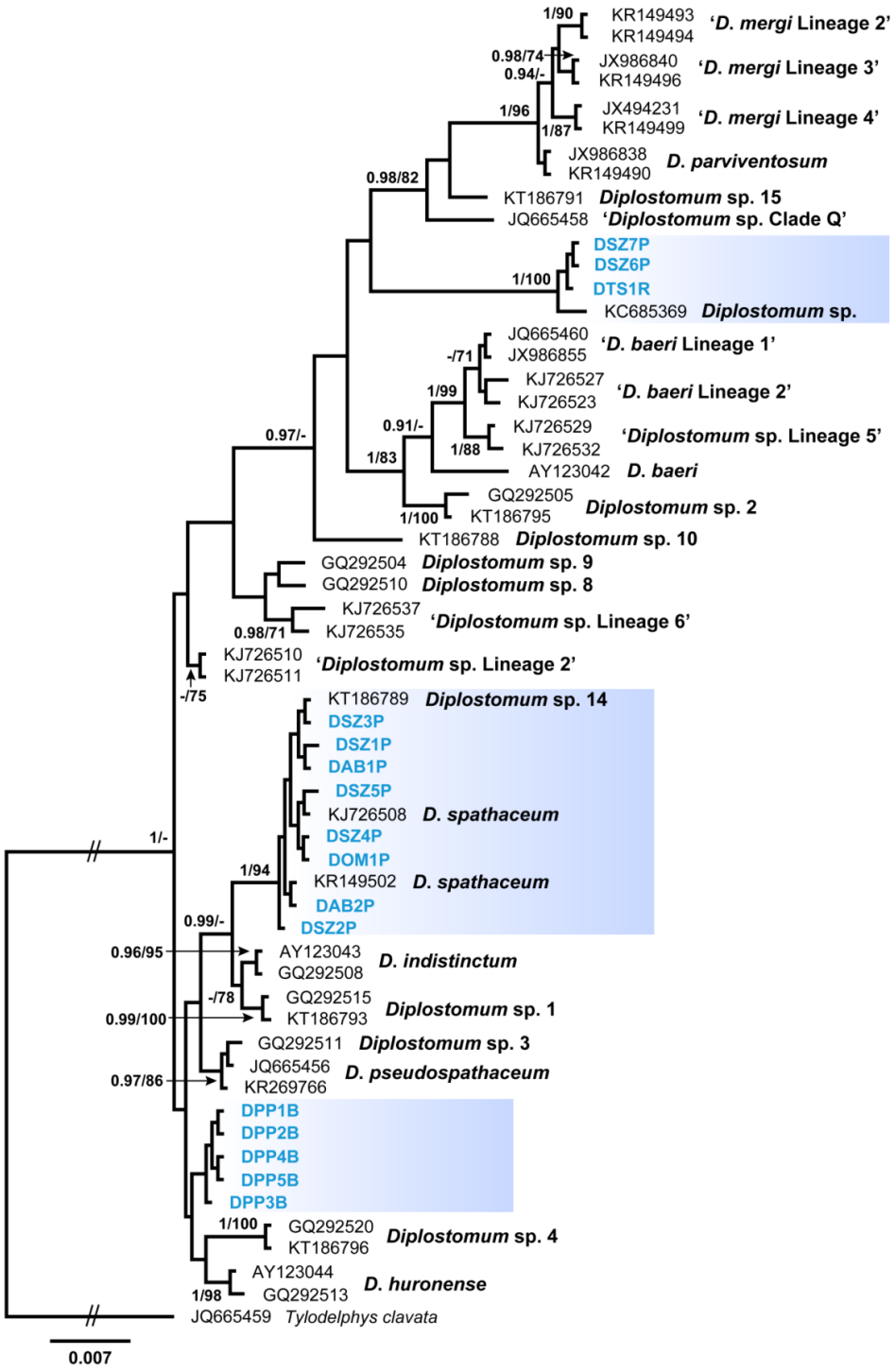


Figure 3-5: Phylogenetic tree for species of *Diplostomum* resulting from Bayesian inference analysis based on the ITS1-5.8S-ITS2 sequences. Nodal support from BI and ML analyses indicated as BI/ML; only values > 0.90 (BI) and > 70 (ML) are displayed. Scale-bar indicates the expected number of substitutions per site. Sequences generated in this study are in bold and indicated by blue rectangles. Codes with isolate information for newly generated sequences are provided in Table 3-4.

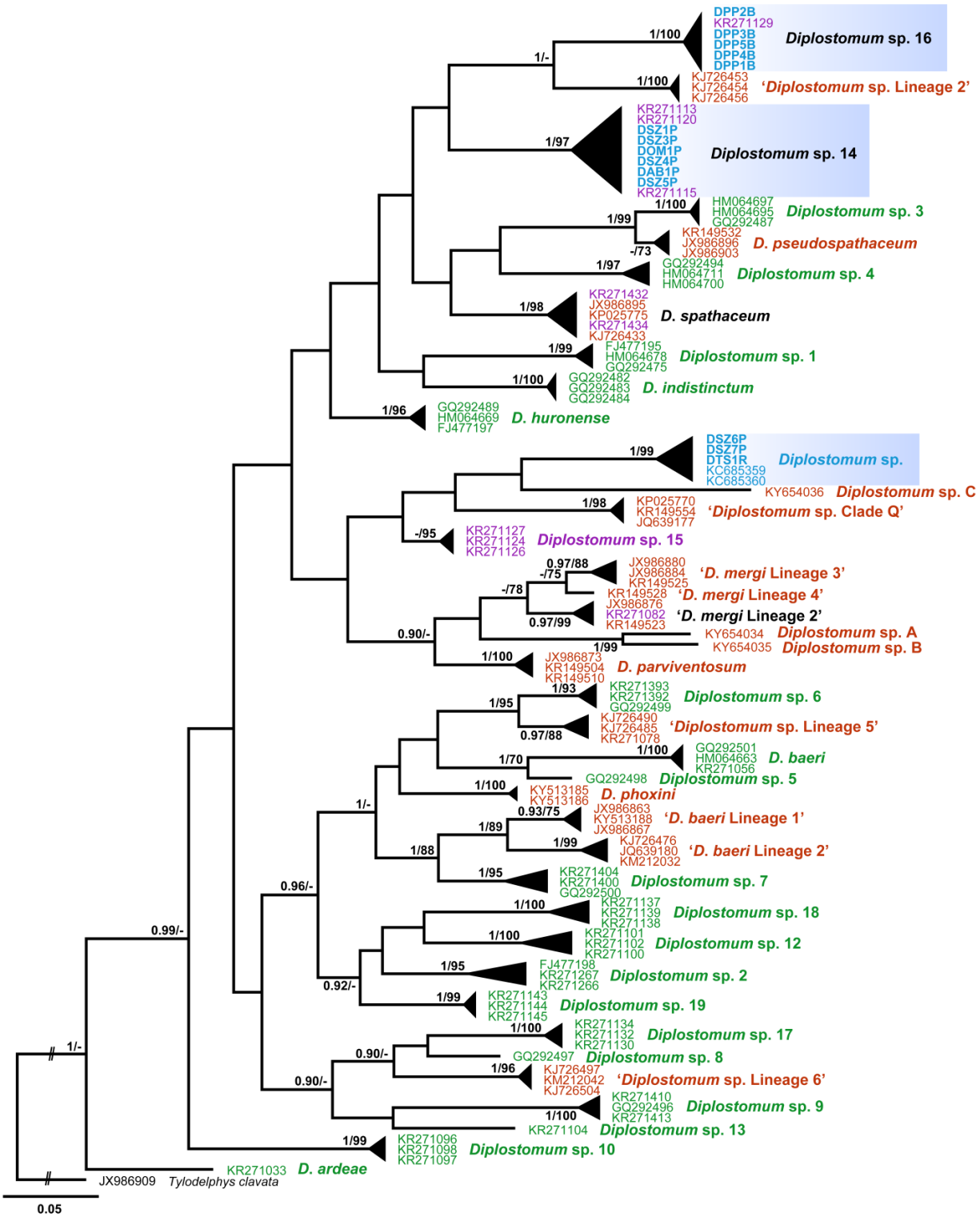


Figure 3-6: Phylogenetic tree for species of *Diplostomum* resulting from Bayesian inference analysis based on the partial *cox*1 sequences. Nodal support from BI and ML analyses indicated as BI/ML; only values > 0.90 (BI) and > 70 (ML) are displayed. Scale-bar indicates the expected number of substitutions per site. Sequences generated in this study are in bold and indicated by blue rectangles. Codes with isolate information for newly generated sequences are provided in Table 3-4.

A total of three species were identified and they appeared to be conspecific to the three species previously reported from Nigeria (*Diplostomum* sp.), China (*Diplostomum* sp. 14) and Iraq (*Diplostomum* sp. 14 and *Diplostomum* sp. 16). Therefore, the three species are referred to as *Diplostomum* sp. *sensu* Chibwana *et al*. (2013), *Diplostomum* sp. 14 *sensu* Locke *et al*. (2015) and *Diplostomum* sp. 16 *sensu* Locke *et al*. (2015). The interspecific divergence between *Diplostomum* sp. and *Diplostomum* sp. 14 was 13.2–14.7% (36–40 nt), *Diplostomum* sp. and *Diplostomum* sp. 16 was 11.8–12.1% (32–33 nt) and *Diplostomum* sp. 14 and *Diplostomum* sp. 16 was 11.8–12.5% (32–34 nt). Although the three species can be well distinguished using molecular sequence data, they also exhibit several prominent characteristics that can be used for the identification based on morphology. Selected voucher material was deposited in the Parasite Collection of the National Museum, Bloemfontein (NMB). The morphological descriptions of the present metacercariae are provided below.

Morphological descriptions of metacercariae

Superfamily: Diplostomoidea Poirier, 1886

Family: Diplostomidae Poirier, 1886

Genus: *Diplostomum* von Nordmann, 1832

***Diplostomum* sp. *sensu* Chibwana *et al*., 2013**

Second intermediate host: Plain squeaker *Synodontis zambezensis* Peters, 1852 (Siluriformes: Mochokidae) from Phongolo River Site 1; Banded tilapia *Tilapia sparrmanii* Smith, 1840 (Perciformes: Cichlidae) from Riet River.

Site in host: Eye lens.

Localities: Phongolo River Site 1 (26°55'46.7"S 32°19'29.7"E) and Riet River (S28°59'59.5", E24°28'50.0"), South Africa.

Prevalence: 5% (*S*. *zambezensis*, Phongolo River Site 1); 1 of 6 (*T*. *sparrmanii*, Riet River).

Intensity of infection: 1 metacercaria per fish (Phongolo River Site 1; Riet River).

Representative DNA sequences: 28S – three sequences, ITS1-5.8S-ITS2 – three sequences, *cox*1 – three sequences.

Description (Fig. 3-7A–C)

[Based on 1 live metacercaria] Body large, oval, 738 × 532, with maximum width at level of ventral sucker. Tegument covered with numerous tiny spines. Forebody subspherical, 590 × 532, larger than hindbody. Hindbody conical, short, 168 × 223, rounded. Forebody/ hindbody length ratio 1:0.28, forebody/ hindbody width ratio 1:0.42. Pseudosuckers sunken, at level of pharynx (Fig. 3-7C). Oral sucker subterminal, oval, 63 × 56. Prepharynx long, 21; pharynx muscular, elongate-oval, 68 × 37; oesophagus short; caeca long, reach posterior to holdfast organ. Ventral

sucker transversely oval, in forebody, 82 × 102, distinctly larger than oral sucker [sucker width ratio 1:1.82]. Distance from ventral sucker to anterior extremity of body, 364, and to posterior extremity of forebody, 237. Holdfast organ large, transversely oval, 103 × 170, in posterior part of forebody. Distance from holdfast organ to ventral sucker, 11. Excretory vesicle large, V-shaped; reserve excretory system of diplostomid type. Excretory granules medium-sized, numerous, scattered throughout forebody. Excretory pore subterminal.

[Based on 3 fixed metacercariae] Body large, subspherical, 379–615 × 456–525 (497 × 491) with maximum width at level of ventral sucker. Tegument covered with numerous tiny spines. Forebody transversely oval, 337–568 × 456–563 (491 × 515), larger than hindbody. Hindbody short, 55–76 × 288–296 (66 × 292), bluntly rounded. Forebody/ hindbody length ratio 1:0.13–1:0.16 (1:0.15), forebody/ hindbody width ratio 1:0.56–1:0.63 (1:0.60). Pseudosuckers sunken, at level of pharynx. Oral sucker subterminal, subspherical, 38–55 × 48–54 (48 × 50). Prepharynx long, 42–92 (60); pharynx muscular, elongate-oval, 42–65 × 31–41 (50 × 35); oesophagus short; caeca long, reach posterior to holdfast organ. Ventral sucker transversely oval, 66–87 × 100–112 (75 × 106), distinctly larger than oral sucker [oral/ ventral sucker width ratio 1:1.98–1:2.29 (1:2.12)]. Distance from ventral sucker to anterior extremity of body, 174–298 (256), and to posterior extremity of forebody, 91–208 (163). Holdfast organ large, transversely oval, 104–123 × 141–196 (115 × 165), in posterior part of forebody, contiguous with ventral sucker. Excretory vesicle large, V-shaped; reserve excretory system of diplostomid type. Excretory granules medium-sized, numerous, scattered throughout forebody. Excretory pore subterminal.

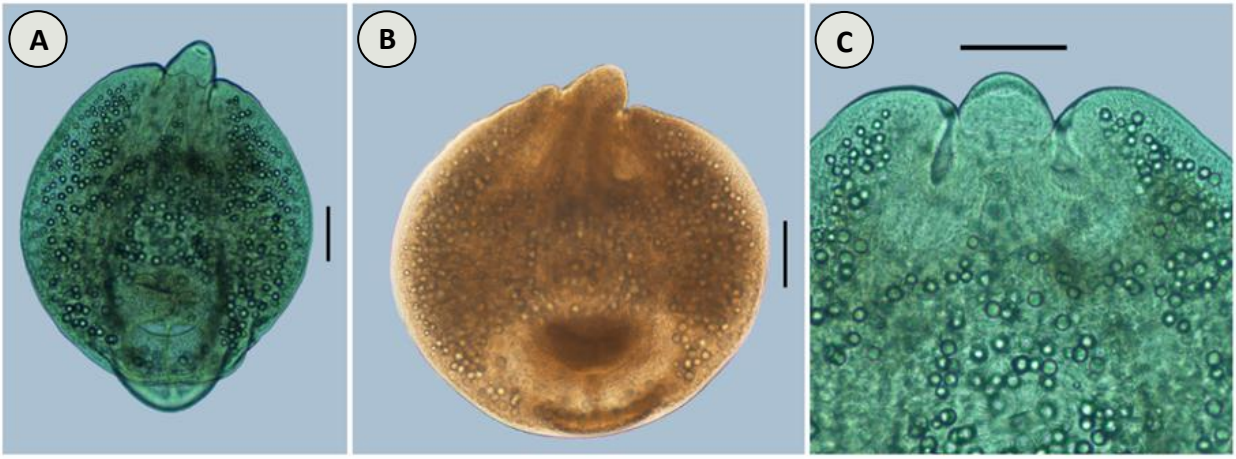


Figure 3-7: Metacercariae of *Diplostomum* sp. *sensu* Chibwana *et al*., 2013 from eye lenses of different fish hosts. A, *Diplostomum* sp. from *T*. *sparrmanii*, live, ventral view; B, *Diplostomum* sp. from *T*. *sparrmanii*, fixed, ventral view; C, *Diplostomum* sp. from *T*. *sparrmanii*, live, indicating sunken pseudosuckers (hologenophore). *Scale-bars*: A–C, 100 µm.

***Diplostomum* sp. 14 sensu Locke *et al*., 2015**

Second intermediate host: African mottled eel *Anguilla labiata* (Peters, 1852) (Anguilliformes: Anguillidae), Plain squeaker *Synodontis zambezensis* Peters, 1852 (Siluriformes: Mochokidae) from Phongolo River Site 1; Mozambique tilapia *Oreochromis mossambicus* (Peters, 1852) (Perciformes: Cichlidae) from Phongolo River Site 2.

Site in host: Eye lens.

Localities: Phongolo River Site 1 (26°55'46.7"S 32°19'29.7"E) and Phongolo River Site 2 (S26°52'57.81", E32°18'41.01"), South Africa.

Prevalence: 55% (*S*. *zambezensis*, Phongolo River Site 1); 3 of 4 (*A*. *labiata*, Phongolo River Site 1); 1 of 3 (*O*. *mossambicus*, Phongolo River Site 2).

Intensity of infection: 1–12 metacercariae per fish (*S*. *zambezensis*, Phongolo River Site 1); 2–6 metacercariae per fish (*A*. *labiata*, Phongolo River Site 1); 1 metacercaria per fish (*O*. *mossambicus*, Phongolo River Site 2).

Representative DNA sequences: 28S – three sequences, ITS1-5.8S-ITS2 – eight sequences, *cox*1 – six sequences.

Voucher material: 14 voucher specimens deposited in NMB as NMB P 526–530 [NMB P 526 (2 specimens), NMB P 527 (2 specimens), NMB P 528 (3 specimens), all from *S*. *zambezensis*, Phongolo River, KwaZulu-Natal, South Africa; NMB P 529 (3 specimens) and NMB P 530 (4 specimens), both from *A*. *labiata*, Phongolo River, KwaZulu-Natal, South Africa].

Description (Fig. 3-8A–C)

[Based on 10 live metacercariae] Body elongate-oval, 300–472 × 175–294 (354 × 229), with maximum width at level of ventral sucker or just anterior to ventral sucker. Tegument covered with numerous tiny spines. Forebody elongate-oval, 277–443 × 178–294 (336 × 235), longer than hindbody. Hindbody rounded, short, 53–84 × 57–100 (68 × 78). Forebody/ hindbody length ratio, 1:0.16–1:0.26 (1:0.21). Forebody/ hindbody width ratio 1:0.24–1:0.47 (1:0.34). Pseudosuckers elongate-oval, 35–57 × 20–25 (42 × 22). Oral sucker subterminal, subspherical (n = 8), 40–51 × 40–54 (45 × 46) or transversely oval (n = 2), 44–48 × 51–54 (46 × 53). Prepharynx very short, 3–10 (5) or absent; pharynx muscular, elongate-oval, 25–37 × 17–26 (31 × 20); oesophagus short; caeca thick, long, reach posterior to holdfast organ. Ventral sucker transversely oval, postequatorial, 34–46 × 42–53 (39 × 46), smaller or equal to oral sucker [oral/ ventral sucker width ratio 1:0.82–1:1.08 (1:0.96)]. Distance from ventral sucker to anterior extremity of body, 146–224 (177) and to posterior extremity of forebody, 96–168 (126). Holdfast organ subspherical, 52–67 × 57–78 (61 × 68). Distance from holdfast organ to ventral sucker, 7–15 (11). Excretory granules, medium- or large sized, scattered in forebody, but generally grouped into two lateral extracaecal and one median field. Excretory vesicle V-shaped; reserve excretory system of diplostomid type. Excretory pore subterminal.

[Based on 14 fixed metacercariae] Body oval, 237–372 × 206–271 (302 × 240), with maximum width just anterior to ventral sucker. Tegument covered with numerous tiny spines. Forebody oval, 216–346 × 206–271 (274 × 237), longer than hindbody. Hindbody rounded, 41–91 × 66–102 (61 × 81), short Forebody/ hindbody length ratio 1:0.08–1:0.26 (1:0.18), forebody/ hindbody width ratio, 1:0.28–1:0.60 (1:0.40). Pseudosuckers elongate-oval, 30–56 × 18–32 (41 × 23). Oral sucker subterminal, subspherical, 36–55 × 35–53 (45 × 43). Prepharynx very short or absent, 3–7 (5); pharynx muscular, elongate-oval, 28–38 × 15–27 (33 × 22); oesophagus short; caeca long, thick, reach posterior to holdfast organ. Ventral sucker subspherical, 31–49 × 34–53 (40 × 44), postequatorial, smaller to larger than oral sucker [oral/ ventral sucker width ratio 1:0.84–1:1.28 (1:1.05)]. Distance from ventral sucker to anterior extremity of body, 85–187 (133) and to posterior extremity of forebody, 89–144 (105). Holdfast organ subspherical, 52–87 × 58–91 (68 × 73), in posterior part of forebody. Distance from holdfast organ to ventral sucker, 5–6 (5) or holdfast organ contiguous with ventral sucker. Excretory granules, medium- (Fig. 3-8B) or large sized (Fig. 3-8C), scattered in forebody, but generally grouped into two lateral extracaecal and one median field. Excretory vesicle V-shaped; reserve excretory system of diplostomid type. Excretory pore subterminal.

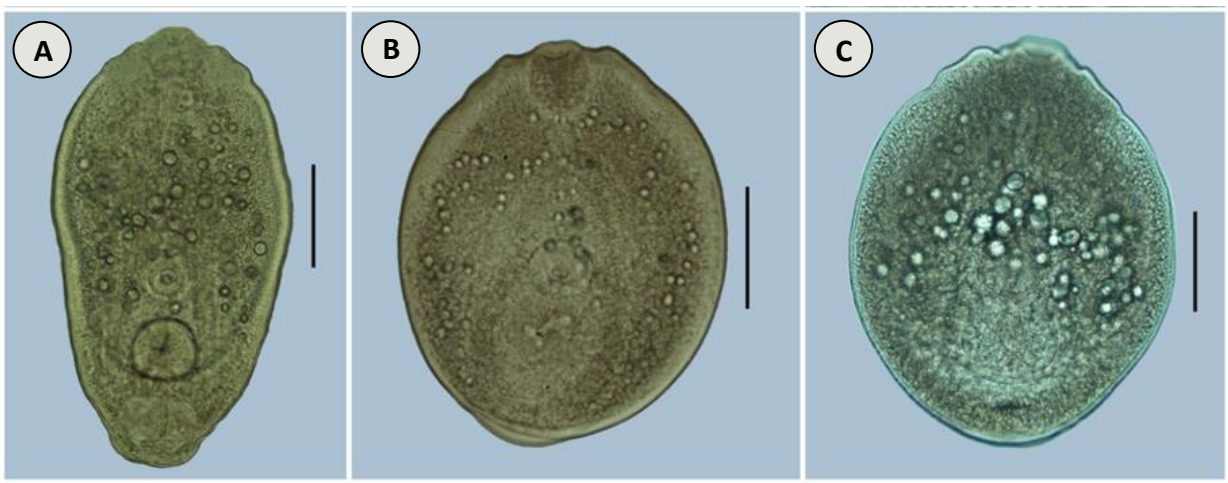


Figure 3-8: Metacercariae of *Diplostomum* sp. 14 *sensu* Locke *et al*., 2015 from eye lenses of different fish hosts. A, *Diplostomum* sp. 14 from *S*. *zambezensis*, live, ventral view (hologenophore); B, *Diplostomum* sp. 14 from *O*. *mossambicus*, fixed, ventral view, small excretory granules (hologenophore); C, *Diplostomum* sp. 14 from *S*. *zambezensis*, fixed, ventral view, large excretory granules (hologenophore). *Scale-bars*: A–C, 100 µm.

***Diplostomum* sp. 16 sensu Locke *et al*., 2015**

Second intermediate host: Southern mouthbrooder *Pseudocrenilabrus philander* (Weber, 1897) (Perciformes: Cichlidae).

Site in host: Eye lens.

Localities: Mooi River – Boskop Dam (26°33'57.9"S 27°07'16.2"E), South Africa.

Prevalence: 90% (9 out of 10).

Intensity of infection: 3–21 metacercariae per fish.

Representative DNA sequences: 28S – two sequences, ITS1-5.8S-ITS2 – five sequences, *cox*1 – five sequences.

Voucher material: 15 voucher specimens deposited in NMB as NMB P 531–533 [NMB P 531 (7 specimens), NMB P 532 (5 specimens), NMB P 533 (3 specimens), all from *P. philander*, Mooi River, North West Province, South Africa].

Description (Fig. 3-9 A–C)

[Based on 15 fixed metacercariae] Body elongate-oval, 284–434 × 212–306 (356 × 254), with maximum width at level of ventral sucker or just anterior to ventral sucker. Tegument covered with numerous tiny spines. Forebody elongate-oval, 293–414 × 231–277 (346 × 250), longer than hindbody. Hindbody rounded, 56–96 × 72–130 (81 × 104), short. Forebody/ hindbody length ratio 1:0.19–1:0.27 (1:0.23), forebody/ hindbody width ratio 1:0.31–1:0.49 (1:0.41). Pseudosuckers elongate-oval, 43–63 × 28–35 (53 × 32), everted (n = 14; Fig. 3-9A) or inverted (n = 1; Fig. 3-9B). Oral sucker subterminal, elongate-oval, 51–62 × 38–59 (55 × 49). Prepharynx short, 8–22 (15); pharynx muscular, elongate-oval, 31–39 × 20–28 (36 × 24); oesophagus short; caeca long, reach posterior to holdfast organ. Ventral sucker transversely oval, 40–55 × 52–68 (49 × 61), equatorial, equal, or larger than oral sucker [oral/ ventral sucker width ratio 1:0.95–1:1.50 (1:1.25)]. Distance from ventral sucker to anterior extremity of body, 128–207 (167) and to posterior extremity of forebody, 105–158 (133). Holdfast organ transversely oval, 77–99 × 84–124 (91 × 101) in posterior part of forebody, contiguous with ventral sucker. Excretory granules, medium-sized, grouped into two lateral extracaecal and one median field. Excretory vesicle V-shaped; reserve excretory system of diplostomid type. Excretory pore subterminal.

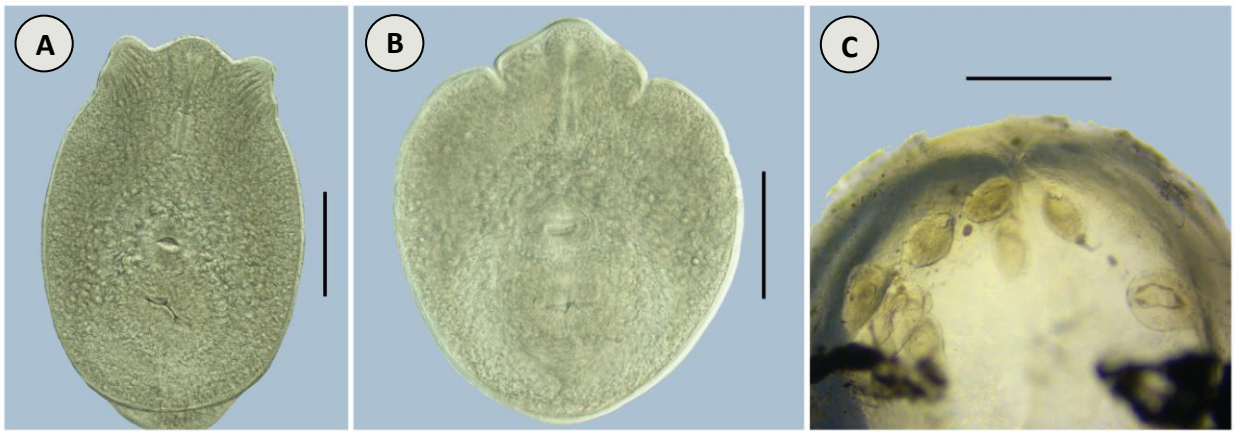


Figure 3-9: Metacercariae of *Diplostomum* sp. 16 *sensu* Locke *et al*., 2015 from eye lenses of *Pseudocrenilabrus philander*. A, *Diplostomum* sp. 16, fixed, ventral view, everted pseudosuckers (hologenophore); B, *Diplostomum* sp. 16, fixed, ventral view, inverted pseudosuckers (hologenophore); C, *Diplostomum* sp. 16, live metacercariae inside of fish lens. Scale-bars: A, B, 100 µm; C, 700 µm.

Remarks

The three species of *Diplostomum* described above represent the species that were previously reported from freshwater fishes in Nigeria (Chibwana *et al*., 2013), Iraq and China (Locke *et al*., 2015) based on the analyses of molecular data. The previous reports were not accompanied with morphological descriptions of the metacercarial isolates and, thus, this study provides the first morphological characterisation of the isolates of *Diplostomum* sp. *sensu* Chibwana *et al*., 2013 (Chibwana *et al*., 2013), *Diplostomum* sp. 14 *sensu* Locke *et al*., 2015 and *Diplostomum* sp. 16 *sensu* Locke *et al*., 2015 (Locke *et al*., 2015). Morphologically metacercariae of the present species are well-distinguishable from each other. The most characteristic feature differentiating metacercariae of *Diplostomum* sp. from two other species in the current study is the presence of pseudosuckers of the sunken type.

Metacercariae of *Diplostomum* sp. differ from both, *Diplostomum* sp. 14 and *Diplostomum* sp. 16 by the shape (subspherical body *vs* elongate-oval *vs* elongate-oval, respectively) and size of body [379–615 × 456–525 (497 × 491) *vs* 237–372 × 206–271 (302 × 240) *vs* 284–434 × 212–306 (356 × 254)], longer prepharynx [42–92 (60) *vs* 3–7 (5) *vs* 8–22 (15)], larger pharynx [42–65 × 31–41 (50 × 35) *vs* 28–38 × 15–27 (33 × 22) *vs* 31–39 × 20–28 (36 × 24)], ventral sucker [66–87 × 100–112 (75 × 106) *vs* 31–49 × 34–53 (40 × 44) *vs* 40–55 × 52–68 (49 × 61)], oral/ ventral suckers ratio [1:1.98–1:2.29 (1:2.12) *vs* 1:0.84–1:1.28 (1:1.05) *vs* 1:0.95–1:1.50 (1:1.25)] and holdfast organ [104–123 × 141–196 (115 × 165) *vs* 52–87 × 58–91 (68 × 73) *vs* 77–99 × 84–124 (91 × 101)]. Furthermore, the size and distribution of the excretory granules in the metacercariae of *Diplostomum* sp., i.e. medium-sized and scattered throughout the forebody differs from the

state observed in the two other species in which the excretory granules are of small to large size and grouped into two lateral extracaecal and one median field.

The metacercariae of *Diplostomum* sp. 14 differ from metacercariae of *Diplostomum* sp. 16 in possessing a low lower limits for the length and width of body [237–372 × 206–271 (302 × 240) *vs* 284–434 × 212–306 (356 × 254)], smaller oral sucker [36–55 × 35–53 (45 × 43) *vs* 51–62 × 38–59 (55 × 49)], shorter prepharynx [3–7 (5) *vs* 8–22 (15)], ventral sucker [31–49 × 34–53 (40 × 44) *vs* 40–55 × 52–68 (49 × 61)] and holdfast organ [52–87 × 58–91 (68 × 73) *vs* 77–99 × 84–124 (91 × 101)].

The metacercariae of *Diplostomum* sp. strongly resemble morphologically the metacercariae of *D*. *longicollis* reported by Zhokhov (2014) from *Enteromius humilis* (Boulenger, 1902) and *Garra dembecha* Getahun & Stiassny, 2007 in Ethiopia in the presence of the pseudosuckers of the sunken type. However, the morphometric data comparison of the fixed metacercariae revealed that the specimens from the present study exhibit shorter body [379–615 (497) *vs* 612–1008 (748)], smaller oral sucker [36–55 × 48–54 (48 × 50) *vs* 66–72 × 66–72 (63 × 65)], shorter prepharynx (42–92 *vs* 72–180), lower low limits for pharynx length (42–65 *vs* 60–66) and ventral sucker length (66–87 *vs* 72–96), and smaller holdfast organ [104–123 × 141–196 (115 × 165) *vs* 132–180 × 150–252 (158 × 183)] (see Table 3-5 for details).

Table 3-5: Comparative metrical data on *Diplostomum* sp. and *Diplostomum longicollis* (fixed specimens).

Species	***Diplostomum* sp.**		***Diplostomum longicollis* Zhokhov, 2014**	
Host	*Synodontis zambezensis*, *Tilapia sparrmanii*		*Enteromius humilis*, *Garra dembecha*	
Country	South Africa		Ethiopia	
Source	Present study		Zhokhov (2014)	
Character	Range	Mean	Range	Mean
BL	379–615	497	612–1,008	748
BW	456–525	491	378–576	490
FL	337–568	491	–	–
FW	456–563	515	–	–
HL	55–76	66	–	–
HW	288–296	292	–	–
OSL	38–55	48	66–72	63
OSW	48–54	50	66–72	65
PPHL	42–92	60	72–180	123
PHL	42–65	50	60–66	63
PHW	31–41	35	30–42	39
VSL	66–87	75	72–96	89
VSW	100–112	106	96–120	104
HOL	104–123	115	132–180	158
HOW	141–196	165	150–252	183
OSW:VSW	1:1.98–2.29	1:2.12	1:1.45–1.67*	1:1.6*

*Estimated from measurements provided in Zhokhov (2014).

The metacercariae of *Diplostomum* sp. 14 are morphologically most similar to the metacercariae of *D*. *montanum* from the eye lenses of *E*. *humilis*, *G*. *dembecha*, *L*. *gorgorensis*, *V*. *beso* and *D*. *tilapiae* from the eye lenses of *O*. *niloticus* collected in Ethiopia (Zhokhov, 2014) based on the shape of the body and pseudosuckers, position and size of the holdfast organ in relation to the ventral sucker and position of the ventral sucker. However, almost all body dimensions of metacercariae in the material of the present study are smaller than of metacercariae of *D*. *montanum* and *D*. *tilapiae* (see Table 3-6 for details).

The metacercariae of *Diplostomum* sp. 16 possess features that agree well with metacercariae of *D*. *garrae* found in the eye lens of *G*. *dembecha* in Ethiopia (Zhokhov, 2014). These include: the shape of the body, pseudosuckers and holdfast organ and position of the ventral sucker. Metacercariae of *Diplostomum* sp. 16 can be further distinguished from *D*. *garrae* in having lower low limits for a number of features, including length and width of body, oral sucker, pharynx, ventral sucker and holdfast organ, and lower low limits for the length of the prepharynx (see Table 3-6 for details).

Although metacercariae belonging to the genus *Diplostomum* have been reported in studies focused on freshwater fish parasites in Africa, morphological or molecular evidence of these records were rarely provided or in many cases, species were left unidentified (Van As & Basson, 1984; Khalil & Polling, 1997; Migiro *et al*., 2012; Jansen van Rensburg *et al*., 2013; Grobbelaar *et al*., 2014).

Table 3-6: Comparative metrical data on *Diplostomum* spp. (fixed specimens).

Species	***Diplostomum* sp. 14**		***Diplostomum* sp. 16**		***Diplostomum garrae* Zhokhov, 2014**		***Diplostomum montanum* Zhokhov, 2014**		***Diplostomum tilapiae* Zhokhov, 2014**	
Host	*Anguilla labiata*, *Oreochromis mossambicus*, *Synodontis zambezensis*		*Pseudocrenilabrus philander*		*Garra dembecha*		*Enteromius humilis*, *Garra dembecha*, *Labeobarbus gorgorensis*, *Varicorhinus beso*		*Oreochromis niloticus*	
Country	South Africa		South Africa		Ethiopia		Ethiopia		Ethiopia	
Source	Present study		Present study		Zhokhov (2014)		Zhokhov (2014)		Zhokhov (2014)	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
BL	237–372	302	284–434	356	306–414	380	432–621	552	531–828	653
BW	206–271	240	212–306	254	252–306	283	240–372	289	198–234	212
FL	216–346	274	293–414	346	–	–	–	–	–	–
FW	206–271	237	231–277	250	–	–	–	–	–	–
HL	41–91	61	56–96	81	–	–	–	–	33–121	61
HW	66–102	81	72–130	104	–	–	–	–	–	–
OSL	36–55	45	51–62	55	54–72	65	60–78	63	53–66	58
OSW	35–53	43	38–59	49	54–66	59	48–72	67	53–57	55
PPHL	3–7	5	8–22	15	7–24	17	–	–	–	–
PHL	28–38	33	31–39	36	36–54	44	30–48	41	29–40	33
PHW	15–27	22	20–28	24	24–30	29	24–36	31	22–29	24
PSL	30–56	41	43–63	53	60–78	60	–	–	33–66	47
PSW	18–32	23	28–35	32	–	–	–	–	–	–
VSL	31–49	40	40–55	49	42–66	56	36–80	60	42–55	46
VSW	34–53	44	52–68	61	60–66	66	48–90	99	48–57	53
HOL	52–87	68	77–99	91	90–120	106	84–120	111	88–121	98
HOW	58–91	73	84–124	101	90–120	112	84–120	115	88–103	72
OSW:VSW	1:0.84–1.28	1.05	1:0.95–1:1.50	1:1.25	1:1–1.11*	1.12*	1:1–1.26*	1.48*	1:0.91–1*	0.96*

*Estimated from measurements provided in Zhokhov (2014)

It should be noted that metacercariae reported in the present study were not compared to the five species of *Diplostomum*, *D*. *heterobranchi*, *D*. *magnicaudum*, *Diplostomum* sp. type I Prudhoe & Hussey, 1977 and *Diplostomum* sp. type II Prudhoe & Hussey, 1977 and *Diplostomum* type 3 collected in the brain or encysted in the mesenteries of *Clarias gariepinus* in Egypt and South Africa (Khalil & Polling, 1997; Prudhoe & Hussey, 1977; Madanire-Moyo *et al*., 2010). Detailed examination of the descriptions and illustrations of the metacercariae of these species suggested that their affiliation with the genus *Diplostomum* was erroneous and should be reconsidered. The two known species of *Diplostomum* in South Africa, *Diplostomum* type I and *Diplostomum* type II were erroneously identified as the members of this genus. Metacercariae of *Diplostomum* type I were found encysted within the thin-walled cyst in the mesenteries of *C*. *gariepinus* (Prudhoe & Hussey, 1977). However, the metacercariae of *Diplostomum* are known to not form a cyst and occur in the eyes or brain of freshwater fishes (Shigin, 1986; Georgieva *et al*., 2013; Blasco-Costa *et al*., 2014). Metacercariae of *Diplostomum* type II was found unencysted in the cranial cavity of *C*. *gariepinus* and was first believed to represent the species *Tylodelphys mashonensis* Beverly-Burton, 1963. However, the presence of distinct constriction between the forebody and the hindbody in specimens led the authors to identify the species as a member of *Diplostomum*. Based on the description and figure illustrating metacercaria of *Diplostomum* type II (Prudhoe & Hussey, 1977), we suggest that it certainly represents a species within the family Diplostomidae but does not belong to the genus *Diplostomum*. *Diplostomum* type 3 found in the eyes and cranial cavity of *C*. *gariepinus* cannot be confirmed to be a valid species of this genus as it still requires morphological descriptions. Therefore, only reports of this species present in these fish hosts exist (Madanire-Moyo *et al*., 2010). Furthermore, detailed examination of the descriptions and illustrations of two species of *Diplostomum* (*D*. *heterobranchi* and *D*. *magnicaudum*) reported in Egypt suggested that their placement within this genus was also inaccurate. Metacercariae of *D*. *heterobranchi* were found in the brain cranial cavity of *Clarias lazera* (= *C*. *gariepinus*). A short and incomplete description together with the illustration of the metacercaria demonstrates a close resemblance of metacercariae identified as *D*. *heterobranchi* to the species of *Tylodelphys*, particularly *T*. *mashonensis* as reported from the brain cranial cavity of *C*. *gariepinus* in South Africa by Beverly-Burton (1963). The statement of erroneous identification of *D*. *heterobranchi* can also be supported by the conclusion of Shigin (1986) who noted that the length of metacercariae of *Diplostomum* does not exceed 1 mm, however metacercariae of *D*. *heterobranchi* was reported to reach a total body length of up to 1 mm. Metacercariae of another species of *Diplostomum* described from Egypt, *D*. *magnicaudum*, were found encysted between the muscles of *Tilapia nilotica* L., 1758 (= *O*. *niloticus*) near the caudal and dorsal fins (El-Naffar, 1979). However, the general morphology of metacercariae illustrated in the paper of El-Naffar (1979) and the reported site of infection in fish plus metacercariae being encysted suggested that this species belongs to another genus within the Diplostomidae.

Moreover, the following ambiguous reports of both identified and unidentified species of *Diplostomum* have been provided from freshwater fishes in Africa (Chibwana, 2018), but due to insufficient morphological descriptions or molecular evidence, these reports should be revised and their association to this genus should be reconsidered: *Diplostomum commutatum* (Diesing, 1850) Dubois, 1937 found in the intestine of *Pseudotolithus elongates* (Bowdich, 1825) and *Cynoglossus senegalensis* (Kaup, 1858) (see Abraham & Akpan, 2004); *D*. *spathaceum* found in the intestine of *C*. *gariepinus* from Nigeria (Goselle *et al*., 2008); *Diplostomum* spp. reported from the eye vitreous humour and lens of *O*. *niloticus* in Kenya (Migiro *et al*., 2012; Ndeda *et al*., 2013); *Diplostomum* sp. from the eyes of *Barbus intermedius* and the cranial cavity of *C*. *gariepinus* in Ethiopia (Gulelat *et al*., 2013); and *Diplostomum* spp. from the eyes and brains of 13 fish species from Botswana (Grobbelaar *et al*., 2014). After revision on the reports of *Diplostomum tregenna* Nazmi & Gohar, 1932 from the cranial cavity of *C*. *gariepinus*, *Channa obscurra* and *Tilapia zilli* (Gervais, 1848) from Sudan, Benin, Ethiopia, Nigeria and Egypt, this species was synonymised with *Dolichorchis tregenna* (Khalil, 1963; Khalil & Polling, 1997; Okaka & Akhigbe, 1999; Zhokhov, 2010, Chibwana *et al*., 2013). Numerous studies of *Diplostomum* in fishes from South Africa also resulted in reports of many unidentified species: *Diplostomum* spp. found in the eye lenses and vitreous humour of *Barbus* spp., *Micropterus* sp., *Salmo* sp., *Tilapia* sp. (Van As & Basson, 1984); *Diplostomum* type 3 from the eyes and cranial cavity of *C*. *gariepinus* (Madanire-Moyo *et al*., 2010); *Diplostomum* sp. from the eyes of *Schilbe intermedius* (Smit & Luus-Powell, 2012); *Diplostomum* spp. in the eyes of *Labeo umbratus* (Smith, 1841), *Labeo capensis* (Smith, 1841) and *Cyprinus carpio* L., 1758 from the Vaal-Orange River system, South Africa (Grobbelaar *et al*., 2014); and *Diplostomum* sp. from the eyes of *Labeobarbus marequensis* (Smith, 1841) and *Barbus trimaculatus* Peters, 1852 (see Mbokane *et al*., 2015). Critical revision of these unidentified species of *Diplostomum* from freshwater fishes in South Africa is required as well as additional sampling effort and dedicated studies focussing on finding/identifying adults of *Diplostomum* from their definitive hosts. This will allow the identification of these parasites to the species level and finally help elucidate the life cycle of unidentified species in Africa.

Therefore, based on the critical review of the literature and data obtained in the present study, there are currently seven species of *Diplostomum* known to exploit freshwater fishes in Africa, namely *D*. *garrae*, *D*. *longicollis*, *D*. *montanum*, *D*. *tilapiae*, *Diplostomum* sp., *Diplostomum* sp. 14 and *Diplostomum* sp. 16. Of these, three species are distributed in South Africa.

3.4 Discussion

Freshwater ecosystems in South Africa are characterised by a rich fish diversity with over 180 species currently recognised (Froese & Pauly, 2019). In the past, parasite diversity in the sharptooth catfish *C*. *gariepinus* (Siluriformes: Clariidae) have been studied extensively in Africa (Khalil & Polling, 1997; Barson *et al*., 2008; Madanire-Moyo & Barson, 2010; Jansen van Rensburg *et al*., 2013; Chibwana & Nkwenguilila, 2010; Chibwana *et al*., 2013; Moema *et al*., 2013; Grobbelaar *et al*., 2015; Otachi *et al*., 2015), but remarkably little attention has been paid to other fish species as potential hosts for *Diplostomum*. Whilst five species of the Diplostomidae known in South Africa have been found parasitising *C*. *gariepinus* (Khalil & Polling, 1997; Kudlai *et al*., 2018; Hoogendoorn *et al*., 2019), cichlid fishes (*T*. *sparrmanii* and *P*. *philander*) have been reported as hosts only for a single diplostomid species (Moema *et al*., 2013). Recently, Hoogendoorn *et al*. (2019) examined *T*. *sparrmanii* in the North West Province, South Africa and reported four diplostomid species (*Bolbophorus* sp. 3, *Posthodiplostomum* sp. 9, *Uvulifer* sp. 4 and Diplostomidae gen. sp.) that were not previously detected neither in cichlids or cyprinids nor in *C*. *gariepinus*. The fish selected for the current project belonged to the Alestidae, Anguillidae, Centrarchidae, Cichilidae, Clariidae, Cyprinidae, Gobiidae, Mochokidae, Mormyridae and Schilbeidae. This study is the first to examine a wider range of fish species (n = 17) for parasitological screenings and the first to report infections with *Diplostomum* spp. in five additional fish species (*A*. *labiata*, *O*. *mossambicus*, *P*. *philander*, *S*. *zambezensis* and *T*. *sparrmanii*) in South Africa. Of the species of *Diplostomum* reported in this study, two species namely *Diplostomum* sp. and *Diplostomum* sp. 14 infected more than one host species from the families Anguillidae, Cichilidae and Mochokidae. The wide host range of *Diplostomum* sp. was unexpected, especially since it has previously been reported from only a single fish host (*S*. *nigrita*) from Nigeria (Chibwana *et al*., 2013). Therefore, the new data of two host species provided in this study (*T*. *sparrmanii* and *S*. *zambezensis*) increases the host range for *Diplostomum* sp. In contrast, the wide host range of *Diplostomum* sp. 14 in South Africa is not surprising as the host range in Iraq and China is even broader and includes members of the Channidae, Cyprinidae, Hemiramphidae, Odontobutidae, Bagridae, Gobiidae, Percichthyidae (Locke *et al*., 2015). In this study, *Diplostomum* sp. 16 was only found parasitising *P*. *philander*, which is similar to its previous record where it was found infecting a single host species *A*. *caeruleus* (cyprinid) in Iraq (Locke *et al*., 2015). The fact that metacercariae of all three species in the present study were found in cichlid fishes suggests that their transmission is mainly associated with this group. Further comprehensive assessment of freshwater fishes in other parts of southern Africa may reveal more information on the host ranges of *Diplostomum*.

The simple morphology of the larval stages remains one of the major challenges for accurate species identification of *Diplostomum*; therefore, both molecular techniques using multiple genetic markers and detailed morphological characterisation were applied for the

identification of species of *Diplostomum* in the present study. The initial delineation between the species and their identification based on morphological characters was later confirmed by sequence data analyses. The *cox*1 sequence analyses revealed conspecificity of three species in the present study with previously reported *Diplostomum* sp., *Diplostomum* sp. 14 and *Diplostomum* sp. 16. It is important to note that the most prominent feature used to delineate between *Diplostomum* sp. (*T*. *sparrmanii* and *S*. *zambezensis*) and the other *Diplostomum* spp. found in the present study was the presence of pseudosuckers of the sunken type. This type of pseudosuckers, to the best of our knowledge, has been previously described only in metacercariae of several species namely, *D*. *gobiorum*, *D*. *pungiti* Shigin, 1965, *D*. *volvens* and *D*. *longicollis* (Shigin, 1986; Zhokhov, 2014).

The importance of the application of multiple genetic markers with the mitochondrial *cox*1 gene as a priority choice were once again showed by the results of the present study. Based on 28S and ITS1-5.8S-ITS2 analyses, metacercariae collected from *A*. *labiata*, *O*. *mossambicus* and *S*. *zambezensis* (NGR) were conspecific with *D*. *spathaceum*. However, based on *cox*1 analyses, these isolates were identified to belong to *Diplostomum* sp. 14. The present study expanded the 28S rDNA, ITS1-5.8S-ITS2 and *cox*1 sequence database for African species of *Diplostomum* by adding sequences of *Diplostomum* sp., *Diplostomum* sp. 14 and *Diplostomum* sp. 16 (9, 17 and 12 isolates, respectively).

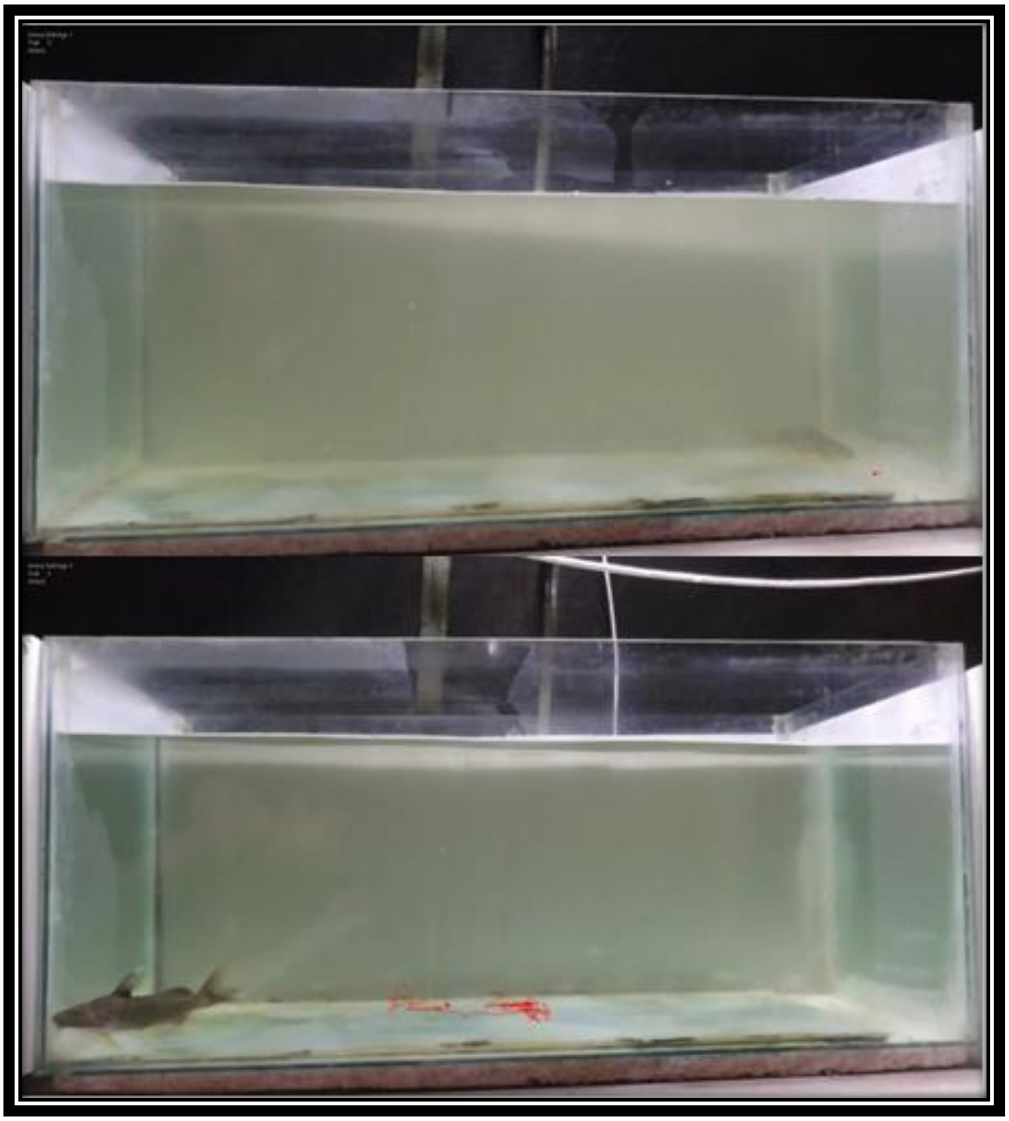
The results of this study not only improve the knowledge on the species diversity within *Diplostomum* in South Africa, but also uncover their geographical range and provide the first molecular evidence for the distribution of *Diplostomum* spp. in both Asia and Africa. The analysis of the data available for *Diplostomum* spp. from four continents (Africa, Asia, Europe, and North America) (see Fig. 3-6), demonstrated a very broad distribution of several species. *Diplostomum* sp. recently reported from *S*. *nigrita* in Nigeria (Chibwana *et al*., 2013) appeared to also parasitise *S*. *zambezensis* and *T*. *sparrmanii* in South Africa, thus this species has a much larger geographical distribution within the Afrotropical region than previously recorded. To date, both *Diplostomum* sp. 14 and *Diplostomum* sp. 16 were only known from the Asian continent (Iraq and China) and the presence of these species in South Africa was rather unexpected, especially since most species of *Diplostomum* have a relatively restricted geographical distribution and have been reported from only one zoogeographical region. Prior to this study, based on molecular data, the only two species *D*. *spathaceum* and *D*. *mergi* Lineage 2 were known to be distributed across two continents, Asia and Europe within the Palaearctic region (Locke *et al*., 2015). The results from the present study provide evidence for species within *Diplostomum* to have a much broader geographical distribution by being common in both Northern (within Palaearctic region) and Southern (within Afrotropical region) hemispheres. The transmission between the continents is primarily associated with the migratory patterns of their definitive hosts – piscivorous birds. Four out of six sampling localities of the present survey were situated within the NGR, an area known

for its high diversity of resident and migratory birds. It accommodates 430 bird species that is 19% of all species present on the African continent (Marnewick *et al*., 2015). Moreover, this area is located within the African-Eurasian flyways for the migratory birds that has previously been shown to be involved in the transmission of numerous bird parasites. These flyways differ depending on the total length of flight path and the number of stops and their duration along the flight path. The transmission of *Diplostomum* sp. in Africa is largely due to the Intra-African migration of waterbirds that are mainly driven by the climatic changes.

To summarise, the discovery of three species demonstrates that the species diversity within the genus *Diplostomum* in Africa is underestimated and higher than previously known. This study is the first to provide detailed morphological descriptions along with molecular evidence. This integrative approach allowed for the following outcomes: (i) identification for three species of *Diplostomum* to the genus level i.e. *Diplostomum* sp., *Diplostomum* sp. 14 and *Diplostomum* sp. 16; (ii) morphological comparison of *Diplostomum* spp. in the present study with species previously reported in Africa; (iii) molecular comparison between *Diplostomum* spp. in the present study and sequences of species available in GenBank; (iv) expand the 28S, ITS1-5.8S-ITS2 and *cox*1 sequence database for African diplostomid species; (v) report additional second intermediate hosts for the species of *Diplostomum* in Africa; and (vi) analysis of the geographical distribution of species of *Diplostomum* based on new data obtained in South Africa.

This chapter has been published in the International Journal of Parasitology: Parasites and Wildlife (Hoogendoorn *et al*., 2020 – see Appendix G).

Chapter 4: Effect of metacercariae on fish behaviour



CHAPTER 4: EFFECT OF METACERCARIAE ON FISH BEHAVIOUR

4.1 Introduction

Animals have natural behavioural traits that are adapted in order to reduce predation risks i.e. by switching between habitats to decrease the likelihood of encountering predators, reducing conspicuous behaviour to become less visible, therefore, less exposed to predators, or spend more time vigilant in the presence of predators (Lima & Dill, 1990; Langerhans, 2007; Reebs, 2008). These anti-predatory behaviours are adaptations stressed by co-evolution to prevent them from being caught and in effect prevent transmission to the definitive hosts in trophically transmitted parasites (Poulin, 2013). In cases where intermediate and definitive host transmission is linked with a predator-prey relationship, parasites have the ability to modify their hosts' anti-predatory behaviour, increasing the success of transmission (Bethel & Holmes, 1973; Poulin, 2010). Parasites have many profound effects on their hosts' physiology, feeding and swimming behaviour (Moore, 2002; Poulin, 2007). If the habitats of the parasite's "next host" i.e. predator (piscivorous birds) or prey (freshwater fish) do not strongly overlap, parasites can potentially modify the host's habitat preference, therefore, increasing the probability of encounters that ultimately result in transmission success (Bethel & Holmes, 1973; Dianne *et al*., 2011). Additionally, parasites have the ability to make their hosts more conspicuous to predators by manipulating their cryptic behaviour (i.e. increasing host's activity) (Lafferty & Morris, 1996; Dianne *et al*., 2011). An increase in the fish's activity will lead to them becoming more attractive to predators; as fish cannot be conspicuous and active simultaneously (Lima & Dill, 1990; Krause & Godin, 1995; Pulkkinen *et al*., 2000). Another common anti-predatory trait in nature is the ability to freeze or be motionless (Brown & Dreier, 2002; Kortet *et al*., 2007; Hemmi & Pfeil, 2010). Fish usually freeze or search for shelter in cases of an aerial predator attack (Voellmy *et al*., 2014). However, altering behaviour from freezing to increased activity too early can also be fatal as this change can make the animal more vulnerable to the predator's repeated attacks (Hammerschmidt *et al*., 2009; Hafer & Milinski, 2015). Numerous studies, primarily done in Europe, associated parasitic infection with changes in the behaviour of a wide range of fish hosts (Barber *et al*., 2000; Barber & Wright, 2005). By infecting the eyes of the fish, metacercariae of *Diplostomum* can cause impairments that affect their visual performance (Shariff *et al*., 1980; Stumbo & Poulin, 2016). In some cases, these parasites caused sensitive optical tissue damage and cataract formation resulting in impaired visual functions (Shariff *et al*., 1980; Pádros *et al*., 2018; Ubels *et al*., 2018). Two genera belonging to the family Diplostomidae (Digenea: Diplostomoidea) i.e. *Tylodelphys* and *Diplostomum* are known to infect the eyes of their intermediate hosts (fish). Consequently, these eye trematodes provide a suitable model to study host manipulation in behavioural studies. Numerous studies on *Diplostomum* and their effect on host behaviour revealed that these parasite influences hosts' feeding behaviour, foraging efficiency, habitat

selection, shoaling nature and anti-predator behaviour (Crowden & Broom, 1980; Owen *et al*., 1993; Seppäla *et al*., 2004, 2005a, b, 2008, Grobbelaar, 2011; Seppäla *et al*., 2012; Gopko *et al*., 2015, 2017; Flink *et al*., 2017). However, these behavioural studies were largely performed on various European host species (*A*. *alburnus*, *Abramis brama* (Linnaeus, 1758), *Blicca bjoerkna* (Linnaeus, 1758), *C*. *lavaretus*, *G*. *aculeatus*, *G*. *cernuus*, *L*. *leuciscus*, *Neogobius melanostomus* (Pallas, 1814, Gobiidae), *O*. *mykiss*, *O*. *eperlanus*, *P*. *phoxinus*, *R*. *rutilus* and *S. alpinus*) with very high intensity of infections with *Diplostomum* spp. reaching up to 87 metacercariae per infected fish reported from Gulf of Gdańsk, southwestern and N.E. Bothnian Bay, Baltic Sea, Hungary, Slovakia and Sweden (Kvach & Skóra, 2007; Kvach & Winkler, 2011; Seppäla *et al*., 2011; Kudlai *et al*., 2017; Flink *et al*., 2017).

Even though these studies have focused on *Diplostomum* from numerous fish hosts belonging to the families Cyprinidae, Gasterosteidae, Gobiidae, Percidae, Osmeridae and Salmonidae. In Africa, there are no data on the effects of *Diplostomum* on bottom dwelling fish such as *S*. *zambezensis* (Mochokidae). Currently, there are over 131 species of *Synodontis* Cuvier, 1816 reported and widely used in the aquarium fishing trade due to the diverse colourations (Friel & Vigliotta, 2011). *Synodontis zambezensis* is the only species within this genus that is found in South African river systems including the Limpopo River and Phongolo floodplain system (Skelton, 2001; Bruwer & van der Bank, 2003). In the Phongolo floodplain, *S*. *zambezensis* is a readily available food source for rural communities (Coetzee *et al*., 2015). These fish are nocturnal, bottom feeders that occur in a wide habitat range (Sanyanga, 1998). Their diet consists of detritus and plant matter as well as small invertebrates and snails (Skelton, 2001). They are easy to keep in aquaria, making them a good laboratory species for investigating changes in behaviour induced by infections with *Diplostomum* metacercariae. To date, thirteen species of parasites; one trichodinid, *Trichodina heterodentata* Duncan, 1977, two monogeneans, *Synodontella synodontii* (Paperna & Thurston, 1968) [syns. *Ancyrocephalus synodontii* Paperna & Thurston, 1968; *Schilbetrema synodontii* (Paperna & Thurston, 1968)], *Synodontella zambezensis* Douëllou & Chishawa, 1995 (Ancyrocephalidae), and five nematodes, *Labeonema synodontisi* (Vassiliadès, 1973) (syn. *Raillietnema synodontisi* Vassiliades, 1973) (Atractidae), *Paracamallanus cyathopharynx* (Baylis, 1923) (Camallanidae), *Synodontisia thelastomoides* Petter, Vassiliadès & Troncy, 1972 (Pharyngodonidae), *Spinitectus polli* Campana-Rouget, 1961 (syn. *Spinitectus zambezensis* (Boomker, 1993) (Cystidicolidae) and *Procamallanus daleneae* (Boomker, 1993) (syn. *Spirocamallanus daleneae* (Boomker, 1993) (Camallanidae) as well as three unindentified nematode species namely, *Capillaria* sp., *Rhabdochona* sp., Philometridae gen. sp.; and two arguloida, *Dolops ranarum* (Stuhlmann, 1892) [syn. *Gyropeltis ranarum* Stuhlmann, 1892], *Ergasilus mirabilis* Oldewage & Van As, 1987 have been reported to infect *S*. *zambezensis* in Africa (Boomker, 1994; Khalil & Polling, 1997; Raphahlelo *et al*., 2016; Scholz *et*

al., 2018). Surprisingly, no digenean trematodes have previously been reported from *S. zambezensis*.

In South Africa only a single study exploring the effect of parasites on their fish hosts have been done (Grobbelaar, 2011; unpublished dissertation). In her dissertation, Grobbelaar (2011) investigated the effects of diplostomid trematodes on the behaviour of *T. sparrmanii* and *C. rendalli* based on predator simulated exposures (*T. sparrmanii*) and different light intensity exposures (*C. rendalli*). However, this study noted no effects of identified "*Diplostomum* spp." on the behaviour of infected and uninfected *T. sparrmanii* and *C. rendalli* based on both the escape response to simulated aerial attacks and light intensity exposures. An overall low mean intensity of the *Diplostomum* infection were recorded during this study and the author concluded that no obvious pathological changes in fish could be reported due to the small number of parasites present in the eye vitreous humour and brain of the fish. However, this can largely be due to the fact that the results obtained by Grobbelaar (2011) relied on visual observations only and numerous environmental and human factors could have influenced the results. Moreover, the identification of the metacercariae during this study may have been erroneous due to the authors not taking previous studies reporting on *Diplostomum* species into consideration. The morphological characteristics and site of infections in host suggest that some of the metacercariae for *Diplostomum* type 1–3 and *Diplostomum* type a, b and d belong to other genera within the Diplostomidae. Thus, reliable data on the effects of metacercariae of *Diplostomum* on their fish host (especially bottom-dwelling fish) in South Africa is non-existent. The development of Ethovision software in the late 1900's contributed significantly to studies on the behaviour of animals enabling reliable and consistent data outputs for statistical analysis that serve as supporting material for the established "visual" theories of previous behavioural studies (Noldus *et al*., 2001). In the present chapter the influence of metacercariae of *Diplostomum* on the behaviour of *S. zambezensis* based on visual observations combined with statistical analyses of video recording data outputs are investigated. It is hypothesised that the metacercariae of *Diplostomum* will have an influence on the behaviour of their naturally infected fish hosts. In order to address this hypothesis, the following research questions were set:

- Will there be a difference in natural behaviour between fish infected with *Diplostomum* spp. and uninfected fish, therefore, making them more susceptible to predation?
- Will *Diplostomum*-infected fish spend more time in the top zones/ surface areas than uninfected fish?
- Will infected fish be more active (measured in distance moved, acceleration and number of zone alternations) than uninfected fish?
- Will infected fish spend less time immobile (in other words scurry more) after an attack?

- Will there be a difference in responses in fish between the different attack-methods (heron vs fly-by)?

4.2 Materials & Methods

Laboratory experiments

The required acclimation times for both group and individual behaviour of *S. zambezensis* were determined in the National Aquatic Bioassay Facility (NABF) of the NWU (26°41'09.2"S 27°05'40.2"E), Potchefstroom Campus prior to exposures in the field. A total of 19 individuals of *S. zambezensis* were collected from laboratory tanks housed for a period of minimum six months in the aquarium of the NWU WRG. Behaviour analysis tanks (90 cm x 40 cm) were filled with aerated RO water and kept at a constant temperature of 21°C prior to testing. Three fish (in triplicate) and individual fish (n = 10) were transported to the behaviour analysis tank in the Behaviour Room of the NABF (SOP NWU-00272-17-A5). The behaviour of the group (n = 3) and individual trials (n = 10) were recorded using a Basler GigE (Germany) camera (placed in front of the tank) for the duration of 4 hours at 25 frames per second, where after the videos were analysed using EthoVision XT 14 software (Noldus, The Netherlands). Video recordings were assessed by physically viewing them while interpolating any missing data points where the software was unable to correctly track movements. Behavioural analysis profiles were set up within the software and the quantitative locomotor activity end points were swimming distance and speed as well as mobility state. The data obtained during the lab experiments were used to establish the required acclimation time (1 h) for individual fish that were used in the field experiments. No fish were dissected during these trials.

Field experiments

Twenty-two fish of *S. zambezensis* were kept in groups separate from other collected fish species for the behavioural experiments and were transported in containers with fresh river water and air pumps from the sampling site to the field laboratory (Fig. 4-1) where they were transferred to large cooler boxes (SOP NWU-00272-17-A5). All fish were acclimatised overnight in aerated large aquaria (acclimation tanks) with fresh river water prior to behavioural analysis. Behavioural analysis tanks were drained and replaced with fresh water daily to avoid any chemical or hormonal influence of the experiments conducted on fish from the previous day. The water in the acclimation tanks and behavioural analysis tanks (90 cm x 40 cm) were aerated and temperature was kept constant at 21°C for the entirety of the behavioural experiments. In the acclimation tanks, a 25% water change was made daily. The experimental setup allowed experiments to be conducted for two fish at the same time (Fig. 4-2). Fish (11 – 21 cm in total length) were individually transported to the behavioural analysis tanks and left for the required acclimatisation period of 1 hour prior to

the experiment; while video recordings were taken aeration was turned off. The videos were recorded using a Panasonic HC-V180 camcorder (Malaysia) at 25 frames per second and recordings started once the fish were placed in the behaviour analysis tank to minimise human influence during the exposures. Following 1 hour, the separated individual fish were exposed to one of two different “attack” stimuli from the outside of the tent using a pulley mechanism. This was done to avoid human interference in the results. One fish was exposed to a water contact stimulus i.e. Grey Heron “attacking” the fish with three strikes, splashing the water (Fig. 4-3 A, B). The other fish was exposed to an aerial, non-contact stimulus of a bird “cut-out” i.e. Gull flying above the tank creating a shadow (Fig. 4-3 C, D). These attacks were repeated 3 times for each fish with 10-minute intervals. All the steps were repeated in a random manner for the fish collected during this study. The videos were saved to an external hard drive for later analysis in the laboratory (NABF) at the NWU, Potchefstroom Campus. Following the behavioural experiments, all fish were euthanized and examined for the presence of metacercariae of *Diplostomum* in the eyes and brain following the same procedures as in Chapter 3, Section 3.2.2. Finally, all metacercariae found in *S. zambezensis* were identified to the genus level (applying morphological and molecular analyses) as in Chapter 3, Section 3.2.

Figure 4-1: Field laboratory at the Ndumo Game Reserve campsite, KwaZulu-Natal Province.

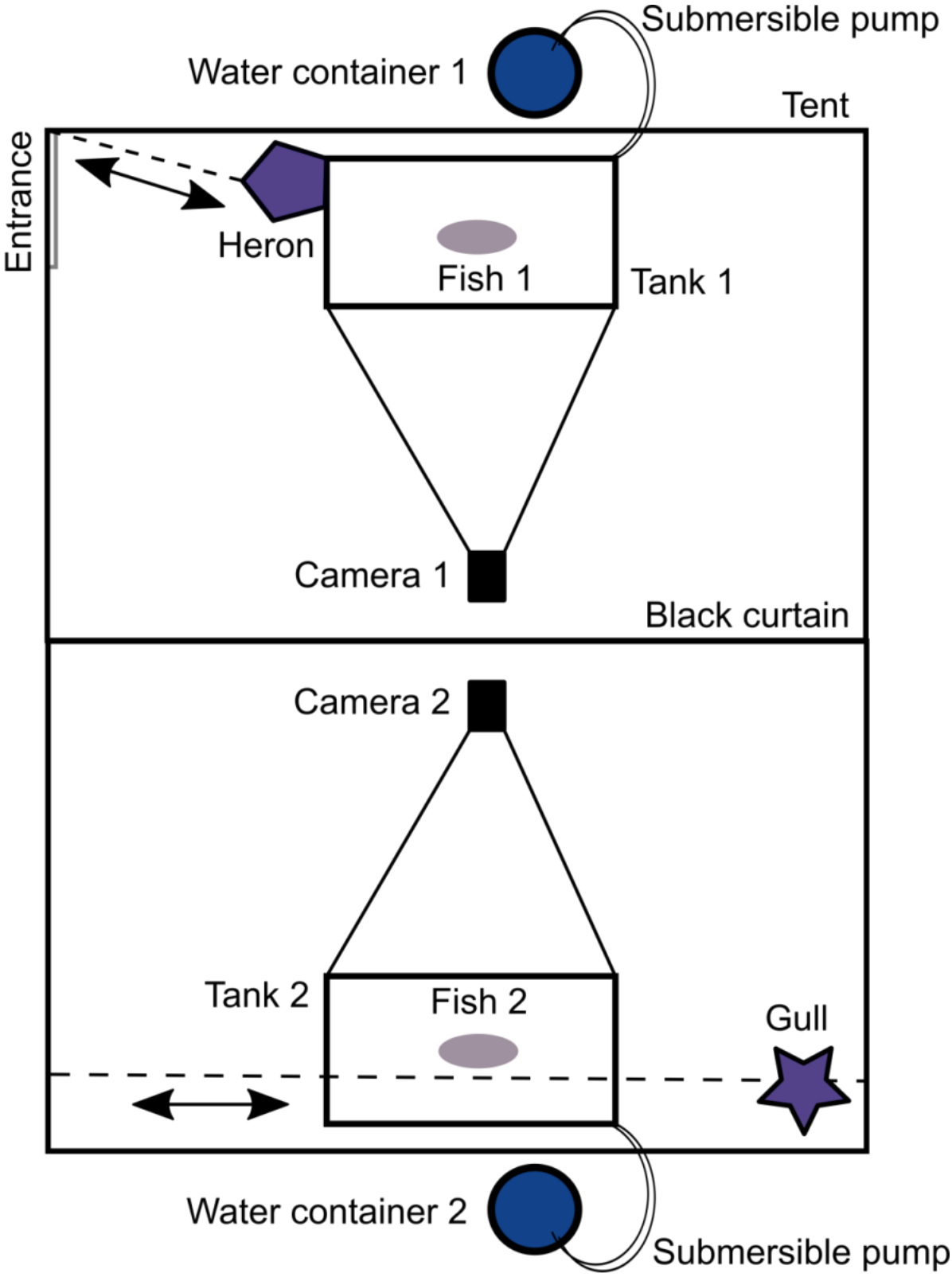


Figure 4-2: Design of the experimental setup in the tent of the behaviour experiments.

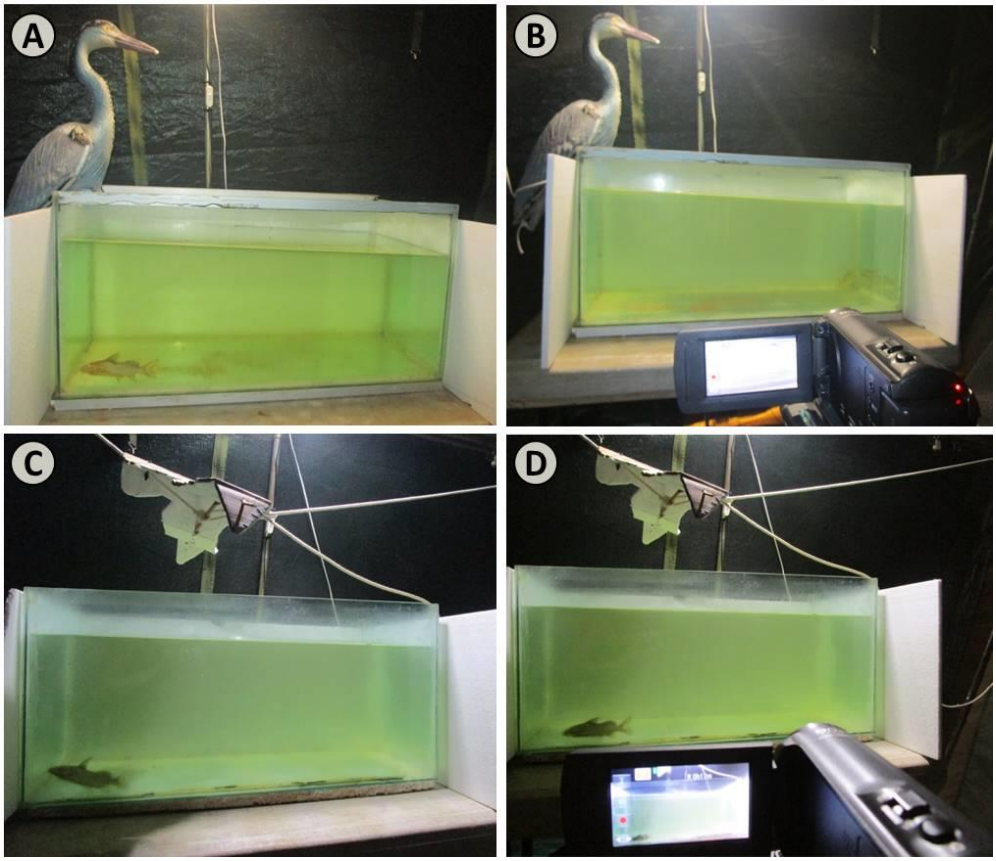


Figure 4-3: Behavioural experiments setup. A, B, heron attack; C, D, fly-by attack.

Video analysis of fish behaviour

The data from the recordings of both laboratory- and field-based experiments were analysed in the NABF using EthoVision XT 14 software (Noldus, The Netherlands). The video files for each fish were uploaded into the program. Next, the arena (tank) was digitally divided into top and bottom zones using EthoVision XT 14 (Fig. 4-4). The top zone made up the top third (12 cm from water line) of the tank and the bottom zone the remaining two thirds of the tank. A digital calibration of the behaviour tank was done using length (90 cm). The detection settings of the fish in the tank/ arena was confirmed and videos were acquired.

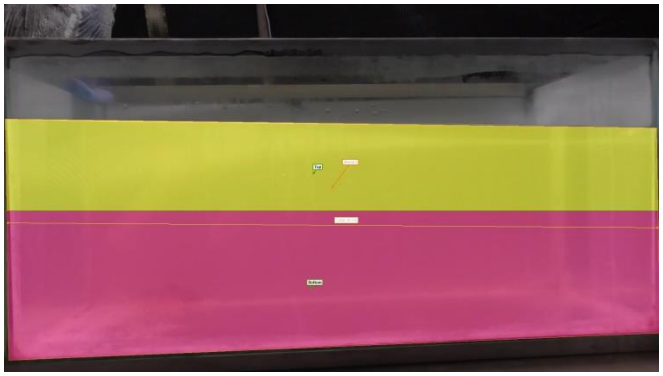


Figure 4-4: Arena settings of behaviour experiments.

The track editor tool allowed confirmation that the fish was tracked during the entire video by physically viewing them and inserting any missing data points by placing the marker on the same position on the fish at the coordinates it was in the arena in that point in time as well as reassigning any incorrect data points where software may have lost track of the fish in the arena. The x- and y coordinates of the fish within the arena (set in 1-minute time bins) were used to determine quantitative endpoints in the data analysis. Data profiles were divided and defined for the acclimation and attack periods in the field experiments. The endpoints set for the analysis profiles of the field experiments included distance moved, swimming speed, minimum and maximum acceleration, time spent in top and bottom zones and number of zone alternations. Data were acquired in EthoVision XT 14 and exported to GraphPad Prism 7 for statistical analysis.

Statistical Analysis

All data were tested for normality using the D'Agostino & Pearson omnibus and Shapiro-Wilk normality test. The laboratory acclimation, field acclimation and attack data were analysed by using unpaired t-test with Welch's correction in order to compensate for unbalanced sample sizes for the relevant parameters including distance, velocity and mobility state (lab acclimation); distance, velocity, minimum acceleration, maximum acceleration, time in top zone and time in bottom zone (field acclimation); and distance, velocity, time in top zone, time in bottom zone and number of zone alternations (attacks). Attack response between the contact (heron) and non-contact (fly-by) attack data were analysed by performing a One-way ANOVA using the Krustal-Wallis test for non-parametric data with post-hoc Tukey's multiple comparison test. This was done for distance, velocity, and mobility state. The significance of the results was established at $p<0.05$. The graphs were compiled in GraphPad Prism 7 and the data reported as mean and SEM (standard error of the mean).

4.3 Results

Laboratory acclimation

The swimming behaviour of the individuals of *S. zambezensis* stabilised after a period of 60 minutes as seen for both the distance moved (Fig. 4-5A), swimming speed (Fig. 4-5B) as well as the mobility state (Fig. 4-5C). Therefore, an acclimation time of one hour was established prior to field exposures based on the analysis of the trials (n = 10) from *S. zambezensis* (previously collected from NGR, 2017) during the laboratory acclimation tests.

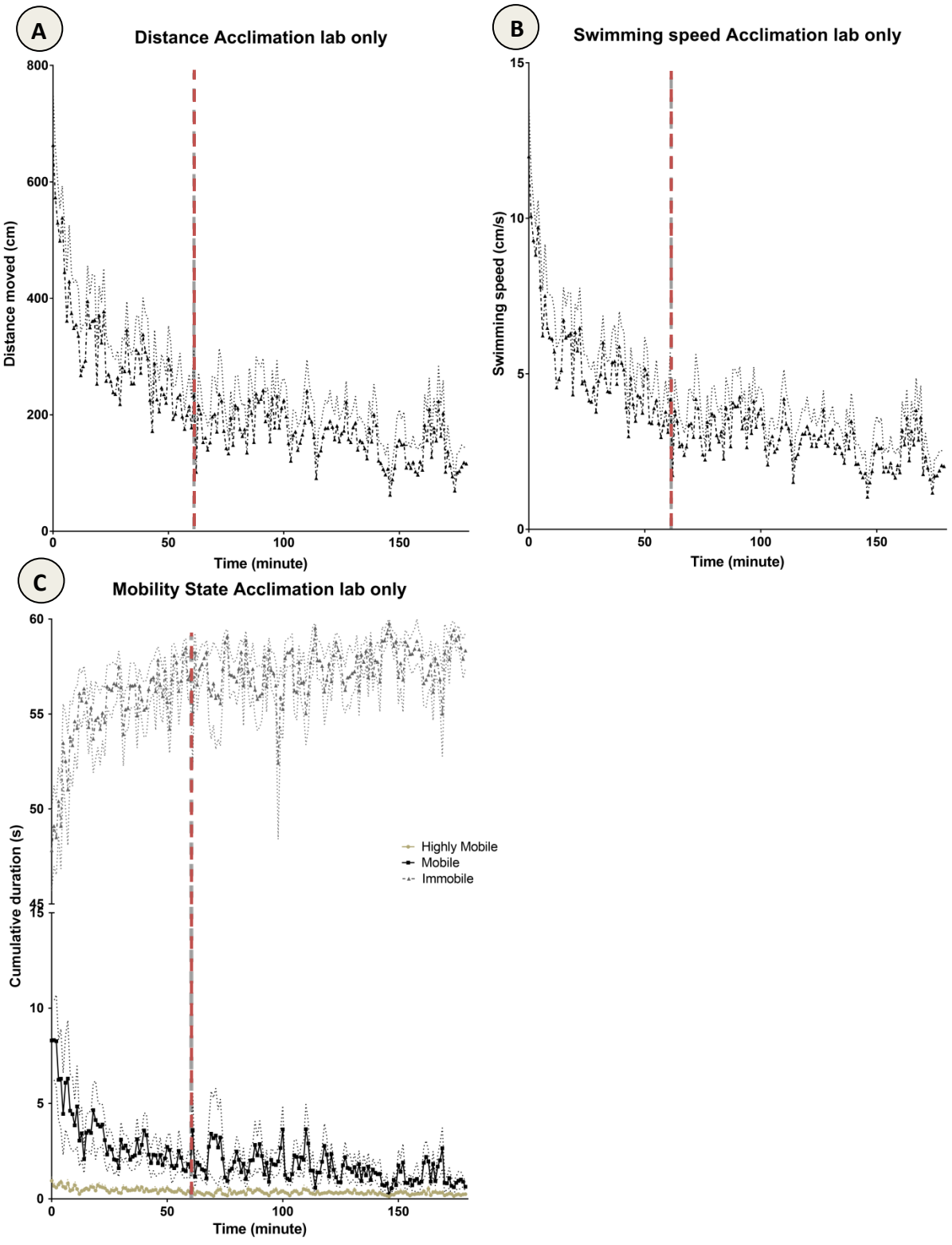


Figure 4-5: Results of the behaviour of *Synodontis zambezensis* in the laboratory acclimation trials analysed over a duration of four hours. A, Distance (cm); B, Swimming speed (cm/s); C, Mobility state (s).

Field acclimation

In the field, 22 individuals of *S. zambezensis* were selected at random for the behavioural experiments. Several observations associated with the behaviour of *S. zambezensis* were compared between infected (n = 15) and uninfected fish (n = 7). The prevalence (5%) and intensity of infection (1 metacercaria per fish) of *Diplostomum* sp. in *S. zambezensis* were very low compared to the high prevalence (55%) and intensity of infection (1–12 metacercariae per fish) of *Diplostomum* sp. 14 in *S. zambezensis* from the Phongolo River, NGR. After performing the D'Agostino & Pearson normality test for all variables (distance, swimming speed, minimum acceleration, maximum acceleration, in zone (top), in zone (bottom) and zone alternation), data distribution for zone alternation was the only variable that passed the normality test indicating that the data is distributed normally. The remaining variables all had abnormal data distributions and non-parametric statistical tests with Welch's correction were used. The Welch's t-test calculated the mean distance travelled (Infected = 172.5 cm; uninfected = 162.6 cm), mean swimming speed (Infected = 2.88 cm/s; uninfected = 2.71 cm/s) and mean minimum acceleration (Infected = -809.8 cm/s^2; uninfected = -667.7 cm/s^2) and showed no significant differences between the infected and uninfected fish (Fig. 4-6A–C). However, significant differences for mean maximum acceleration (Infected = 933.4 cm/s^2; uninfected = 735.9 cm/s^2) with a p-value of 0.0147 was calculated between infected and uninfected fish (Fig.4-6D).

The largest variations were noted from the time spent in top and bottom zones (p = 0.0002), where the mean duration spent in the top zone was significantly higher for the infected fish (Infected = 6.71 s; uninfected = 4.50 s) and the mean duration spent in the bottom zone were significantly higher for the uninfected fish (Infected = 53.29 s; uninfected = 55.50 s) (Fig. 4-6E, F) within a one minute time bin. This was further confirmed by the significant difference (p = 0.0016) in the total number of zone alternations within the infected fish (Infected = 1.87 ± 0.20; uninfected = 1.15 ± 0.74) (Fig. 4-6G). Therefore, the infected fish were most likely found in the top zones and significantly alternate between zones during acclimation proposing a significant difference in host behaviour between infections.

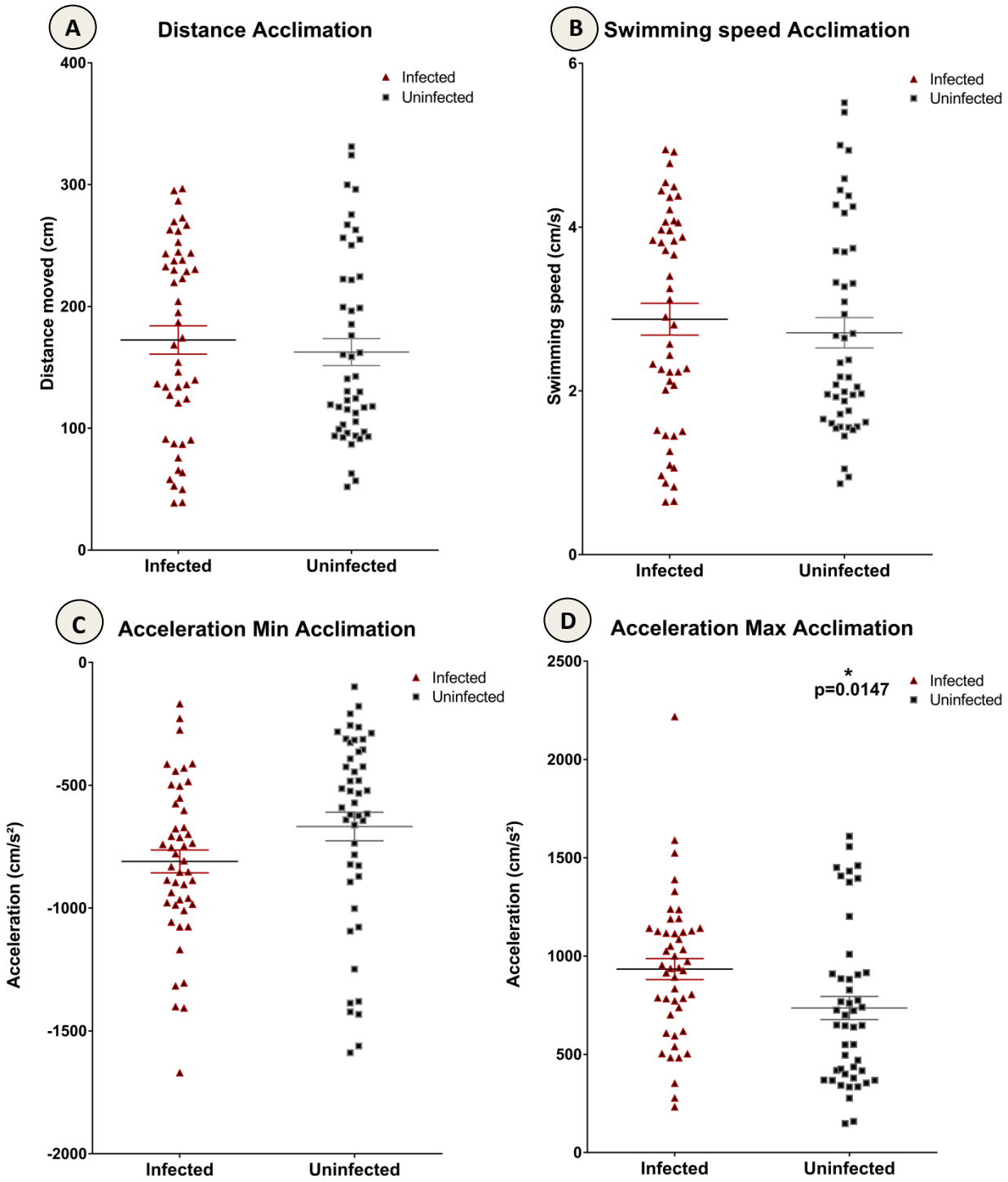


Figure 4-6: Mean ± SEM activity during field acclimation of infected *vs* uninfected fish of *Synodontis zambezensis*. A, Distance (cm); B, Swimming speed (cm/s); C, Minimum acceleration (cm/s^2); D, Maximum acceleration (cm/s^2). *Asterisks indicate significant differences between the infected and uninfected *S. zambezensis* (Welch's t-tests; $p < 0.05$).

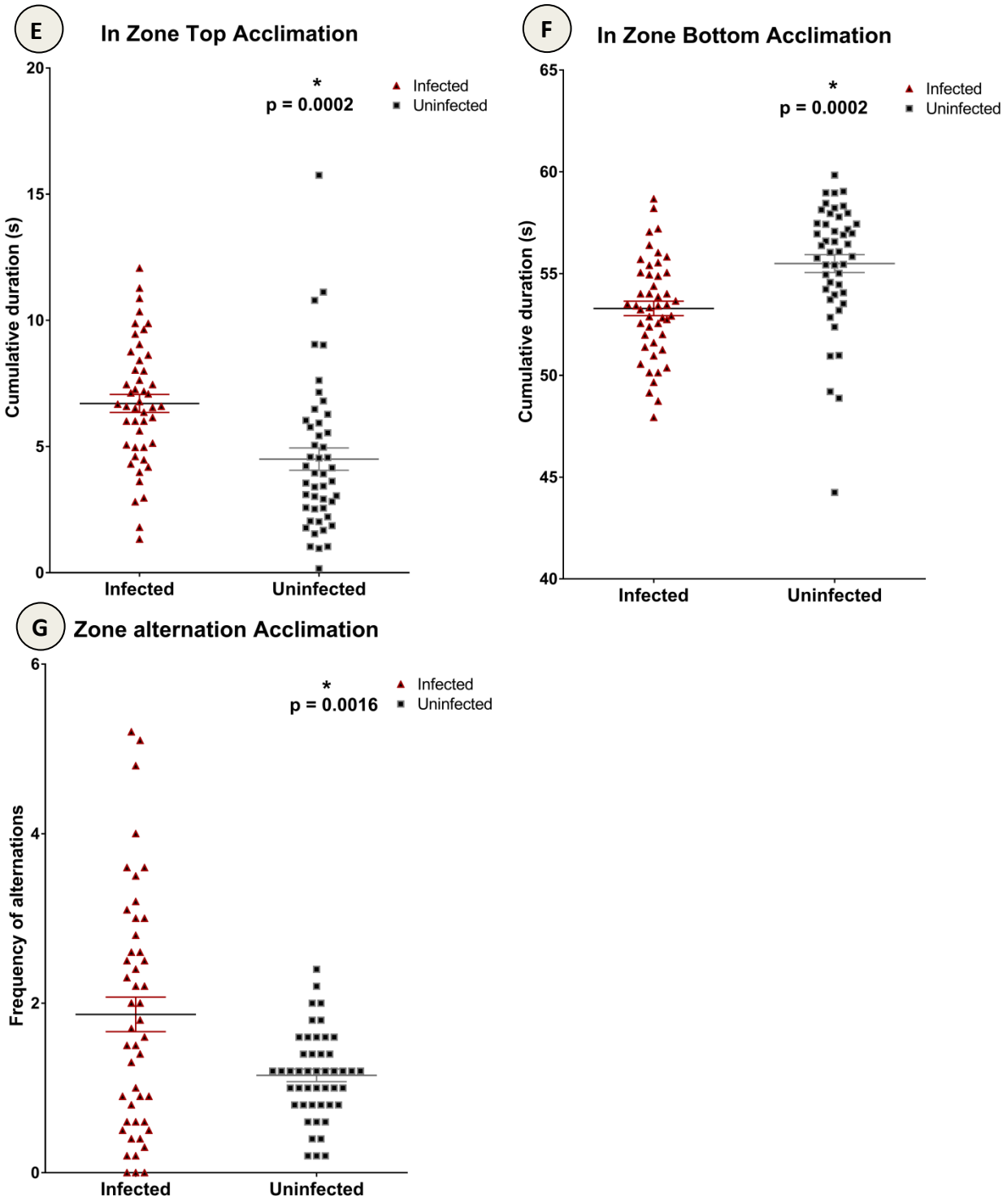


Figure 4-6 (continued): Mean ± SEM activity during field acclimation of infected *vs* uninfected fish of *Synodontis zambezensis*. E, In Zone (Top) (s); F, In Zone (Bottom) (s); G, Zone alternation. *Asterisks indicate significant differences between the infected and uninfected *S. zambezensis* (Welch's t-tests; $p < 0.05$).

Heat maps illustrated differences between uninfected and infected fish of *S. zambezensis* during acclimation, where uninfected fish spent most time in the bottom zone (Fig. 4-7A). Infected fish also spent most time in the bottom zone overall, but alternations between zones occurred more frequently between the top and bottom zones (highly active and high frequency of zone alternations) (Fig. 4-7B).

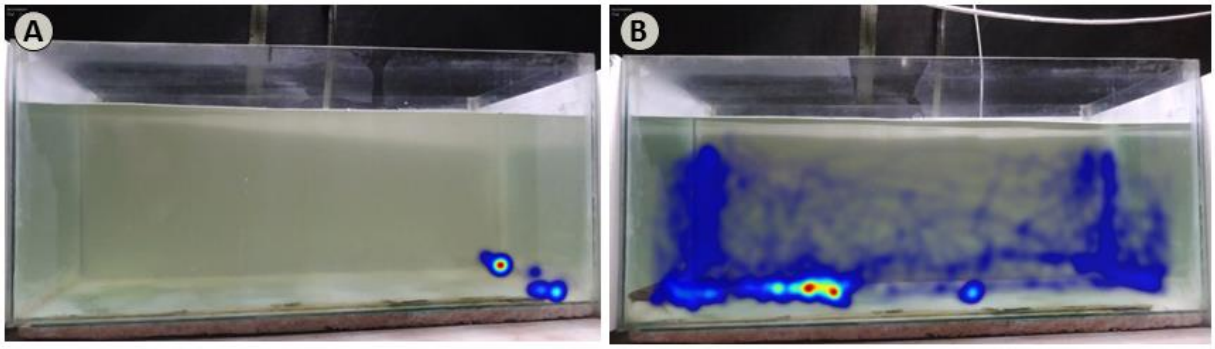


Figure 4-7: Heat maps of movement patterns of *Synodontis zambezensis* during acclimation. A, uninfected fish; B, infected fish.

Attacks (combined)

All three attacks showed significant differences in the behaviour between infected and uninfected fish of *S. zambezensis* in mean distance moved (Infected = 31.47 cm; uninfected = 70.57 cm; p = 0.0001) and mean swimming speed (Infected = 0.55 cm/s; uninfected = 1.18 cm/s; p = 0.0002) where uninfected fish moved a larger distance and at higher swimming speed compared to the infected fish (Fig. 4-8A, B). There were no significant differences ($p > 0.05$) between infected and uninfected fish based on the position of fish in the top zone (Infected = 2.87 s; uninfected = 2.23 s) and bottom zone (Infected = 57.54 s; uninfected = 57.77 s) of the tank as all fish preferred to remain in the bottom zone for the duration of the experiment (Fig. 4-8C, D). In contrast, a significant difference in alternation between the top and bottom zones were recorded (Infected = 0.115; uninfected = 0.48; p = 0.0002) in which uninfected fish were more mobile and moved between zones (also confirmed by the distance moved and swimming speed of uninfected fish) (Fig. 4-8E).

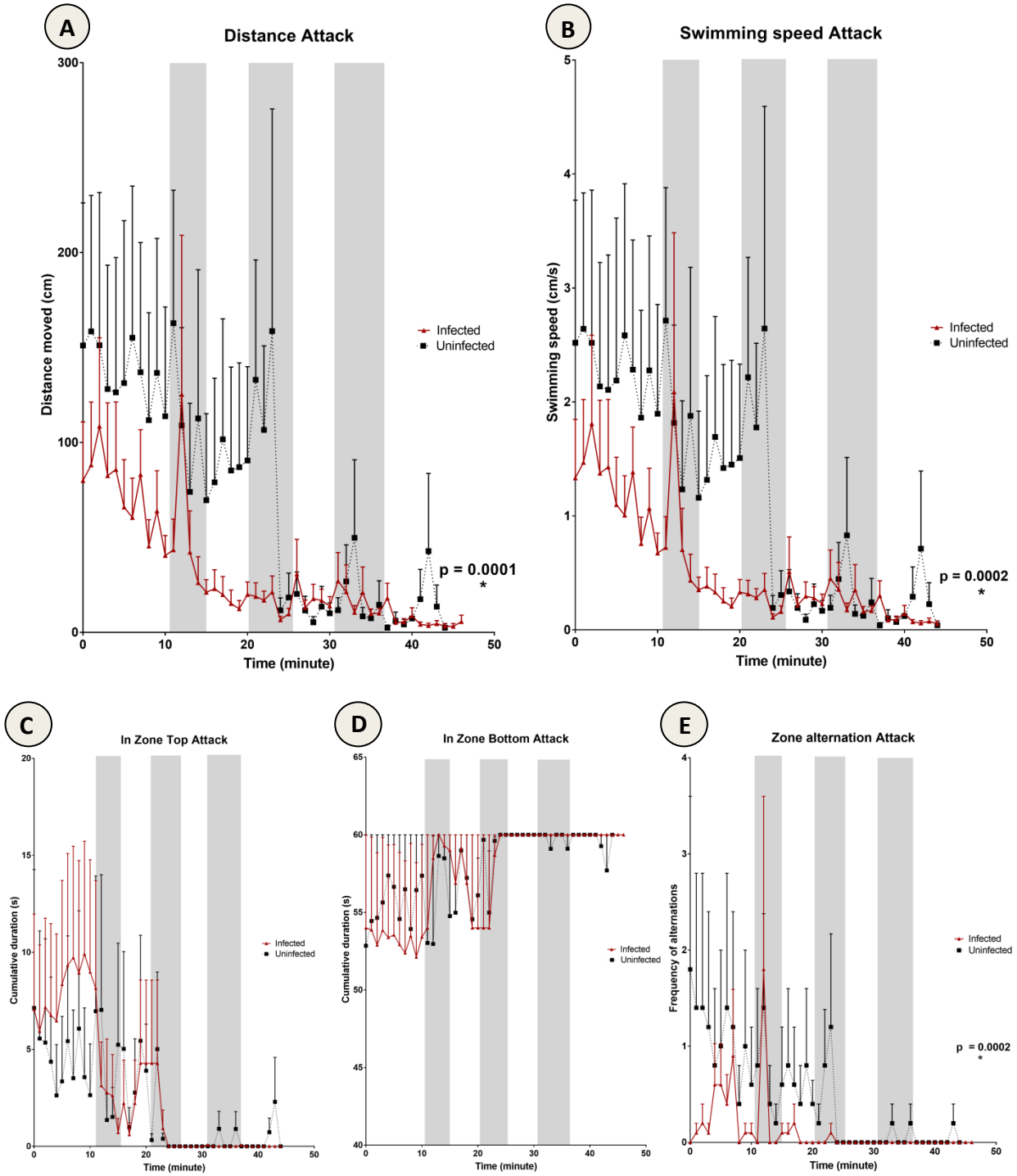


Figure 4-8: Mean ± SEM activity for all attacks (heron and fly-by combined) on infected and uninfected fish of *Synodontis zambezensis* during field exposures. A, Distance (cm); B, Swimming speed (cm/s); C, In Zone (Top) (s); D, In Zone (Bottom) (s); E, Zone alternation where shaded areas indicate bird attack intervals over the trials. *Asterisks indicate significant differences between the infected and uninfected *S. zambezensis* (Welch's t-tests; $p < 0.05$).

Heat maps illustrated differences between uninfected and infected *S. zambezensis* in 1-minute intervals during attack 1 (Fig. 4-9A, D); attack 2 (Fig. 4-9B, E) and attack 3 (Fig. 4-9C, F). The uninfected fish (Fig. 4-9A–C) were more mobile during experiments and more frequent zone alternations were observed. In contrast, the infected fish were mostly immobile and preference of the bottom zone in tank (Fig. 4-9D–F).

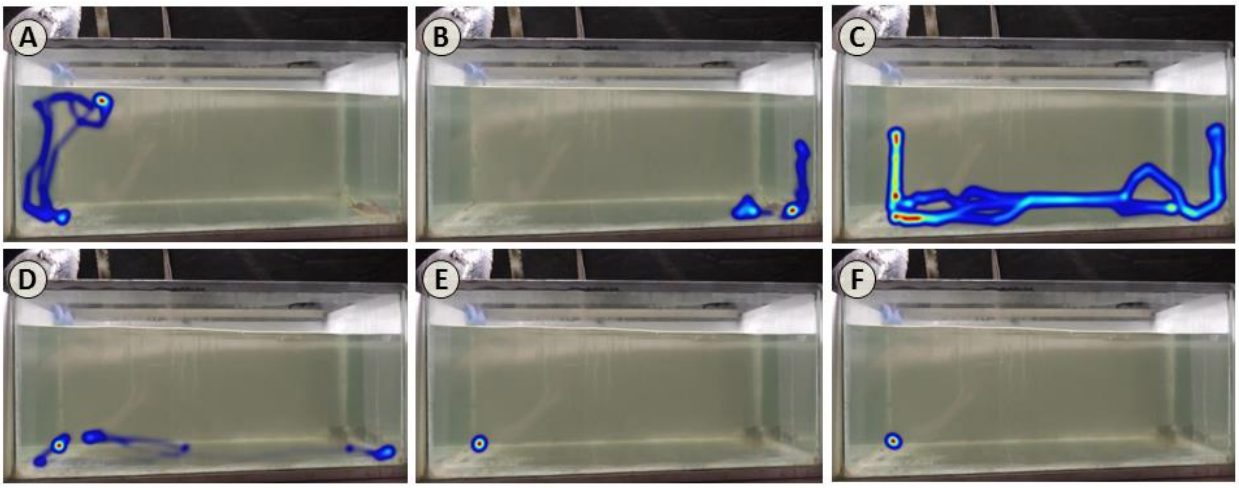


Figure 4-9: Heat maps of movement patterns of uninfected *vs* infected *Synodontis zambezensis* during three heron attacks. A, attack 1 (U); B, attack 2 (U); C, attack 3 (U); D, attack 1 (I); E, attack 2 (I); F, attack 3 (I). Abbreviations: U, uninfected fish; I, infected fish.

A summary of the visual observations is presented in Table 4-1. Uninfected fish (n = 5) showed the same pattern of behaviour following each attack as most fish (71.4%) were immobile after all three attacks. Infected fish (n = 10, 66.6%) scurried and were swimming erratically following the first attack. After the second attack, the majority of the fish (54%) continued to scurry, but more fish became immobile (46% from 33%) and finally after the third attack, most fish were immobile (54%) and fewer fish scurried (46%).

Table 4-1: Visual observations between infected and uninfected *Synodontis zambezensis*.

	Infected (n = 15)		**Uninfected (n = 7)**	
	Scurry	**Immobile**	**Scurry**	**Immobile**
Attack 1	10 (66.6%)	5 (33.3%)	2 (29.6%)	5 (71.4%)
Attack 2	8 (54%)	7 (46%)	2 (29.6%)	5 (71.4%)
Attack 3	7 (46%)	8 (54%)	2 (29.6%)	5 (71.4%)

The track visualisation illustrated the differences between uninfected and infected fish (scurry and immobility) after attack 1 (Fig. 4-10A, D); attack 2 (Fig. 4-10B, E) and attack 3 (Fig. 4-10C, F). As indicated in Table 4-1, the uninfected fish spent most time immobile for the duration of the experiment (Fig. 4-10A–C) whereas a higher percentage infected fish scurried after the first attack but became more immobile after each attack (Fig. 4-10D–F).

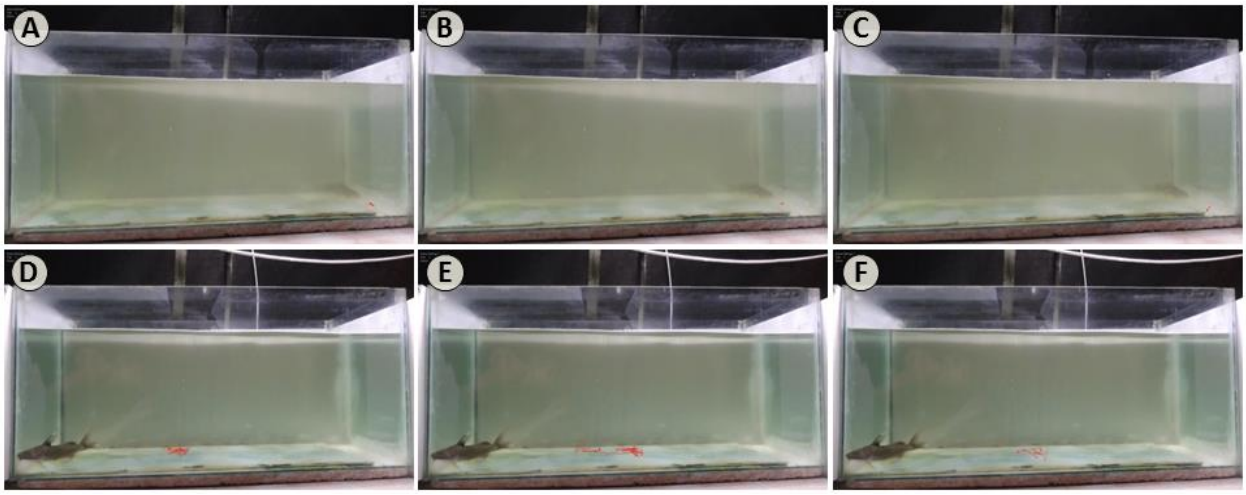


Figure 4-10: Track visualisation of uninfected *vs* infected *Synodontis zambezensis* after three fly-by attacks. A, attack 1 (U); B, attack 2 (U); C, attack 3, (U); D, attack 1 (I); E, attack 2 (I); F, attack 3 (I). Abbreviations: U, uninfected fish; I, infected fish.

Attack Response (heron *vs* fly by)

A One-way ANOVA (Kruskal-Wallis test) showed significant differences between infected fish and uninfected fish of the heron attack for all but mobility state. Significant differences in distance moved (mean rank difference = -44.23; p = 0.0004), swimming speed (mean rank difference = -40.93; p = 0.0012), highly mobile - mobility state (mean rank difference = -31; p = 0.024) and mobile – mobility state (mean rank difference = -32.81; p = 0.017) can be visualised in Fig. 4-11A–D. Based on the measurements from Ethovision XT 14, significant differences were calculated for all variables except when comparing between infected fish in the heron attack *vs* infected fish in the fly-by attack (Fig. 4-11E). Most of the fish (both infected and uninfected) in the fly-by attacks (Fig. 4-11E) did not illicit a response by the shadow created overhead of the bird cut-out and the majority of the fish were immobile during the experiment (as can be seen in Table 4-1 and Fig. 4-10A–C).

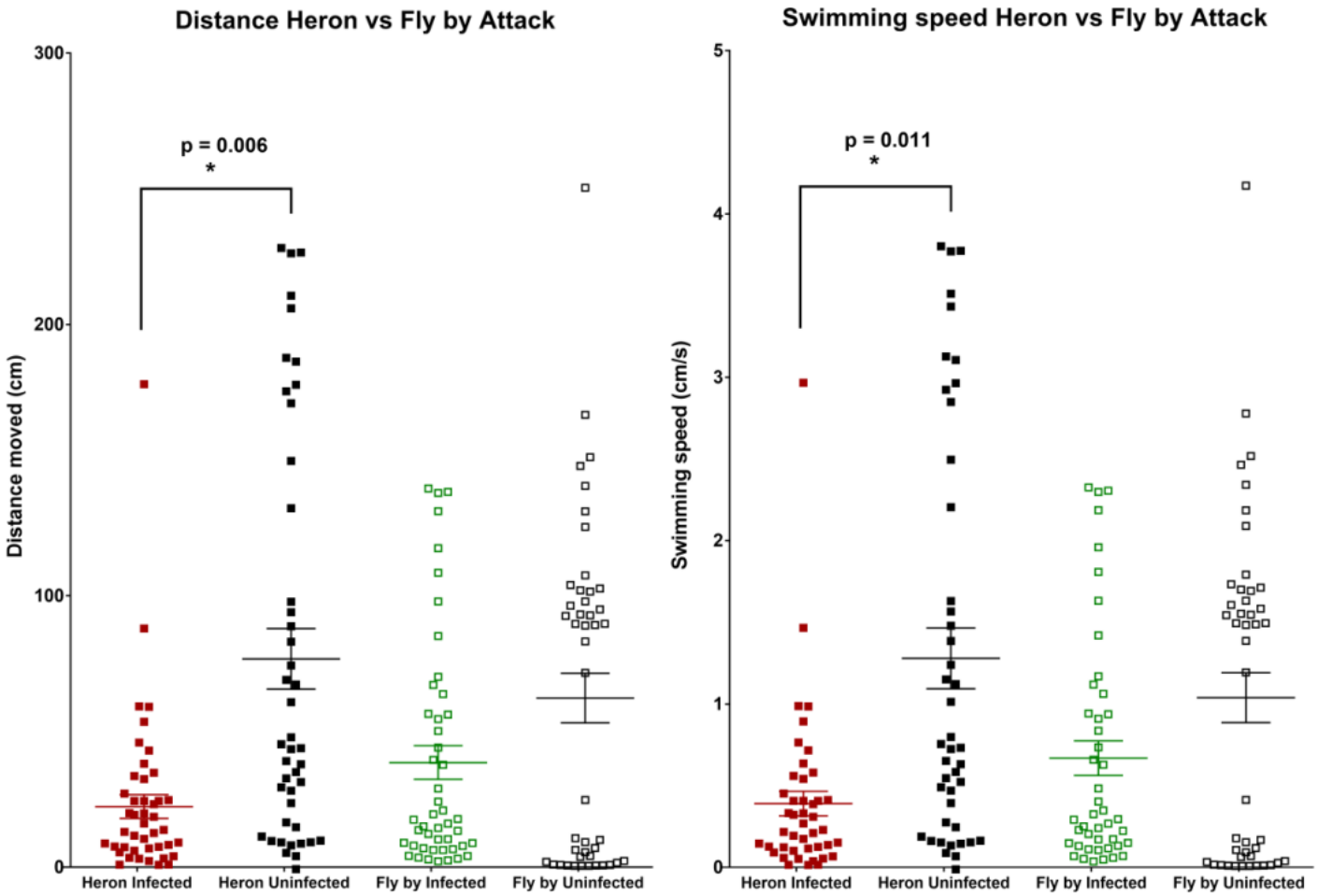


Figure 4-11: Mean ± SEM activity between heron *vs* fly-by attacks of infected and uninfected fish of *Synodontis zambezensis* during field exposures. A, Distance (cm); B, Swimming speed (cm/s). *Asterisks indicate significant differences between the infected and uninfected *S. zambezensis* (Dunnett T3; $p < 0.05$). Hash indicates no significant differences between the variables all other variables show significant difference.

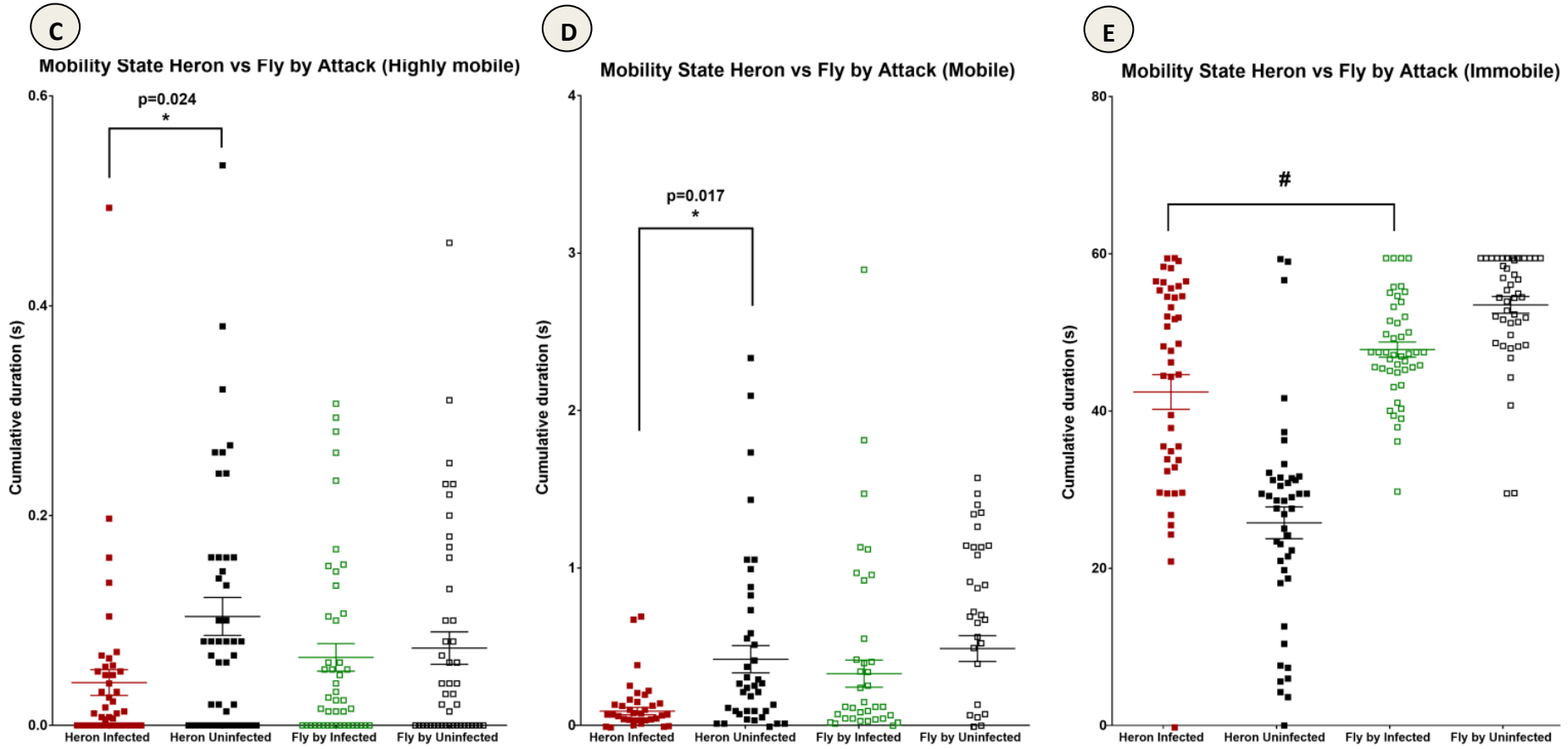


Figure 4-11 (continued): Mean ± SEM activity between heron *vs* fly-by attacks of infected and uninfected fish of *Synodontis zambezensis* during field exposures. C, In Zone (Top) (s); D, In Zone (Bottom) (s); E, Zone alternation. *Asterisks indicate significant differences between the infected and uninfected *S. zambezensis* (Dunnett T3; $p < 0.05$). Hash indicates no significant differences between the variables all other variables show significant difference.

To summarise, the results provide evidence of significant changes during normal swimming patterns induced by *Diplostomum* spp. in infected hosts *vs* uninfected hosts. During acclimations infected *S. zambezensis* preferred spending time in the top zone and had more varied zone alternations towards the top zones in the arena with altered acceleration movement. During attacks, these findings were the opposite, with uninfected fish moving further distances at higher swimming speed, spending more time in the top zone, and had altered movement acceleration. However, during visual observations, infected fish scurried more during an attack, where uninfected fish preferred to stay immobile. Refined analyses between different attack methods (i.e. heron *vs* fly-by), revealed significant differences only between infected and uninfected hosts during a heron attack.

4.4 Discussion

The most common behavioural changes induced by trophically transmitted parasites involve interference with the host's escape response and predation avoidance in order to increase parasite fitness and facilitate transmission by means of impairing the vision of fish hosts (Crowden & Broom, 1980; Owen *et al*., 1993; Seppälä *et al*., 2004, 2005a, b, 2008; Voutilainen *et al*., 2008; Seppälä *et al*., 2012; Gopko *et al*., 2017). The natural anti-predatory behavioural traits in the second intermediate hosts, *S. zambezensis* were affected by the infections with two species of *Diplostomum* identified in the present study (*Diplostomum* sp. and *Diplostomum* sp. 14). Initially, it was suspected that no parasite-induced behavioural changes of infected fish will be observed due to the relative low intensity of infections with digenean trematodes in fish hosts previously recorded in the study areas. However, significant differences between infected and uninfected fish were observed and calculated during both acclimation periods and two different simulated avian attacks, indicating that even low infections with metacercariae of *Diplostomum* in *S. zambezensis* does affect the host's natural behaviour. In order to facilitate parasite transmission, the host's behaviour was altered by means of increased activity within the water column, increased time spent in top areas and more cases of erratic movements during attacks. It is generally accepted that an increase in activity makes fish more susceptible to predators (Krause & Godin, 1995).

Interestingly, previous studies investigating the effect of *Diplostomum* spp. on rainbow trout revealed that the fish infected with immature (not-ready-to-infect) eye fluke larvae were less active compared to control (uninfected) fish. Therefore, mature and immature metacercariae of *Diplostomum* altered host's behaviour in opposite directions, in other words, making the host less vulnerable to predators until the parasite reaches maturity/ infective stage or enhancing predation risk once the eye fluke has reached infective stage. This complex adaptation of parasites enhancing or suppressing predation risk of their hosts was suggested for numerous trophically transmitted parasites (Parker *et al*., 2009; Dianne *et al*., 2011; Hafer & Milinski, 2015). Parasites

also have the ability to change the host's preference to different habitats (e.g. Curtis, 1987; Miura *et al*., 2006; Miura & Chiba, 2007). Fish infected with metacercariae of *Diplostomum* in the present study preferred the top zones of the water column compared to the uninfected fish. Fish in shallow waters are especially susceptible to avian predation, as they are more conspicuous and easier to capture even by birds that cannot dive deep into the water. Nonetheless, the influence of parasites on depth preference has rarely been reported. Crowden & Broom (1980) found that the dace, *L*. *leuciscus*, Cyprinidae, infected with *Diplostomum* metacercariae changed their behaviour by spending more time in the surface layers. However, their study did not compare difference among infected and uninfected fish, but this approach cannot exclude possibilities of inverse relationships between fish depth preference and intensity of parasite infections (Gopko *et al*., 2017). This was later tested by Seppälä *et al*. (2004) who investigated whether Rainbow trout (*O*. *mykiss*) infected with *D. pseudospathaceum* preferred shallow water compared to uninfected fish. However, contrary to the findings of the present study, no significant differences were observed. Later, Gopko *et al*. (2017) found an increase in depth preference in infected rainbow trout agreeing with the findings of the present study that infected *S*. *zambezensis* preferred the top zones in the water column. Although a difference in host and arena size were determined as possible explanations for the differences in the findings (Gopko *et al*., 2017), we suggest that another factor may play a role in these differences since the fish used in the present study has a larger average body length of 16 cm *vs* 9.9 cm (Seppälä *et al*., 2004) or 9.4 cm (Gopko *et al*., 2017) and smaller tank size of 90 cm x 40 cm *vs* 314 cm^2 (Seppälä *et al*., 2004) or 1200 cm^2 (Gopko *et al*., 2017).

Fish activity and depth preference (i.e. top zones) were moderately correlated with each. If parasites have the ability to change two linked anti-predatory behaviours, i.e. cryptic colouration as well as freezing reaction, they will be able to double their chances on transmission ensuring that the host fails avoiding predators one way or another (Poulin, 2010). In the present study, significantly higher maximum acceleration values as well as the slightly increased distance moved and swimming speed during acclimation of infected fish *vs* uninfected fish were recorded. Infected fish also preferred to spend most of their time in the top zone of the arena. However, the opposite effects were observed during attacks, where uninfected fish travelled further distances at higher swimming speeds and travelled between top and bottom zones more frequently compared to infected *S*. *zambezensis*. Although the maturity of the metacercariae during the present study were not determined, this phenomenon could be explained by the proven ability of immature metacercariae to decrease predator susceptibility (Gopko *et al*., 2015), and could be considered in future studies investigating parasite induced changes on host behaviour in South Africa. Mature and immature parasites have different effects on the host's phenotype (Parker *et al*., 2009; Cézilly *et al*., 2014). While immature parasites enhance anti-predatory behaviour until infective stages, mature parasites suppress it in order to increase hosts' susceptibility to predation. In theory, immature parasites will try to "sabotage" changes in host behaviour (Hafer & Milinski, 2015). The

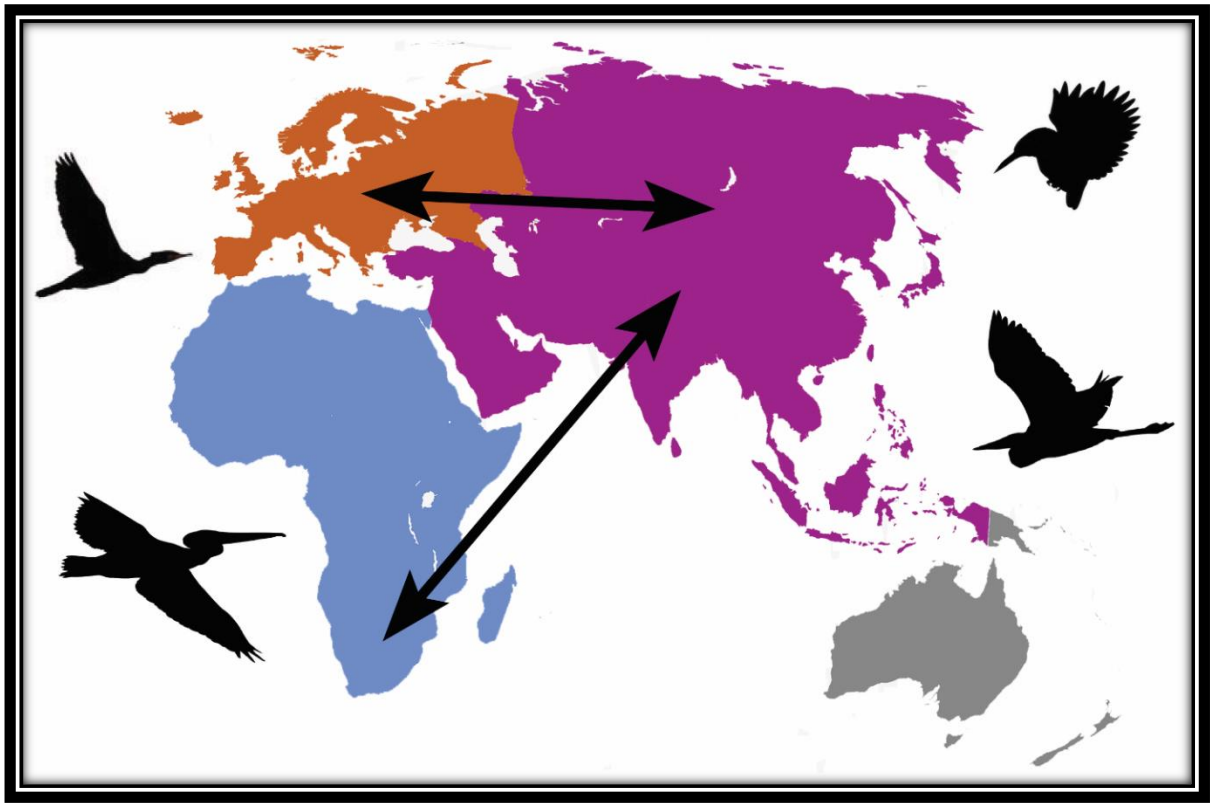
maturity of the metacercariae of *Diplostomum* was not recorded during this study; we therefore assume that these findings conform to previous studies and may serve as an explanation for the current findings.

Significant differences for the distance moved, swimming speed and mobility state between infected and uninfected *S. zambezensis* were best observed during simulations of "heron attacks". It is, therefore, important to select the correct simulation method closest resembling natural occurrences (i.e. avian predator splashing water during dive) for comprehensive results. This study did not investigate the parasite's influence on host's susceptibility to a real avian predator. Moore (2013) suggested that changes in behaviour were side effects of the pathology, is a questionable conclusion and should be discarded without any clear evidence. Additionally, even though pathology is linked to parasite transmission, it is unlikely that natural selection has been blind to pathology (Thomas *et al*., 2005; Cézilly *et al*., 2013). Finally, Karvonen *et al*. (2004) suggested vision deterioration as a mechanism of manipulation of *D. pseudospathaceum* metacercariae as a result of cataract formation on the eye lens of infected fish. Although, it was suggested that not all modifications induced by metacercariae of *Diplostomum* are explained by decreased vision (i.e. decreases in fish activity during immature larval stages, followed by increased activity after parasite maturation as well as observed short recovery time following simulated attacks cannot be explained by simple vision deterioration mechanisms). Therefore, some studies suggest a chemical influence on host behaviour (generally common among various host-parasite interactions) that should also be considered during the interpretation of results (Lafferty & Shaw, 2013; Hafer & Mikinski, 2015).

Finally, it is worth noting the following possible limitations or factors that may have played a role in the outcomes of the study: (i) movement or noise in or around the campsite where field experiments were carried out may have influenced the acclimation period of the fish; and (ii) the time (morning, afternoon or late afternoon) that the experiment was carried out may also influence the outcomes of the experiment due to the natural behaviour of the model fish species, *S. zambezensis* which is highly active at night (Skelton, 2001). Even with low intensity of infections (1–12 metacercariae per fish), changes in the natural behaviour of *S. zambezensis* (differences between infected and uninfected fish) were observed. Also, the presence of parasites from other groups within *S. zambezensis* should be taken into consideration and may also have an influence on the outcomes of behavioural studies. In this study, an unidentified caryophyllidian species was found in the intestines of nine *S. zambezensis* with a low intensity of infection of 1–6 cestodes per fish and a prevalence of 25% (Dr. Schaeffner, personal communication, 19 February 2020, Potchefstroom). Of these, six individuals were infected with both cestodes and trematodes, a factor that should be considered in the interpretation of the results as it is not known what the influence of other parasites in the same host may be and could also have effects on the behaviour of their hosts.

The results of the present study conform to that previously reported from Gopko *et al*. (2017), which suggest that even low intensity of infections show significant differences between infected and uninfected fish. Metacercariae of *Diplostomum* showed definite influences on the behaviour of *S*. *zambezensis* by means of habitat preference (upper layers/ top zones) and activity (mobility state) which as a result affects parasite transmission to definitive hosts.

Chapter 5: Summutative Discussion



CHAPTER 5: SUMMUTATIVE DISCUSSION

5.1 General Discussion

The plants and animals that currently live on Earth have continued to evolve for millions of years since the last mass extinction. Today, due to human activities including, pollution, agricultural development, overexploitation, habitat destruction, or distribution of invasive species, the biodiversity is at high risk of extinction. Many of the species may become extinct without being taxonomically described or even noticed. This is why studies on biological diversity are extremely important.

The African continent is home to a variety of animals with many of them being endemic, but our knowledge on the true biodiversity is limited. Parasites, despite their rather small size, play an essential role in ecosystems as they are present in all trophic levels within their hosts whether it be in the first intermediate-, second intermediate- or definitive host. Diversity on parasites in freshwater ecosystems in Africa remain poorly known, especially regarding trematodes. To date, 69 adult trematodes have been reported in freshwater fishes in Africa and even less is known for the larval stages. The simple morphology of larval stages makes reliable species identification almost impossible based on morphological analysis only (Kudlai *et al*., 2018).

In South African fishes, focus of the present study, there are three species based on adults (*Emoleptalea nwanedi* King, Smit, Baker & Luus-Powell, 2018, *Phyllodistomum bavuri* Boomker, 1984 and *Phyllodistomum vanderwaali* Prudhoe & Hussey, 1977) and 10 species of larvae trematodes currently known (Kudlai *et al*., 2018; Hoogendoorn *et al*., 2019). The larval stages of trematodes from the Diplostomidae, due to their pathogenicity, were reported in several studies that have been done in South Africa thus far (Prudhoe & Hussey, 1977; Mashego & Saayman, 1989; Khalil & Polling, 1997; Barson & Avenant-Oldewage, 2006; Madanire-Moyo *et al*., 2010; Chibwana *et al*., 2013; Moema *et al*., 2013; Hoogendoorn *et al*., 2019). There are nine unidentified and one identified species currently known from the Diplostomidae from freshwater fishes in South Africa: *Bolbophorus* sp. 3 (Hoogendoorn *et al*., 2019); *Diplostomum* sp. type I, *Diplostomum* sp. type II (Prudhoe & Hussey, 1977); *Diplostomum* type 3 (Madanire-Moyo *et al*., 2010); *Neodiplostomum* sp. (Prudhoe & Hussey, 1977); *Ornithodiplostomum* sp. (Barson & Avenant-Oldewage, 2006); *Posthodiplostomum* sp. 9 (Hoogendoorn *et al*., 2019); *Tylodelphys* sp. (Moema *et al*., 2013); *Tylodelphys mashonensis* (Mashego & Saayman, 1989; Khalil & Polling, 1997; Chibwana *et al*., 2013; Moema *et al*., 2013); *Uvulifer* sp. 4 (Hoogendoorn *et al*., 2019); and Diplostomidae gen. sp. (Hoogendoorn *et al*., 2019). Of these, only three unidentified species of *Diplostomum* were reported from the eyes, brain, and mesenteries of *C. gariepinus* (Prudhoe & Hussey, 1977; Madanire-Moyo *et al*., 2010). However, after intensive evaluation on the genus *Diplostomum* during the present study, it could be concluded that the placement of these metacercariae within the genus *Diplostomum* was erroneous and the species may belong to some

other genera within the Diplostomidae. Therefore, with knowledge on the diversity of *Diplostomum* parasitising freshwater fishes virtually lacking and the presence of almost 190 freshwater fishes reported from South Africa, it can be expected that we do not currently know the full extent of the biodiversity of these parasites from this region.

The present study investigated the diversity of *Diplostomum* in freshwater fishes from three provinces in South Africa (KwaZulu-Natal, Northern Cape, and North West Province) and the effect of infections with metacercariae on the behaviour of the plain squeaker, *Synodontis zambezensis*.

The first aim of this study was to identify and record the diversity of metacercariae of *Diplostomum* from the Mooi, Phongolo, Usuthu and Riet rivers (Chapter 3). This was achieved by conducting parasitological examinations of the eyes and brains of numerous fishes from different habitats within these systems. Based on phylogenetic and morphological analyses, three species of *Diplostomum* from five fishes were identified: *Diplostomum* sp. from *S*. *zambezensis* and *T*. *sparrmanii* (NGR and MNP, respectively) – previously reported from *S*. *nigrita* (Nigeria); *Diplostomum* sp. 14 from *A*. *labiata*, *O*. *mossambicus* and *S*. *zambezensis* (NGR) – previously reported from members of the Channidae, Cyprinidae, Hemiramphidae, Odontobutidae, Bagridae, Gobiidae, Percichthyidae (Iraq and China); and *Diplostomum* sp. 16 from *P*. *philander* (BDNR) – previously reported from *A*. *caeruleus* (Iraq). Interestingly, all three species of *Diplostomum* were found to infect cichlid fish, which suggests that this group of hosts may play an important role in transmission of these parasites to their bird definitive host. The unexpected distribution of two species of *Diplostomum* in this study between Asia and Africa was also an interesting find, since *Diplostomum* sp. 14 and *Diplostomum* sp. 16 were previously only reported from Asia (China & Iraq). Therefore, the discovery of these species in South Africa broadens their geographical distribution. The novel information obtained in this study has unlocked new opportunities to explore the migratory patterns of piscivorous birds occurring in these areas in order to gain more knowledge on the transmission and distribution of diplostomid parasites on a global scale. The results from the present study exceeded expectations as it was hypothesised that a low diversity of *Diplostomum* in freshwater fishes will be recorded, but based on the discovery of three species of *Diplostomum* from five fishes during the present study, this hypothesis was not supported.

An overall intensity of infection with metacercariae of *Diplostomum* recorded from the eye lenses of fishes (1–21 metacercariae per fish) in the present study was low in comparison with higher values provided in some of the previous studies done in Europe, where intensity of infections with *D*. *spathaceum* reported from *N*. *melanostomus* ranged between 1–50, 31, 12–57 and 1–74 metacercariae per fish from four localities in the Gulf of Gdańsk, Baltic sea (Kvach & Skóra, 2007) as well as 1–87 (Stettiner Haff) and 13–76 metacercariae (Peenemünde) from southwestern Baltic Sea (Kvach & Winkler, 2011). Average value of intensity of infection with

metacercariae of *D*. *baeri*, *D*. *mergi* and *D*. *paracaudum* reported from *N*. *melanostomus* in Sweden was 58 metacercariae per infected fish (in some cases fish were infected with both *D*. *mergi* and *D*. *paracaudum*) (Flink et al., 2017). In the study of Seppäla *et al*. (2011), intensity of infection with *Diplostomum* spp. in *A*. *alburnus*, *C*. *lavaretus, G*. *aculeatus, G*. *cernuus*, *L*. *leuciscus*, *O*. *eperlanus*, and *R*. *rutilus* collected from N.E. Bothnian Bay, Baltic Sea was above 20 metacercariae per fish (range of 2–79). Overall intensity of infection with *D*. *spathaceum*, *D*. *pseudospathaceum* and '*D*. *mergi* Lineage 2' reported from *A*. *brama* in Slovakia ranged between 25–43 and infection with *D*. *spathaceum*, *D*. *pseudospathaceum*, '*D*. *mergi* Lineage 2' and *Diplostomum* sp. A reported from *B*. *bjoerkna* were as high as 27 metacercariae in a single fish from Slovakia and Hungary (Kudlai *et al*., 2017). A low intensity of infection with *Diplostomum* metacercariae in freshwater fishes in South Africa observed in the present study support the second hypothesis stipulated in Chapter 1. This low infection rate may be as a result of South African climate and droughts experienced in recent years that directly affects snail (first intermediate) hosts, their density and distribution and in effect – reduces transmission of *Diplostomum* between first and second intermediate hosts.

Very little is known on the effects of *Diplostomum* on behaviour of freshwater fishes in southern Africa, for previous behavioural studies investigating these effects with aid of statistical evidence were predominantly performed in Europe. These studies mainly focused on the effects of *D*. *spathaceum* and *D*. *pseudospathaceum* and their effects on the behaviour of fish hosts belonging to the families Cyprinidae, Gasteosteidae, Gobiidae, Percidae, Osmeridae and Salmonidae. A gap in studies using bottom feeding fish as model species for behavioural experiments were identified and therefore *S*. *zambezensis* (Mochokidae) were selected as model species for the quantitative behavioural experiments during this study. Thus, the second aim of this study was to determine the influence of metacercariae of *Diplostomum* on the behaviour of their naturally infected fish hosts, *S*. *zambezensis*. This was achieved by means of analysis of video recordings captured during the behavioural experiments, performing statistical analysis on all data endpoints, and integrating results based on visual observations and statistical outputs during the study. Provided in Chapter 4 is a detailed assessment on previous research investigating parasite-induced changes on host behaviour, which formed a basis for comparisons of changes in behaviour of infected *vs* uninfected *S*. *zambezensis* from the Phongolo River, NGR. Based on the findings of the present behavioural study and possible explanations for the results provided by literature, metacercariae of *Diplostomum* may have affected their naturally infected fish hosts either by enhancing or suppressing anti-predatory behaviour in order to facilitate or impede transmission to the definitive host. However, modifications suppressing anti-predatory behaviour and as a result facilitating transmission to the definitive host i.e. changes in preference of natural habitat (top zones) or changes in swimming activity (traveling higher distances, at higher swimming speed and more frequently between zones), were only seen during acclimation periods

where no simulated avian attacks were performed and, therefore, conclusions should be based on the findings that occurred during simulated attacks. These findings showed that *S. zambezensis* infected with *Diplostomum* did not travel large distances, reach high swimming speed, or alternate between zones, but rather preferred the bottom zones during attacks when compared to their uninfected counterparts in this study. These are all characteristics of enhanced anti-predatory behaviour, where fish remain at the bottom and immobile making them inconspicuous and unlikely targets to predators. The findings of the present study conform to those of Gopko *et al*. (2015), who explained by means of experiments that immature metacercariae (not yet infective) enhances anti-predatory behaviour in order to impede transmission to the definitive host until the parasite reaches infective stages. Based on the statistical analysis from the present study, changes in behaviour between infected and uninfected *S. zambezensis* were observed, even in cases of low infections, therefore supporting the third hypothesis stipulated in Chapter 1. This study represents the first behavioural study exploring the effects of *Diplostomum* on the behaviour of natural hosts, *S. zambezensis* based on visual and statistical evidence in South Africa.

In conclusion, the present study highlighted the importance of the assessment of parasite diversity using both morphological descriptions as well as molecular analyses based on multiple genetic markers in order to provide reliable identification. The results presented here also expanded data on the geographical distribution of *Diplostomum* sp. 14 and *Diplostomum* sp. 16 between Africa and Asia as well as Intra-African distribution of *Diplostomum* sp. This study contributes to the global knowledge on the biodiversity of *Diplostomum* by reporting three species of *Diplostomum* from five new fish hosts from southern Africa. Even though the report of three species of *Diplostomum* from five fishes were unexpected during this study, data for cercariae (from first intermediate hosts) and adult worms (from definitive hosts) for these species are yet to be discovered and identified. In the behavioural studies, we found that even with low intensity of infections with metacercariae reported in the eye lenses of *S. zambezensis*, changes in their natural behaviour (such as distance moved, swimming speed and mobility state) were observed and differed in comparison to uninfected fish. This study is the first to investigate the effects of *Diplostomum* spp. on the behaviour of natural fish hosts applying quantitative statistical analyses not only in South Africa, but in the African continent as well.

5.2 Recommendations for Future Research

Morphological descriptions and novel sequence data generated during the present study will contribute to the elucidation of the life cycle of *Diplostomum* spp. and advance further research of diplostomids in South Africa. It is anticipated that the methods used during this study would be used in future studies focusing on the taxonomy of *Diplostomum* in Africa, subsequently elucidating the true diversity of these parasites in this continent. However, further detailed

descriptions of adults from piscivorous birds are required for species identification. A detailed list of all piscivorous birds, their habitat occupancy (i.e. where they occur in South Africa) and their migratory patterns should be compiled that can be used to determine potential definitive hosts for *Diplostomum* sp., *Diplostomum* sp. 14 and *Diplostomum* sp. 16.

Comprehensive studies on the migratory patterns of piscivorous birds between Africa and Asia as well as Africa and Europe should be considered for the study on the distribution and transmission of *Diplostomum* between these continents as learned during this study or may even reveal new species that have not yet been discovered. Even though the diversity of metacercariae of *Diplostomum* from freshwater fishes found during this study was surprising, gaps in the knowledge on the first and definitive hosts for these parasites remain. These life stages are also important for the elucidation of the life cycles for these species. Therefore, intensive surveys on freshwater snail hosts present in the Mooi, Phongolo, Usuthu and Riet rivers in the NGR, MNP, and BDNR should be considered in future studies as well as their parasite diversity. An alternative approach is to study the stomach contents of some freshwater fish species i.e. *S*. *zambezensis* during parasitological screenings in order to determine the snail species ingested by fish i.e. the study by Sanyanga (1998). This could also give an indication of possible first intermediate hosts for *Diplostomum* occurring within these systems. Moreover, extensive experimental studies on the larval stages of *Diplostomum* are recommended in order to determine other factors such as the “infectiveness” of metacercariae (stage where fish hosts will be most susceptible to predation/stage of maturity where the parasite will be ready for transmission) or the importance of intensity of infections that play a role in changes of host behaviour i.e. habitat preference, feeding, shoaling, group behaviour etc.

Finally, extensive studies on piscivorous birds present in areas where *Diplostomum* infections were found/recorded in intermediate hosts should be carried out in order to identify species to the lowest possible taxonomic level and complete the life cycles of *Diplostomum*.

This knowledge on the life cycles of *Diplostomum* will allow fish farmers to target specific host species in the prevention of transmission of *Diplostomum* in fish farms i.e. prevent fish-eating birds from catching the fish, in doing so preventing the distribution of these parasites. It is also advised that a cautionary approach during the identification of species of *Diplostomum* is taken and both molecular and morphological analysis are used in order to prevent any further confusion on the diversity and composition of this genus in the future.

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Appendices

Appendix A – Publication: Molecular and morphological characterisation of four diplostomid metacercariae infecting *Tilapia sparrmanii* (Perciformes: Cichlidae) in the North West Province, South Africa

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FISH PARASITOLOGY - ORIGINAL PAPER

Molecular and morphological characterisation of four diplostomid metacercariae infecting *Tilapia sparrmanii* (Perciformes: Cichlidae) in the North West Province, South Africa

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Abstract
Despite their pathogenic effects on fish, the diversity of trematodes from the family Diplostomidae remains vastly unexplored in Africa and specifically South Africa. To date, only six species of diplostomids have been reported from freshwater fishes in this country, with only two species being molecularly characterised. In this study, combined morphological and molecular analyses were used to identify and describe metacercariae of the Diplostomidae (Digenea) parasitising banded tilapia *Tilapia sparrmanii* (Perciformes: Cichlidae) collected within the North West Province, South Africa. Metacercariae found on the body surface and muscles of the fish were separated into four groups based on the infection site, the colour of the cysts and the morphology of excysted specimens. Isolates from each group were further identified through molecular analyses. Comparative analyses of the newly generated 28S rDNA, ITS1-5.8S-ITS2 and *cox*1 sequences revealed the presence of four species of which three were identified as *Bolbophorus* sp. 3 (28S rDNA, ITS1-5.8S-ITS2 and *cox*1), *Posthodiplostomum* sp. 9 (28S rDNA and ITS1-5.8S-ITS2) and *Uvulifer* sp. 4 (28S rDNA, ITS1-5.8S-ITS2 and *cox*1), respectively, and the fourth species belonging to the Diplostomidae gen. sp. (28S rDNA, ITS1-5.8S-ITS2 and *cox*1). Morphology of metacercariae of *Posthodiplostomum* sp. was compared with metacercariae of this genus previously reported in fishes in Africa. This study presents the first molecular data for species of *Bolbophorus* Dubois, 1935, *Posthodiplostomum* Dubois, 1936 and *Uvulifer* Yamaguti, 1934 from Africa, and it highlights the need for future research on the diversity of diplostomid parasites in South Africa and in Africa as whole.

Keywords Digenea · Metacercariae · *Tilapia sparrmanii* · DNA · South Africa

Introduction

The Diplostomidae Poirier, 1886 (Digenea) is a large and diverse family of widely distributed digeneans that infect numerous mammals and bird species. The life cycles of diplostomids typically include freshwater snails, fish and amphibians as intermediate hosts and mammals and fish-eating birds as definitive hosts (Niewiadomska 2002). The metacercarial stages of some species of the genera *Bolbophorus* Dubois, 1935, *Crassiphiala* Van Haitsma, 1925, *Diplostomum* von Nordmann, 1832, *Posthodiplostomum* Dubois, 1936, *Tylodelphys* Diesing, 1850 and *Uvulifer* Yamaguti, 1934 are known to be pathogenic to their fish intermediate hosts and can cause severe morbidity and occasionally mortalities in cases of high infection intensities (Terhune et al. 2003; López-Jiménez et al. 2017; Blasco-Costa and Locke 2017 and references therein). Although this group, particularly its type genus *Diplostomum*, has become in recent years a focus of intensive studies in Europe and North America (Blasco-Costa and Locke 2017), our knowledge of these parasites in Africa remains incomplete due to the absence of dedicated studies and presence of numerous ambiguous reports that lack of morphological and molecular evidence (Van As and Basson 1984; Khalil and Polling 1997; Florio et al. 2009; Akoll et al.

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Appendix B – Table B1: Summary of nominal species of *Diplostomum* worldwide, with known hosts and zoogeographical distribution.

Nominal *Diplostomum* spp.	Hosts			Zoogeo-graphical region	Source
	1st intermediate	2nd intermediate	Definitive		
D. adamsi Lester & Huizinga, 1977	*Lymnaea elodes; Lymnaea stagnalis*	*Perca flavescens*	*Larus argentatus*	Nearctic	Lester & Huizinga (1977)
D. amygdalum Dubois & Pearson, 1965	–	–	*Egretta garzetta Mesophoyx intermedia plumifera;*	Australian	Dubois & Pearson (1965)
D. antarcticum Feiler, 1986	–	–	*Nyctocorax caledonicus; Larus dominicanus*	Antarctic	Feiler (1986)
D. ardeae Dubois, 1969	–	–	*Ardea herodias*; *Ardea goliath*	Nearctic Palearctic	Dubois (1969); El-Naffar *et al*. (1980); Moszczynska *et al*. (2009); Locke *et al*. (2015)
D. (*Dolichorchis*) *auriculosum* Dubois & Pearson, 1967	–	–	*Anhinga novaehollandiae*	Australian	Dubois & Pearson (1967)
D. auriflavum Molin, 1858	–	–	*Nycticorax nycticorax* (= *Ardea nycticorax*)	Palearctic	Molin (1858)
D. baeri Dubois, 1937[a]	–	*Notropis hudsonius; Oncorhynchus mykiss*; *Perca flavescens*	*Larus delawarensis*; *Larus* sp.; *Stercorarius longicaudus*	Nearctic Palearctic	Dubois (1937); Galazzo *et al*. (2002); Moszczynska *et al*. (2009); Locke *et al*. (2010a, b, 2015); Behrmann-Godel (2013); Mateos-Gonanzalez *et al*. (2015); Flink *et al*. (2017); Ubels *et al*. (2018)
D. (*Dolichorchis*) *buteii* Vidyarthi, 1937	–	–	(Accipitridae); *Ardea cocoi*; *Buteo rufinus*; *Haliastur indus*; *Milvus migrans*; *Milvus mgrans govinda*	Oriental Neotropical	Dubois (1970b); Yamaguti (1971); Gupta & Mishra (1975); Smith & Hickman, (1983); Lunaschi & Drago (2006)
D. chromatophorum (Brown, 1931) Shigin, 1986[b]	–	(Cyprinidae); (Salmonidae)	(Laridae)	Palearctic	Shigin (1986)

Table B1 (continued)

Nominal *Diplostomum* spp.	Hosts			Zoogeo-graphical region	Source
	1st intermediate	2nd intermediate	Definitive		
D. commutatum (Diesing, 1850) Dubois, 1937	*Radix ovata*	*Pseudotolithus elongatus*	*Cynoglossus senegalensis*; *Hydrocheidon nigra*; *Larus minitus*; *Larus ridibundus*; *Sterna albifrons*; *Sterna hirundo*; *Sterna paradisaea*	Afrotropical Nearctic Palearctic	Dubois (1969); Shigin, (1986); Abraham & Akpan (2004)
D. (*Austrodiplostomum*) *compactum* (Lutz, 1928) Dubois, 1970	–	*Caquetaia kraussii*; *Cichla monoculus*; *Geophagus brasiliensis;* *Hoplias malabaricus*; *Oreochromis aureus*; *Oreochromis mossambicus*; *Plagioscion squamosissimus*; *Rhamdia guatemalensis;* *Satanoperca pappaterra*	*Phalacrocorax olivaceus*	Neotropical	Dubois (1970a); Novaes *et al*. (2006); Olivero-Verbel *et al*. (2012); Ramos *et al*. (2013)
D. crassum Chandler & Rauch 1948	–	–	*Quiscalus versicolor*	Nearctic	Chandler & Rauch (1948)
D. *dominicanum* Feiler, 1986	–	–	*Larus dominicanus*	Antarctic	Feiler (1986)
D. *erythrophthalmi* Shigin, 1965[a]	–	(Cyprinidae)	?	Palearctic	Shigin (1976)
D. *flexicaudum* (Cort & Brooks, 1928) Haitsma, 1931[a]	*Lymmaea emarginata angulata;* *Lymnaea stagnalis appressa;* *Lymnaea humilis modicella*; *Lymnaea stagnalis perampla*	*Mastacembelus mastacembelus*	–	Palearctic Nearctic	Cort & Brooks (1928); Bashe & Abdullah (2010)
D. (*Dolichorchis*) *galaxiae* Smith & Hickman, 1983	–	*Galaxias auratus*	*Ardea novaehollandiae*; *Anas platyrhynehos* (exp)	Australian	Smith & Hickman (1983)
D. *garrae* Zhokhov, 2014	–	*Garra dembecha*	–	Afrotropical	Zhokhov (2014)
D. gasterostei Williams, 1966	*Lymnaea peregra* = *Radix peregra*	(Percidae); *Gasterosteus aculeatus*	(Anatidae)(exp); *Columba livia domestic* (exp)	Palearctic	Williams (1966); Dubois (1970b)

Table B1 (continued)

Nominal *Diplostomum* spp.	Hosts			Zoogeo-graphical region	Source
	1st intermediate	2nd intermediate	Definitive		
D. gavium (Guberlet, 1922) Hughes, 1929	–	–	*Gavia immer*; *Gavia arctica*	Palearctic Nearctic	Guberlet (1922); Dubois & Rausch (1950); Shigin (1986)
D. ghanense Ukoli,1968	–	–	*Anhinga rufa rufa*	Afrotropical	Ukoli (1968)
D. gobiorum Shigin, 1965	*Radix auricularia*	(Gasterosteidae); (Gobiidae)	(Anatidae)(exp)	Palearctic	Shigin (1969)
D. helveticum (Dubois, 1929) Shigin, 1977	*Radix auricularia*	(Cyprinidae); (Percidae)	(Laridae)	Palearctic	Shigin (1986)
D. heterobranchi Wedl, 1861	–	*Clarias gariepinus*	–	Palearctic	Khalil & Polling (1997)
D. hupehensis Pan & Wang, 1963	*Radix swinhoei*	*Aristichthys nobilis; Cirrhina molitorella; Ctenpharyngoden idella; Gambusia affinis; Hypophthalmichthys molitrix; Monopterus albus; Oreochromis mossambicus*; *Paramisgurnus dabryanus*	*Larus ridibundus ridibundus*	Palearctic	Junyi (1990)
D. huronense (La Rue, 1927) Hughes, 1929	–	*Ambloplites rupestris; Catostomus commersonii; Lepomis gibbosus; Morone americana; Notemigonus crysoleucas; Osmerus mordax; Perca flavescens; Petromyszon marinus*	*Larus argentatus; Larus delawarensis*	Nearctic Palearctic	La Rue (1927); Gibson (1996); Galazzo *et al*. (2002); Locke *et al*. (2010a, b, 2015)
D. indistinctum (Guberlet, 1923)	*Lymnaea elodes; Radix auricularia; Radix ovata; Radix pereger*	(Catostomidae); (Cyprinidae); *Abramis brama*; *Alburnus alburnus; Ballerus ballerus*; *Ballerus sapa; Blicca bjoerkna*; *Carassius carassius; Catostomus commersonii; Esox lucius; Gymnocephalus cernua*; *Leuciscus idus; Leuciscus leuciscus; Lota lota*; *Neogobius melanostomus; Notemigonus crysoleucas*; *Pelecus cultratus*; *Rutilus rutilus*; *Scardinius erythrophthalmus*	*Larus* sp.	Nearctic Palearctic	Shigin (1968a, b); Galazzo *et al*. (2002); Moszczynska *et al*. (2009); Locke *et al*. (2010a, b, 2015); Gordy *et al*. (2016)

Table B1 (continued)

Nominal *Diplostomum* spp.	Hosts			Zoogeo-graphical region	Source
	1st intermediate	2nd intermediate	Definitive		
D. (*Dolichorchis*) *ketupanense* Vidyarthi, 1937	–	*Aplocheilus lineatus; Catla catla; Mystus malabaricus; Pseudosphromenus cupanus; Puntius arulius arulius; Puntius fasciatus fasciatus; Rasbora daniconius*	*Ardeola grayii*; *Ketupa zeylonensis hardwicki*	Oriental	Yamaguti (1971); Roopa & Janardanan (2001); Pandey & Agrawal (2013)
D. kronschnepi Bychovskaja-Pavlovskaja, 1953	–	–	*Numenius arquatus; Numenius madagascariensis*; (Charadriiformes)	Palearctic	Belopolskaya (1975); Dronen & Badley (1979)
D. longicollis Zhokhov, 2014	–	*Enteromius humilis*; *Garra dembecha*	–	Afrotropical	Zhokhov (2014)
D. *macrostomum* Shigin, 1965[a]	–	?	(Scolopacidae) *Gallinago* sp.	Palearctic	Shigin (1965a); Shigin (1968a)
D. magnicaudum El-Naffar, 1979	–	*Gallus gallus domesticus* (exp) *Oreochromis niloticus*	*Columba livia* (exp) *Gallinula chloropus chloropus;*	Palearctic	El-Naffar (1979)
D. mahonae Dubois, 1953	–	–	*Uña aalge*	Palearctic	Dubois (1953)
D. marshalli Chandler 1954	–	–	*Totanus flavipes*	Nearctic	Chandler (1954)
D. mergi Dubois, 1932	*Lymnaea stagnalis*; *Radix auricularia*; *Radix balthica*	(Cyprinidae); (Percidae)	(Anatidae) *Mergus merganser*	Palearctic Nearctic	Shigin (1965b); Haarder *et al*. (2013); Behrmann-Godel (2013); Sitko & Rzad (2014)
D. micradenum (Cort et Brackett 1938) Oliver 1940	*Lymnaea elodes*	*Rana pipiens* (exp)	*Columba livia* (exp)	Nearctic	Olivier (1940)
D. minutum Szidat, 1964	–	*Puntius* sp.	*Larus dominicanus* *Larus maculipennis*	Antarctic Neotropical Oriental	Niewiadomska *et al*. (1989); Pandey & Agrawal (2013)
D. montanum Zhokhov, 2014	–	*Enteromius humilis*; *Garra dembecha*; *Labeobarbus gorgorensis*; *Varicorhinus beso*	–	Afrotropical	Zhokhov (2014)
D. murrayense (Johnston & Cleland, 1938) Johnston & Simpson, 1939	*Lymnaea lessoni*	(Cyprinidae)	(Laridae); *Chlidonias hybrida; Chlidonias leucopareia*	Australian	Johnston & Angel (1941); Dubois & Pearson (1965)
D. nemachili Zhatkanbaeva & Shigin, 1986	–	(Cyprinidae); *Nemachilus* sp.	–	Palearctic	Shigin (1986); Shakaraliyeva (2017)

Table B1 (continued)

Nominal *Diplostomum* spp.	Hosts			Zoogeo-graphical region	Source
	1st intermediate	2nd intermediate	Definitive		
D. niedashui Pan & Wang, 1963[a]	*Radix swinhoei*	*Aristichthys nobilis; Cirrhina molitorella; Ctenpharyngoden idella; Gambusia affinis; Hypophthalmichthys molitrix; Monopterus albus; Oreochromis mossambicus*; *Paramisgurnus dabryanus*	*Larus ridibundus ridibundus*	Palearctic	Junyi (1990)
D. nordmanni Shigin & Shapirov, 1986	*Lymnaea bactriana*, *Lymnaea fontinalis*, *Lymnaea lagotis*	(Cyprinidae)	(Laridae)	Palearctic	Shigin (1986)
D. numericum Niewiadomska, 1988[a, b]	–	*Gymnocephalus cernuus; Scardinius erythrophthalmus*	–	Palearctic	Niewiadomska (1988); Höglund & Thulin (1992)
D. oedicnemum Singh, 1956	–	–	*Burhinus oedicnemus indicum*	Oriental	Singh (1956); Yamaguti (1971)
D. (*Adenodiplostomum*) *odeningi* Gupta & Mishra, 1975	–	–	*Milvus migrans*	Oriental	Gupta & Mishra (1975)
D. orientale Yamaguti, 1934	–	–	*Mergus merganser merganser*	Palearctic	Kamegai & Ichihara (1973)
D. paracaudum (Iles, 1959) Shigin, 1977[a]	*Galba palustris* *Lymnaea peregra;*	*Abramis brama* (exp); *Coregonus lavaretus; Gasterosteus aculeatus* (exp)	*Larus fuscus; Larus ridibundus; Gallus gallus domesticus* (exp)	Palearctic	Niewiadomska (1987); Niewiadomska & Našincová (1990); Pojmańska *et al*. (2012); Behrmann-Godel (2013)
D. paraspathaceum Shigin, 1965[a, b]	*Lymnaea* sp.	(Cyprinidae)	(Laridae)	Palearctic	Shigin (1968a)
D. parviventosum Dubois, 1932	*Lymnaea lagotis*; *Radix auricularia*; *Radix ovata*	(Cyprinidae)	(Anatidae); *Mergus merganser*; *Melanitta fusca*	Palearctic	Dubois (1970b); Shigin (1986); Sitko & Rzad (2014); Selbach *et al*. (2015)
D. parvulum Dubois & Angel,1972	–	–	*Hydroprogne caspia; Pelecanus conspicillanus*	Australian	Dubois & Angel (1972)
D. pelmatoides Dubois, 1932[a]	–	*Phoxinus phoxinus*	(Anatidae)(exp)	Palearctic	Rees (1955)

Table B1 (continued)

Nominal *Diplostomum* spp.	Hosts			Zoogeo-graphical region	Source
	1st intermediate	2nd intermediate	Definitive		
D. petromyzifluviatilis Diesing, 1850 = *Tylodelphys petromyzifluviatilis*	*Bithynia tentaculata*	(Petromyzonidae) *Lampetra fluviatilis*; *Lampetra planeri*	(Anatidae)(exp); *Gallus gallus domesticus* (exp); *Larus argentatus; Mus musculus* (exp); *Rattus norvegicus domestica* (exp)	Palearctic	Sweeting (1976)
D. phoxini (Faust, 1918) Arvy & Buttner, 1954	*Radix auricularia*; *Radix balthica; Radix peregra*	(Cyprinidae) *Phoxinus phoxinus*	(Anatidae) *Mergus merganser*	Palearctic	Rees (1955); Olson *et al*. (2003); Sitko & Rzad (2014); Soldánová *et al*. (2017)
D. podicepsi Shigin & Kostadinova, 1995	–	–	*Podiceps griseigena*	Palearctic	Filimonova & Zinov'yeva (1998)
D. pseudobaeri Razmashkin & Andrejuk, 1978[a]	*Radix ovata*; *Lymnaea peregra*	(Percidae); *Oncorhynchus mykiss*;	*Gallus gallus domesticus*	Palearctic	Shigin (1986); Field & Irwin (1995)
D. pseudomergi Belopolskaya, 1975	–	–	*Squatarola squatarola*	Palearctic	Belopolskaya (1975)
D. pseudospathaceum Niewiadomska, 1984[a]	*Lymnaea stagnalis*; *Radix labiata*; *Stagnicola palustris*	*Abramis brama; Ballerus sapa; Blicca bjoerkna; Cyprinus carpio; Gasterosteus aculeatus; Gymnocephalus schraetser; Leuciscus aspius; Lota lota; Rutilus rutilus; Silurus glanis*; *Vimba vimba*	*Larus argentatus*; *Larus cachinnans*; *Larus ridibundus*	Palearctic	Niewiadomska (1984); Georgieva *et al*. (2013) Behrmann-Godel (2013); Pérez-del-Olmo *et al*. (2014); Brabec *et al*. (2015); Locke *et al*. (2015); Selbach *et al*. (2015); Kudlai *et al*. (2017); Enabulele *et al*. (2018)
D. pungitis Shigin, 1965	–	*Gasterosteus* sp.; *Pungitius platygester*	*Aythya fuligula; Bucephala clangula; Clangula hyemalis; Somateria mollissima*	Palearctic	Filimonova & Zinov'yeva (1998); Sitko & Rzad (2014)
D. (*Hemistomum*) *pusillum* (Dubois, 1928) Nazmi Gohar, 1932	–	(Cobitidae)	(Anatidae); (Laridae); (Mamamalia); *Mergus merganser*	Nearctic Palearctic	Dubois (1970b); Iksanov (1968); Sitko & Rzad (2014)

Table B1 (continued)

Nominal *Diplostomum* spp.	Hosts			Zoogeo-graphical region	Source
	1st intermediate	2nd intermediate	Definitive		
D. repandum Dubois & Rausch, 1950	–	*Dallia admirabilis*	*Sterna hirundo*; *Stercorarius longicaudus*	Nearctic Palearctic	Dubois & Rausch (1950); Atrashkevich *et al*. (1996)
D. rutili Razmashkin, 1969 [b]	*Lymnaea bactriana*; *Lymnaea fontinalis*; *Lymnaea lagotis*	(Cyprinidae); (Salmonidae)	(Laridae)	Palearctic	Razmashkin (1969); Shigin (1986)
D. scudderi (Oliver, 1941) Dubois, 1966	*Lymnaea elodes*; *Stagnicola palustris*	(Gasterosteidae); *Culaea inconstans; Gasterosteus aculeatus*	(Anatidae)	Nearctic	Lester (1974); Shigin (1986)
D. shigini Zhatkanbaeva, 1978[a]	–	–	*Chlidonias niger*	Palearctic	Fonteneau *et al*. (2009); Georgieva *et al*. (2013)
D. sobolevi Shigin,1959	–	–	*Saxicola rubetra*	Palearctic	Shigin (1986)
D. spathaceum (Rudolphi, 1819) Olsson, 1876	*Radix auricularia*; *Radix peregra*	*Abramis brama; Acanthobrama marmid; Acipenser ruthenus; Alburnus caeruleus; Blicca bjoerkna; Carasobarbus luteus; Chondrostoma nasus; Clarias gariepinus; Cyprinus carpio*; *Cyprinion macrostomum; Gasterosteus aculeatus; Leuciscus aspius; Misgurnus anguillicaudatus; Perca fluviatilis; Pseudochondrostoma willkommii; Rutilus pigus*; *Rutilus rutilus*; *Salvelinus alpinus*; *Silurus glanis*	(Laridae) *Anas platyrhynchos; Larus ridibundus; Larus argentatus*; *Larus cachinnans; Larus fuscus taimyrensis*	Afrotropical Oriental Palearctic Nearctic	Gupta & Mishra (1975); Shigin, (1977); Goselle *et al*. (2008); Georgieva *et al*. (2013); Sitko & Rzad (2014); Blasco-Costa *et al*. (2014); Pérez-del-Olmo *et al*. (2014); Selbach *et al*. (2015); Brabec *et al*. (2015); Locke *et al*. (2015); Kudlai *et al*. (2017)
D. sterni Gupta, 1958	–	–	*Sterna aurantia*	Oriental	Gupta (1958)
D. sudarikovi Shigin, 1960[a] = *D. volvens*	–	–	?	Palearctic	Shigin (1986); Georgieva *et al*. (2013)
D. tilapiae Zhokhov, 2014	–	*Oreochromis niloticus*	–	Afrotropical	Zhokhov (2014)
Diplostomulium (*Dolichorchis*) *tregenna* Nazmi & Gohar, 1932	–	*Coptodon zilli*	–	Afrotropical	Goselle *et al*. (2008)
D. (*Adenodioplostomum*) *triangulare* (Johnston, 1904) Hughes, 1929 & Dubois, 1937	–	–	*Dacelo novaeguineae* (=*Dacelo gigas*)	Australian	Dubois & Pearson (1967)

Table B1 (continued)

Nominal *Diplostomum* spp.	Hosts			Zoogeo-graphical region	Source
	1st intermediate	2nd intermediate	Definitive		
D. vanelli Yamaguti, 1935	–	–	*Vanellus vanellus*	Palearctic	Yamaguti (1935); Dubois (1970b); Kamegai & Ichihara (1973); Chandler (1954)
D. variabile (Chandler, 1932) = *Didelphodiplostomum variabile*	*Menetus dilatatus*	*Ambystoma opacum*	*Didelphis virginiana*	Nearctic	Chandler & Rausch (1946); Harris *et al*. (1967)
D. (*Glossodiplostomoides*) *vidyarthii* Gupta & Mishra, 1975	–	–	*Milvus migrans*	Oriental	Gupta & Mishra (1975)
D. vitreophilum Shigin & Stanislavez, 1989[a, b] = *D. gavium*	–	*Rutilus rutilus*	–	Palearctic	Filimonova & Zinov'yeva (1998)
D. yogenum (Cort & Brackett, 1937) Shigin, 1977[a] = *D. volvens*	–	(Percidae)	–	Palearctic	Shigin (1977); Lebedeva (2008); Georgieva *et al*. (2013)
D. volvens von Nordmann, 1832	*Radix auricularia*	(Percidae); (Godiidae); *Gymnocephalus cernua*; *Perca fluviatilis*; *Sander lucioperca*	(Laridae)	Palearctic	Shigin (1986); Lebedeva (2008);

Species considered **not valid** by [a]Shigin (1993); [b]Niewiadomska (2010). ?, Information on host/s unavailable.

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Appendix C – Permit for sampling in Ndumo Game Reserve

EZEMVELO KZN WILDLIFE
Conservation, Partnerships & Ecotourism

ORIGINAL

ORDINARY PERMIT

Fee:	R 50,00	Permit No:	OP 1582/2018
Receipt No:	1689/2018	Contact:	Miss S.M. Hughes

This permit is issued in pursuance of the provisions of the Nature Conservation Ordinance No 15 of 1974 , Chapter 9 and the Regulations framed thereunder.

The permit is issued to:

ID Number: 9312205072085

Mr Divan Van Rooyen
North West University
Zoology,School of Biological Science
Private Bag X6001
Potchefstroom
2520

Residential Address
North West University
Zoology,School of Biological Science
22-34 College Ave Varsity Villa
Potchefstroom
2520

Province: North West Province

In the capacity of Researcher

To Collect and Export the samples the following species of Freshwater Fish

CATFISH *(CLARIAS GARIEPINUS)*

40 (Forty) Individuals per species. Collect and export muscle tissue throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: and Ndumo Game Reserve.

TILAPIA *(OREOCHROMIS MOSSAMBICA)*

40 (Forty) Individuals per species. Collect and export muscle tissue throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: and Ndumo Game Reserve.

Please read the Terms and Conditions under which this Permit is issued

ISSUED at PIETERMARITZBURG, KwaZulu-Natal, on 13 April 2018

for CHIEF EXECUTIVE

Permit Holder

EZEMVELO KZN WILDLIFE PERMITS OFFICE
PO Box 13053, Cascades, 3202, Pietermaritzburg, KwaZulu-Natal.
Tel +27 33 845 1320 / 1324. Fax: +27 33 845 1747. Fax to Email: 086 529 3320
Email: permits@kznwildlife.com. Website: www.kznwildlife.com

EZEMVELO KZN WILDLIFE
Conservation, Partnerships & Ecotourism

ORIGINAL

REDBREASTED TILAPIA *(TILAPIA RENDALLI)*

40 (Forty) Individuals per species. Collect and export muscle tissue throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: and Ndumo Game Reserve.

PLAIN SQUEAKER *(SYNODONTIS ZAMBEZENSIS)*

40 (Forty) Individuals per species. Collect and export muscle tissue throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: and Ndumo Game Reserve.

RIVER SARDINE *(MESOBOLA BREVIANALIS)*

40 (Forty) Individuals per species. Collect and export muscle tissue throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: and Ndumo Game Reserve.

RIVER GOBY *(GLOSSOGOBIUS GIURIS)*

40 (Forty) Individuals per species. Collect and export muscle tissue throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: and Ndumo Game Reserve.

SILVER CATFISH *(SCHILBE INTERMEDIUS)*

40 (Forty) Individuals per species. Collect and export muscle tissue throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: and Ndumo Game Reserve.

THREESPOT BARB *(BARBS TRIMACULATUS)*

40 (Forty) Individuals per species. Collect and export muscle tissue throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: and Ndumo Game Reserve.

LOWVELD SUCKERMOUTH *(CHILOGLANIS SWIERSTRAI)*

40 (Forty) Individuals per species. Collect and export muscle tissue throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: and Ndumo Game Reserve.

Please read the Terms and Conditions under which this Permit is issued

ISSUED at PIETERMARITZBURG, KwaZulu-Natal, on 13 April 2018

for CHIEF EXECUTIVE

Permit Holder

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EZEMVELO KZN WILDLIFE
Conservation, Partnerships & Ecotourism

ORIGINAL

BANDED TILAPIA *(TILAPIA SPARMANTII)*

40 (Forty) Individuals per species. Collect and export muscle tissue throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: and Ndumo Game Reserve.

SOUTHERN MOUTHBROODER *(PSEUDOCRENILABRUS PHILANDER)*

40 (Forty) Individuals per species. Collect and export muscle tissue throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: and Ndumo Game Reserve.

IMBERI *(BRYCINUS IMBERI)*

40 (Forty) Individuals per species. Collect and export muscle tissue throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: and Ndumo Game Reserve.

AQUATIC INVERTEBRATE SPECIES

10 (Ten) Collect samples, identify and keep specimens for reference collection and laboratory identification throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: Tembe Elephant Park and Ndumo Game Reserve.

ALL SPECIES OF INDIGENOUS FISH

3 (Three) Individuals per species. Capture, identify and release throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas but including the following protected areas: and Ndumo Game Reserve.

Please read the Terms and Conditions under which this Permit is issued

ISSUED at PIETERMARITZBURG, KwaZulu-Natal, on 13 April 2018

for CHIEF EXECUTIVE

Permit Holder

EZEMVELO KZN WILDLIFE PERMITS OFFICE
PO Box 13053, Cascades, 3202, Pietermaritzburg, KwaZulu-Natal.
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Email: permits@kznwildlife.com. Website: www.kznwildlife.com

EZEMVELO KZN WILDLIFE
Conservation, Partnerships & Ecotourism

ORIGINAL

TERMS AND CONDITIONS UNDER WHICH THIS PERMIT IS ISSUED

1. It is valid only:

   (i) from : 11 April 2018
   to : 10 April 2019

   (ii) in the original

   (iii) if all **5** pages are signed by the permit holder named above

   (iv) to the permit holder named above and the following Nominees :

   Prof. N J Smit

   Mr R.J Gerber

   Prof. V Wepener

   Mr R Pienaar

   Prof. L Brendonck

2. Please provide us with your collection data annually.

3. This permit/licence/certificate is issued subject to compliance with all other relevant legislation and does not preclude the permit holder from adherence thereto.

4. By signing the permit or licence the holder accepts, and agrees to comply with the conditions under which it is issued.

5. Permit to be returned to E KZN Wildlife, P O Box 13053, Cascades, 3202, upon expiry for renewal or cancellation.

6. Permit shall be carried by holder, or the specified nominees, at all times during use.

7. Outside of E KZN Wildlife areas, use of this permit is subject to landowner's or controlling authority's written permission.

8. Prior to collecting in areas under the control of the E KZN Wildlife the holders shall contact the Officer-in-Charge of the area at least 48 (Forty-eight) hours before commencing, and shall comply with any conditions which the Officer may impose at his discretion. The officer may refuse collection or capture at his or her discretion.

9. At least one representative specimen (preferably at least one male and one female) of each species collected from each locality must be lodged with a recognised South African museum/herbarium. Holotype specimens, and half the number of paratype specimens, of any

Please read the Terms and Conditions under which this Permit is issued

ISSUED at PIETERMARITZBURG, KwaZulu-Natal, on 13 April 2018

for CHIEF EXECUTIVE

Permit Holder

EZEMVELO KZN WILDLIFE PERMITS OFFICE
PO Box 13053, Cascades, 3202, Pietermaritzburg, KwaZulu-Natal.
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EZEMVELO KZN WILDLIFE
Conservation, Partnerships & Ecotourism

ORIGINAL

new species MUST BE DEPOSITED with a recognised South African museum/herbarium, and may only leave South Africa on a loan basis. These specimens are to be deposited in the SA museums within a year of publishing the description of the new species. The holder shall provide the Chief Executive Officer, KZNNCS with the name of the museum at which the specimens have been lodged, and the accession number of each specimen. This condition relates to unavoidable by-catch of non-target organisms as well.

10. A copy or copies of any publication arising from the authority herein contained will be made available to E KZN Wildlife.

11. (i) Reserving accommodation within E KZNWildlife areas is entirely the responsibility of the permit holder. Booking is obtainable at the Central Booking Office, Telephone 033 8451000.(ii) Any assistance required from Board staff will be subject to other demands on the Officer's time and must be arranged in advance with him/her.

12. Holders shall provide the Chief Executive, with a named list of every specimen collected (including the class, order, family, gender and age), the geographical co-ordinates (to seconds accuracy) of each collection locality and dates of collection, as laid out in the following table. A Global Positioning System with the WGS84 Datum should be used wherever possible to determine the geographical co-ordinates of the collection sites; please state the method used.

13. SPECIMEN - COLLECTION DATE - SPECIES - LOCALITY - LATITUDE - LONGITUDE
(museum (ddmmyy) (Seconds (Seconds
Accession) Accuracy) Accuracy).
Holders are requested to provide additional information, such as the habitat in which each specimen was collected and abundance or relative abundance data (providing standardised sampling methods are used) with the list.

14. Fish may be caught by electroshocking

15. No collecting is permitted within the road reserve which is a strip 30 (thirty) metres either side of a public road, no matter how small or remote the road may be.

Please read the Terms and Conditions under which this Permit is issued

ISSUED at PIETERMARITZBURG, KwaZulu-Natal, on 13 April 2018

for CHIEF EXECUTIVE

Permit Holder

Appendix D – Permit for sampling in the North West Province

MCPO 00833/2/2019 | NW 8065/03/2019 | Application ID 8065

North West Province

Issued in terms of the provisions of:
The National Environmental Management Biodiversity Act, Act 10 of 2004 as amended

Biodiversity North West Integrated Permit Ordinary

Issued in terms of the provisions of:
(1) Bophuthatswana Nature Conservation Act, Act No.3 of 1973; (2) Transvaal Nature Conservation Ordinance, No.12 of 1983; (3) Cape Nature and Environmental Conservation Ordinance, 19 of 1974

Angle, Catch, Import, Transport, Keep, Research Immobilised, Boat, Net

Conduct Research or Scientific Project

APPROVED SPECIES AND NUMBERS, RESTRICTED ACTIVITIES AND CONDITIONS AS PER ADDENDUM AND PAGES ATTACHED

PERMIT HOLDER

Details		Physical Address		Postal Address	
Surname:	Erasmus	Building:	E6 J.S. van der Merwe, North-West University	Private Bag	X6001
Full Name:	Johannes Hendrik	Street:	11 Hoffman Street	Post Office:	
Id Number:	9111115030088	Suburb:	Bult		
Passport:		Town:	Potchefstroom	Town:	Potchefstroom
Cell Home:	0767455807	Area Code:	2531	Postal Code:	2520
Tel Home:		Division/Region:	Potchefstroom	District/Region:	Potchefstroom
Tel Work:	0182852279	Province/State:	North West Province	Province/State:	North West Province
Fax Home:		Country:	SOUTH AFRICA	Country:	SOUTH AFRICA
Email:	22119809@nwu.ac.za				

REGION

NorthWest

LOCATION

Facility

Property
Property Name: Vaal River
Building:
Street:
Suburb:
Town:
Area Code:
Division/Region:
Province/State:
Country:

NATURE CONSERVATION PERMIT OFFICE
NORTH WEST PROVINCE
06 Mar 2019, 03:06 PM
Private Bag X2039 Mmabatho 2735
Tel: +27 (0)18 389 5331 Fax: +27(0)18 389 5640

Stamp of Issuing authority

VALIDITY PERIOD
FROM 20/02/2019 TO 28/02/2022

Stamp if applicable

Permit holder / Dealer

Signature of Issuing Authority
(Basie Diole)
06 Mar 2019, 03:06 PM

Signature of Permit Holder
(Johannes Hendrik Erasmus)
06 Mar 2019, 03:06 PM

North West Department of Rural, Environment and Agricultural Development. (READ), Cnr. Dr. James Moroka Drive & Stadium Road, Mmabatho
Contact Information: Tel:+27 (0)18 389 5130, Fax:+27 (0)18 389 5130, E-mail:jdenga@nwpg.gov.za
Postal Address: Private Bag X 15, Mmabatho, 2735

ADDENDUM

PROPERTIES

Name	Town	Province	Country
Vaal River			
Mooi River			
Hex River			
Crocodile River			
Marico River			
Baberspan			
Boskop Dam			
Bospoort Dam			
Elands River			
Potchefstroom Dam			
Klipdrift Dam			
Olifantsnek Dam			
Vaalkop Dam			
Roodekopjes Dam			

SPECIES INFORMATION

Scientific Name	Common Name	Number	Gender	Description/Markings
Cyprinus carpio (excluding Koi)	Common Carp	30	Both (Male and/or Female)	None
Oreochromis mossambicus	Mozambique tilapia / Duplicate	30	Both (Male and/or Female)	None
Clarias gariepinus	Sharptooth catfish	30	Both (Male and/or Female)	None
Labeobarbus aneus	Vaal-Orange Smallmouth Yellowfish	30	Both (Male and/or Female)	None
Barbus anoplus	Chubbyhead barb	20	Both (Male and/or Female)	None
Chetia spp.		20	Both (Male and/or Female)	None
Labeo capensis	Orange River mudfish	20	Both (Male and/or Female)	None
Labeo umbratus	Moggel / Mud Millet	20	Both (Male and/or Female)	None
Pseudocrenilabrus philander	Southern mouthbrooder	20	Both (Male and/or Female)	None
Tilapia sparrmanii	Banded tilapia, banded bream	20	Both (Male and/or Female)	None
Micropterus salmoides	Large-mouth bass / Green bass / Green trout	20	Both (Male and/or Female)	None
Barbus paludinosus	Straightfin Barb / Slender Serrate Minnow	20	Both (Male and/or Female)	None
Barbus trimaculatus	Threespot Barb / Threespot Minnow	20	Both (Male and/or Female)	None
Labeobarbus marequensis	Lowveld Largescale Yellowfish	20	Both (Male and/or Female)	None
Labeo cylindricus	Redeye Labeo	20	Both (Male and/or Female)	None
Labeo molybdinus	Leaden Labeo	20	Both (Male and/or Female)	None

Signature of Issuing Authority
(Basie Diole)
06 Mar 2019, 03:06 PM

Signature of Permit Holder
(Johannes Hendrik Erasmus)
06 Mar 2019, 03:06 PM

North West Department of Rural, Environment and Agricultural Development. (READ), Cnr. Dr. James Moroka Drive & Stadium Road, Mmabatho
Contact Information: Tel:+27 (0)18 389 5130, Fax:+27 (0)18 389 5130, E-mail:jdenga@nwpg.gov.za
Postal Address: Private Bag X 15, Mmabatho, 2735

ADDENDUM

OTHER PARTIES INVOLVED

Full Name	ID Number	Contact	Involvement	Physical Address
Nicolas Jacobus Smit	7206075012084	Tel:0182992128	Research Director	
Marliese Truter	9303090077081	Tel:0182852279	Research support team	
Wynand Malherbe	8111285015084	Tel:0182992367	Field supervisor	
Ruan Jan-Isak Lodewyk Gerber	8504045235085	Tel:0182992367	Field supervisor	
Marelize Labuschagne	9407040044081	Tel:0182852279	Research support team	

ACTIVITIES

Activity Name
Angle
Catch
Import
Transport
Keep
Research

METHODS

Method Name
Immobilised
Boat
Net

STANDARD CONDITIONS

GENERAL CONDITIONS - ALL PERMITS

1) The Issuing Authority for this licence is : The Northwest Department of Rural,Environment and Agricultural Development (READ), Chief Directorate Environmental Services, Private Bag X 2039, Mmabatho, 2735, hereafter named "the Issuing Authority". 2) This permit, unless otherwise stated, is only valid within the boundaries of the North West Province (hereafter named "the Province") and then specifically as specified on the permit. 3) This permit is valid only : - a) for the specific species, sex and numbers as specified on this permit. b) for the specific activity / activities authorised. c) for the specified methods or instruments authorised. d) for the specific property / locality as specified. e) for the specific day, time or period stipulated. 4) This permit is only deemed valid : - a) in the original format and with the content as issued by the Issuing Authority. b) once it has been printed and the signature of the permit holder has been endorsed thereon in ink. 5) the Issuing Authority reserves the right to amend, withhold, withdraw or cancel any permit at any time. 6) This permit is not transferable to any individual, natural person, juristic person or any other legal identity. 7) Any alterations or attempt thereto, whether electronically or in any other way, shall immediately render it invalid. 8) This permit shall lapse and be deemed invalid when it is altered, lost or destroyed and no copy thereof shall be issued. 9) This permit shall also become invalid as soon as the permit holder loses possession of any animal, plant or derivative as the case may be, as specified on the permit. 10) This permit does not grant the permit holder automatic access to any Protected Area, National Park, Provincial Nature Reserve or privately owned land and : - a) the permit holder must beforehand obtain all other relevant written permissions, documents, rights and licences. b) the permit holder must comply with any other / further conditions or restrictions that the manager / landowner may stipulate at his / her discretion. 11) The permit holder must at all times while performing any restricted activity authorised by this permit, have the permit and all other relevant documentation in his / her possession and without delay make it available upon request by any authorized person. 12) An authorized person must also be allowed access onto the property at any reasonable time for any inspection needed and can remain on such property as long as it is needed to do the inspection. 13) The permit holder must immediately after completion of any activity authorised by this permit, record the required particulars in the space provided therefore or on the annexure or document attached hereto or in the prescribed register related to the permit. 14) The permit holder must return the original signed permit to the Issuing Authority within (14) fourteen days : - a) after performing or completing the authorised restricted activity, or b) after the date of expiry thereof whichever happens first, and c) if applicable furnish the Issuing Authority with a prescribed written feedback report on the results of every activity conducted. 15) The permit holder must retain a copy of the permit together with all other relevant written permissions, documents, rights and licenses for a period of at least (2) two years from date of issue or for as long as the permit holder is in possession of the animal, plant or derivative, whichever period is the longer. 16) If applicable, the permit holder shall apply for the renewal of the permit to the Issuing Authority, on the appropriate application form, at least (3) three months prior to the expiry date thereof. 17) Applications for renewal of this permit will only be processed after the original signed permit together with the prescribed written feedback report has been returned to the Issuing Authority. 18) This permit, during the period of validity thereof, is also subject to: - a) all applicable norms and standards in existence at the time of issuance. b) the provisions of any law in force, in respect of the specific species, activity, method or instrument to which this permit applies. 19) It is the permit holder's responsibility to obtain the correct information on any other legislation, specification, requirement or changes thereto that may be applicable or are required by any other Issuing Authority / Organization / Institute, relating to this permit. 20) By signing this permit, the permit holder declares that he / she is aware of the fact that : - a) any transgression or failure to return the original permit or failure to render the required reports can lead to criminal prosecution and also jeopardize any future applications by or in the name of the permit holder. b) if the permit holder contravenes or fails to comply with any permit condition or requirement, he / she shall be guilty of an offence. 21) The prescribed fees paid to the Issuing Authority for the issue of this permit shall not be refunded.

HARVESTING - COLLECT FOR SCIENTIFIC RESEARCH

1) The permit holder must ensure that : - a) specimens collected in terms of this permit is utilised for scientific purposes and only by the applicable institution or Issuing Authority as specified on this permit. b) specimens collected in term of this permit is not sold, bartered or given away. c) the written permission of the owner or occupier of land is obtained before any plant is harvested / picked. d) quantities of soil, rock or specimens is limited to the absolute minimum necessary for the research project. e) no endangered or specially protected specimens is collected. f) detailed record is kept on the specimens, quantities and localities where specimens are collected.

OCCUPATION - RESEARCH - (Non-Bioprospecting)

1) Should the research be conducted by a group, numbers specified are for the group as a whole and not per individual. 2) Written permission of the landowner or relevant authority must be obtained and must always be carried on such person with the valid permit. 3) Care should be taken not to harm

Signature of Issuing Authority
(Basie Diole)
06 Mar 2019, 03:06 PM

Signature of Permit Holder
(Johannes Hendrik Erasmus)
06 Mar 2019, 03:06 PM

North West Department of Rural, Environment and Agricultural Development. (READ), Cnr. Dr. James Moroka Drive & Stadium Road, Mmabatho
Contact Information: Tel:+27 (0)18 389 5130, Fax:+27 (0)18 389 5130, E-mail:jdenga@nwpg.gov.za
Postal Address: Private Bag X 15, Mmabatho, 2735

ADDENDUM

non-target species. 4) An update report must be forwarded to the Issuing Authority which includes the permit holders name and contact details, species scientific and common name, numbers, date and locality (grid reference and description), Latitude and longitude as well as a copy of the written permission of the landowner where the activity had been conducted. 5) If applicable, at least one specimen per species should be lodged / housed at a South African Institute / Herbarium / Museum. 6) A copy of all completed reports, publications, or articles resulting from the project must be submitted free of charge to the Issuing Authority. 7) Should a report, publication, article or thesis arise from this project, an acknowledgement to the Issuing Authority. 8) Unless otherwise specifically indicated in writing, no material or specimens collected with this permit or material or specimens bred or propagated, from material or specimens collected with this permit, may be donated, sold or used for any commercial purpose by any party. 9) Type-specimens of any newly described / discovered species or other taxon collected must be lodged with a recognized South African institution / museum / herbarium (preferably within the Province), where such material will be available to other researchers. 10) This permit does not authorize the collection within the boundaries of any Protected area, National Park, Provincial Nature Reserve, unless specified in the permit. 11) No Critically Endangered or Endangered species may be collected unless specified on the permit. The permit holder should take photographic records of rare / endangered species (where recognized). 12) No habitat must be disturbed at all. 13) Please ensure the validity of all other certificates and permits issued by other Institutes during the validity of this permit 14) This permit is only valid if a valid ethics letter for the relevant project is obtained and supplied to the Issuing Authority.

OCCUPATION - RESEARCH

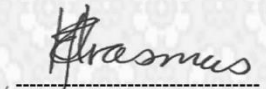
1) "Plant and Quality Control" and any other regulations must be adhered to when exporting material out of the country. 2) No live specimens / material may leave the province, unless otherwise specified. 3) No habitat must be disturbed at all.

TRANSPORT - EXPORT - RESEARCH

1) An update report of species translocated to be forwarded to the Issuing Authority with the application for renewal of a research permit.

Signature of Issuing Authority
(Basie Diole)

06 Mar 2019, 03:06 PM



Signature of Permit Holder
(Johannes Hendrik Erasmus)

06 Mar 2019, 03:06 PM

North West Department of Rural, Environment and Agricultural Development. (READ), Cnr. Dr. James Moroka Drive & Stadium Road, Mmabatho
Contact Information: Tel:+27 (0)18 389 5130, Fax:+27 (0)18 389 5130, E-mail:jdenga@nwpg.gov.za
Postal Address: Private Bag X 15, Mmabatho, 2735

Appendix E – Ethics Approval for sampling

Private Bag X6001, Potchefstroom
South Africa 2520

Tel: 018 299-1111/2222
Web: http://www.nwu.ac.za

Health Sciences Ethics Office for Research, Training and Support

North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC)
Tel: 018 299 2234
Email: Tiaan.Brink@nwu.ac.za

Prof NJ Smit
Unit for Environmental Sciences and Management

05 February 2019

Dear Prof Smit

APPROVAL OF YOUR APPLICATION BY THE NWU-ANIMCARAREC COMMITTEE OF THE FACULTY OF HEALTH SCIENCES

Ethics number: NWU-00160-18-S5

Kindly use the ethics reference number provided above in all future correspondence or documents submitted to the administrative assistant of the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC).

Study title: Study of *Diplostomum* von Nordmann, 1832 (Digenea: Diplostomoidea) in South Africa: Diversity and effect of *metacercariae* on fish behaviour

Study leader: Prof NJ Smit

Student: C Hoogendoorn

Application type: Single study

Project Category *(impact on animal wellbeing)*	**NA**	**0**	**1**	**2**	**3**	**4**	**5**
							X

Expiry date: 29 February 2020 (Monitoring reports are due at the end of August and February annually until completion of the research)

You are kindly informed that after review by the NWU-AnimCareREC, Faculty of Health Sciences, North-West University, your ethics approval application has been successful and was determined to fulfil all requirements for approval. Your study is approved for a year and may commence from 05/02/2019. Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation. A monitoring report should be submitted two months prior to the reporting dates as indicated i.e. annually for Category 0-4 studies, six-monthly for category 5 studies, to ensure timely renewal of the study. A final report must be provided at completion of the study or the NWU-AnimCareREC, Faculty of Health Sciences must be notified if the study is temporarily suspended or terminated. The monitoring report template is obtainable from the Faculty of Health Sciences Ethics Office for Research, Training and Support at Ethics-AnimMonitoring@nwu.ac.za. Annually, a number of studies may be randomly selected for an internal audit.

The NWU-AnimCareREC, Faculty of Health Sciences requires immediate reporting of any aspects that warrants a change of ethical approval. Any amendments, extensions or other modifications to the proposal or other associated documentation must be submitted to the NWU-AnimCareREC, Faculty of Health Sciences prior to implementing these changes. These requests should be submitted to Ethics-AnimCare@nwu.ac.za with a cover letter with a specific subject title indicating "Amendment request: NWU-XXXXX-XX-XX". The letter should include the title of the approved study, the names of the researchers involved, the nature of the amendment/s being made (indicating what changes have been made as well as where they have been made), which documents have been attached and any further explanation to clarify the amendment request being submitted. The amendments made should be indicated in yellow highlight in the amended documents (or in the fillable MSWord format application forms where a yellow highlighter may not be visible, change the text colour to red). The *e-mail*, to which you attach the documents that you send, should have a *specific subject*

line indicating that it is an amendment request e.g. "Amendment request: NWU-XXXXX-XX-XX". This e-mail should indicate the nature of the amendment. This submission will be handled via the expedited process.

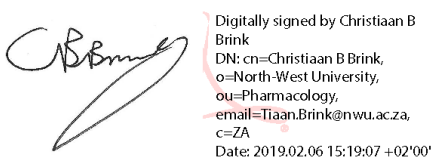
Any adverse/unexpected/unforeseen events or incidents must be reported on either an adverse event report form or incident report form to Ethics-AnimCareIncident-SAE@nwu.ac.za. The *e-mail*, to which you attach the documents that you send, should have a specific subject line indicating that it is a notification of a serious adverse event or incident in a specific project e.g. "SAE/Incident notification: NWU-XXXXX-XX-XX".

Please note that the NWU-AnimCareREC, Faculty of Health Sciences has the prerogative and authority to ask further questions, seek additional information, require further modification or monitor the conduct of your research. The NWU-AnimCareREC, Faculty of Health Sciences reserves the right to visit sites where approved studies will be conducted and any animal housing facility under the authority of NWU as often as it deems necessary, either announced or unannounced.

The NWU-AnimCareREC, Faculty of Health Sciences complies with the South African National Health Act 61 (2003), the Regulations on Research with Human Participants (2014), the Ethics in Health Research: Principles, Structures and Processes (2015), the South African National Standard (SANS) document 10386:2008 entitled, "The care and use of animals for scientific purposes", the Belmont Report and the Declaration of Helsinki (2013).

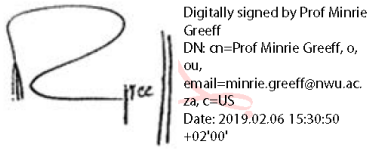
We wish you the best as you conduct your research. If you have any questions or need further assistance, please contact the Faculty of Health Sciences Ethics Office for Research, Training and Support at Ethics-AnimCare@nwu.ac.za.

Yours sincerely



Prof Christiaan B Brink

Chair: NWU-AnimCareREC



Prof Minrie Greeff

Head: Ethics Office

Current details: (13009230) G:\My Drive\9.1.5.1.1 Ethics / 2018 / NWU-00160-18-S5 /9.1.5.4.1_Approval_letter_AnimCare.docm 05 February 2019

File reference: 9.1.5.4.1

Appendix F – Table F1: Summary data for sequences for *Diplostomum* spp. retrieved from GenBank.

Species as in GenBank	Stage	Host	GenBank accession numbers			Country	Source
			28S	ITS1–8.5S–ITS2	*cox*1		
Diplostomum ardeae	A	*Ardea herodias*	–	–	KR271033	Canada	Locke *et al*. (2015)
Diplostomum baeri	A	*Larus delawarensis*	–	AY123042	–	Canada	Galazzo *et al*. (2002)
Diplostomum baeri	A	*Larus* sp.	–	–	GQ292501	Canada	Locke *et al*. (2010a)
Diplostomum baeri	M	*Perca flavescens*	–	–	HM064663	Canada	Locke *et al*. (2010b)
Diplostomum baeri	A	*Larus delawarensis* fed *Perca flavescens*	–	–	KR271056	Canada	Moszczynska *et al*. (2009)
Diplostomum baeri = '*Diplostomum baeri* Lineage 1'	M	*Gobio gobio*	–	JX986855	–	Germany	Georgieva *et al*. (2013)
Diplostomum baeri = '*Diplostomum baeri* Lineage 1'	M	*Salmo trutta fario*	–	–	JX986863	Germany	Georgieva *et al*. (2013)
Diplostomum baeri = '*Diplostomum baeri* Lineage 1'	M	*Salmo trutta fario*	–	–	JX986867	Germany	Georgieva *et al*. (2013)
Diplostomum baeri = '*Diplostomum baeri* Lineage 1'	M	*Perca fluviatilis*	–	JQ665460	–	Germany	Behrmann-Godel (2013)
Diplostomum baeri = '*Diplostomum baeri* Lineage 2'	M	*Perca fluviatilis*	–	–	JQ639180	Germany	Behrmann-Godel (2013)
Diplostomum baeri complex sp. 2 = '*Diplostomum baeri* Lineage 2'	M	*Gasterosteus aculeatus*	–	–	KM212032	Norway	Kuhn *et al*. (2015)
Diplostomum huronense	A	*Larus delawarensis*	–	AY123044	–	Canada	Galazzo *et al*. (2002)
Diplostomum huronense	M	*Catostomus commersonii*	–	GQ292513	–	Canada	Locke *et al*. (2010a)
Diplostomum huronense	A	*Larus delawarensis* fed *Notemigonus crysoleucas*	–	–	FJ477197	Canada	Moszczynska *et al*. (2009)

Table F1 (continued)

Species as in GenBank	Stage	Host	GenBank accession numbers			Country	Source
			28S	ITS1–8.5S–ITS2	*cox*1		
Diplostomum huronense	M	*Catostomus commersonii*	–	–	GQ292489	Canada	Locke *et al*. (2010a)
Diplostomum huronense	A	*Larus delawarensis fed Notemigonus crysoleucas*	–	–	HM064669	Canada	Locke *et al*. (2010b)
Diplostomum indistinctum	A	*Larus* sp.	–	AY123043	–	Canada	Galazzo *et al*. (2002)
Diplostomum indistinctum	M	*Catostomus commersonii*	–	GQ292508	–	Canada	Locke *et al*. (2010a)
Diplostomum indistinctum	M	*Neogobius melanostomus*	–	–	GQ292482	Canada	Locke *et al*. (2010a)
Diplostomum indistinctum	M	*Catostomus commersonii*	–	–	GQ292483	Canada	Locke *et al*. (2010a)
Diplostomum indistinctum	A	*Larus* sp.	–	–	GQ292484	Canada	Locke *et al*. (2010a)
Diplostomum mergi = *'Diplostomum mergi* Lineage 2'	C	*Radix auricularia*	–	KR149493	–	Germany	Selbach *et al*. (2015)
Diplostomum mergi = *'Diplostomum mergi* Lineage 2'	C	*Radix auricularia*	–	KR149494	–	Germany	Selbach *et al*. (2015)
Diplostomum mergi = *'Diplostomum mergi* Lineage 2'	C	*Radix auricularia*	–	–	JX986876	Germany	Georgieva *et al*. (2013)
Diplostomum mergi = *'Diplostomum mergi* Lineage 2'	C	*Radix auricularia*	–	–	KR149523	Germany	Selbach *et al*. (2015)
Diplostomum mergi complex sp. 2 = *'Diplostomum mergi* Lineage 2'	M	*Abramis brama*	–	–	KR271082	China	Locke *et al*. (2015)
Diplostomum mergi = *'Diplostomum mergi* Lineage 3'	M	*Gobio gobio*	–	JX986840	–	Germany	Georgieva *et al*. (2013)
Diplostomum mergi = *'Diplostomum mergi* Lineage 3'	C	*Radix auricularia*	–	KR149496	–	Germany	Selbach *et al*. (2015)
Diplostomum mergi = *'Diplostomum mergi* Lineage 3'	M	*Salmo trutta fario*	–	–	JX986880	Germany	Georgieva *et al*. (2013)

Table F1 (continued)

Species as in GenBank	Stage	Host	GenBank accession numbers			Country	Source
			28S	ITS1–8.5S–ITS2	*cox*1		
Diplostomum mergi = '*Diplostomum mergi* Lineage 3'	M	*Gobio gobio*	–	–	JX986884	Germany	Georgieva *et al*. (2013)
Diplostomum mergi = '*Diplostomum mergi* Lineage 3'	C	*Radix auricularia*	–	–	KR149525	Germany	Selbach *et al*. (2015)
Diplostomum mergi = '*Diplostomum mergi* Lineage 4'	C	*Radix balthica*	–	JX494231	–	Denmark	Haarder *et al*. (2013)
Diplostomum mergi = '*Diplostomum mergi* Lineage 4'	C	*Radix auricularia*	–	KR149499	–	Germany	Selbach *et al*. (2015)
Diplostomum mergi = '*Diplostomum mergi* Lineage 4'	C	*Radix auricularia*	–	–	KR149528	Germany	Selbach *et al*. (2015)
Diplostomum mergi = *Diplostomum parviventosum*	C	*Radix auricularia*	–	JX986838	–	Germany	Georgieva *et al*. (2013)
Diplostomum parviventosum	C	*Radix auricularia*	–	KR149490	–	Germany	Selbach *et al*. (2015)
Diplostomum mergi = *Diplostomum parviventosum*	C	*Radix auricularia*	–	–	JX986873	Germany	Georgieva *et al*. (2013)
Diplostomum parviventosum	C	*Radix auricularia*	–	–	KR149504	Germany	Selbach *et al*. (2015)
Diplostomum parviventosum	C	*Radix auricularia*	–	–	KR149510	Germany	Selbach *et al*. (2015)
Diplostomum phoxini	M	*Phoxinus phoxinus*	AY222173	–	–	UK, Wales	Olson *et al*. (2003)
Diplostomum phoxini	C	*Radix balthica*	–	–	KY513185	Norway	Soldánová *et al*. (2017)
Diplostomum phoxini	M	*Phoxinus phoxinus*	–	–	KY513186	Norway	Soldánová *et al*. (2017)
Diplostomum pseudospathaceum	A	*Larus ridibundus*	KR269766	KR269766	–	Czech Republic	Brabec *et al*. (2015)
Diplostomum pseudospathaceum	M	*Gymnocephalus cernua*	–	JQ665456	–	Germany	Behrmann-Godel (2013)
Diplostomum pseudospathaceum	A	*Larus cachinnans*	–	–	JX986896	Czech Republic	Georgieva *et al*. (2013)
Diplostomum pseudospathaceum	M	*Gasterosteus aculeatus*	–	–	JX986903	Germany	Georgieva *et al*. (2013)

Table F1 (continued)

Species as in GenBank	Stage	Host	GenBank accession numbers			Country	Source
			28S	ITS1–8.5S–ITS2	*cox*1		
Diplostomum pseudospathaceum	C	*Lymnaea stagnalis*	–	–	KR149532	Germany	Selbach *et al*. (2015)
Diplostomum spathaceum	A	*Larus ridibundus*	KR269765	–	–	Czech Republic	Brabec *et al*. (2015)
Diplostomum spathaceum	M	*Gasterosteus aculeatus*	–	KJ726508	–	Iceland	Blasco-Costa *et al*. (2014)
Diplostomum spathaceum	C	*Radix auricularia*	–	KR149502	–	Germany	Selbach *et al*. (2015)
Diplostomum spathaceum	A	*Larus cachinnans*	–	–	JX986895	Czech Republic	Georgieva *et al*. (2013)
Diplostomum spathaceum	M	*Gasterosteus aculeatus*	–	–	KJ726433	Iceland	Blasco-Costa *et al*. (2014)
Diplostomum spathaceum	A	*Larus ridibundus*	–	–	KP025775	Spain	Pérez-del-Olmo *et al*. (2014)
Diplostomum spathaceum	M	*Barbus luteus*	–	–	KR271432	Iraq	Locke *et al*. (2015)
Diplostomum spathaceum	M	*Abramis brama*	–	–	KR271434	China	Locke *et al*. (2015)
Diplostomum sp. Lineage 2	M	*Salmo trutta fario*	–	KJ726510	–	Iceland	Blasco-Costa *et al*. (2014)
Diplostomum sp. Lineage 2	M	*Salmo trutta fario*	–	KJ726511	–	Iceland	Blasco-Costa *et al*. (2014)
Diplostomum sp. Lineage 2	M	*Salmo trutta fario*	–	–	KJ726453	Iceland	Blasco-Costa *et al*. (2014)
Diplostomum sp. Lineage 2	M	*Gasterosteus aculeatus*	–	–	KJ726454	Iceland	Blasco-Costa *et al*. (2014)
Diplostomum sp. Lineage 2	C	*Radix peregra*	–	–	KJ726456	Iceland	Blasco-Costa *et al*. (2014)
Diplostomum sp. Lin 3 = '*Diplostomum baeri* Lineage 1'	M	*Salmo trutta*	–	–	KY513188	Norway	Soldánová *et al*. (2017)
Diplostomum sp. Lin 4 = '*Diplostomum baeri* Lineage 2'	C	*Radix peregra*	–	KJ726523	–	Iceland	Blasco-Costa *et al*. (2014)
Diplostomum sp. Lin 4 = '*Diplostomum baeri* Lineage 2'	M	*Gasterosteus aculeatus*	–	KJ726527	–	Iceland	Blasco-Costa *et al*. (2014)

Table F1 (continued)

Species as in GenBank	Stage	Host	GenBank accession numbers			Country	Source
			28S	ITS1–8.5S–ITS2	*cox*1		
Diplostomum sp. Lin 4 = '*Diplostomum baeri* Lineage 2'	C	*Radix peregra*	–	–	KJ726476	Iceland	Blasco-Costa *et al*. (2014)
Diplostomum sp. Lineage 5	M	*Salmo trutta fario*	–	KJ726529	–	Iceland	Blasco-Costa *et al*. (2014)
Diplostomum sp. Lineage 5	M	*Salvelinus alpinus*	–	KJ726532	–	Iceland	Blasco-Costa *et al*. (2014)
Diplostomum sp. Lineage 5	M	*Salmo trutta fario*	–	–	KJ726485	Iceland	Blasco-Costa *et al*. (2014)
Diplostomum sp. Lineage 5	M	*Salvelinus alpinus*	–	–	KJ726490	Iceland	Blasco-Costa *et al*. (2014)
Diplostomum sp. Lineage 5	M	*Gasterosteus aculeatus*	–	–	KR271078	Norway	Locke *et al*. (2015)
Diplostomum sp. Lineage 6	M	*Gasterosteus aculeatus*	–	KJ726535	–	Iceland	Blasco-Costa *et al*. (2014)
Diplostomum sp. Lineage 6	C	*Radix peregra*	–	KJ726537	–	Iceland	Blasco-Costa *et al*. (2014)
Diplostomum sp. Lineage 6	C	*Radix peregra*	–	–	KJ726497	Iceland	Blasco-Costa *et al*. (2014)
Diplostomum sp. Lineage 6	M	*Gasterosteus aculeatus*	–	–	KJ726504	Iceland	Blasco-Costa *et al*. (2014)
Diplostomum sp. Lineage 6	M	*Gasterosteus aculeatus*	–	–	KM212042	Norway	Kuhn *et al*. (2015)
Diplostomum sp. Clade Q	C	*Radix auricularia*	–	JQ665458	–	Germany	Behrmann-Godel (2013)
Diplostomum mergi = *'Diplostomum* sp. Clade Q'	M	*Rutilus rutilus*	–	–	JQ639177	Germany	Behrmann-Godel (2013)
Diplostomum sp. Clade Q	M	*Cyprinus carpio*	–	–	KP025770	Spain	Pérez-del-Olmo *et al*. (2014)
Diplostomum sp. Clade Q	C	*Radix auricularia*	–	–	KR149554	Germany	Selbach *et al*. (2015)
Diplostomum sp. A	M	*Blicca bjoerkna*	–	–	KY654034	Slovakia	Kudlai *et al*. (2017)
Diplostomum sp. B	M	*Carassius gibelio*	–	–	KY654035	Slovakia	Kudlai *et al*. (2017)

Table F1 (continued)

Species as in GenBank	Stage	Host	GenBank accession numbers			Country	Source
			28S	ITS1–8.5S–ITS2	*cox*1		
Diplostomum sp. C	M	*Rutilus rutilus*	–	–	KY654036	Slovakia	Kudlai *et al*. (2017)
Diplostomum sp.	M	*Synodontis nigrita*	–	KC685369	–	Nigeria	Chibwana *et al*. (2013)
Diplostomum sp.	M	*Synodontis nigrita*	–	–	KC685359	Nigeria	Chibwana *et al*. (2013)
Diplostomum sp.	M	*Synodontis nigrita*	–	–	KC685360	Nigeria	Chibwana *et al*. (2013)
Diplostomum sp. 1	A	*Larus delawarensis*	–	GQ292515	–	Canada	Locke *et al*. (2010a)
Diplostomum sp. 1	M	*Notropis hudsonius*	–	KT186793	–	Canada	Locke *et al*. (2015)
Diplostomum sp. 1	A	*Larus marinus*	–	–	FJ477195	Canada	Moszczynska *et al*. (2009)
Diplostomum sp. 1	A	*Larus delawarensis*	–	–	GQ292475	Canada	Locke *et al*. (2010a)
Diplostomum sp. 1	A	*Larus argentatus*	–	–	HM064678	Canada	Locke *et al*. (2010b)
Diplostomum sp. 2	M	*Pimephales notatus*	–	GQ292505	–	Canada	Locke *et al*. (2010a)
Diplostomum sp. 2	M	*Notropis atherinoides*	–	KT186795	–	Canada	Locke *et al*. (2015)
Diplostomum sp. 2	M	*Pimephales notatus*	–	–	FJ477198	Canada	Moszczynska *et al*. (2009)
Diplostomum sp. 2	M	*Pimephales promelas*	–	–	KR271266	USA	Locke *et al*. (2015)
Diplostomum sp. 2	M	*Notropis atherinoides*	–	–	KR271267	Canada	Locke *et al*. (2015)
Diplostomum sp. 3	M	*Micropterus salmoides*	–	GQ292511	–	Canada	Locke *et al*. (2010a)
Diplostomum sp. 3	M	*Micropterus salmoides*	–	–	GQ292487	Canada	Locke *et al*. (2010a)
Diplostomum sp. 3	A	*Larus delawarensis*	–	–	HM064695	Canada	Locke *et al*. (2010b)
Diplostomum sp. 3	M	*Ambloplites rupestris*	–	–	HM064697	Canada	Locke *et al*. (2010b)
Diplostomum sp. 4	A	*Larus delawarensis*	–	GQ292520	–	Canada	Locke *et al*. (2010a)
Diplostomum sp. 4	M	*Gasterosteus aculeatus*	–	KT186796	–	USA	Locke *et al*. (2015)
Diplostomum sp. 4	A	*Larus delawarensis*	–	–	GQ292494	Canada	Locke *et al*. (2010a)

Table F1 (continued)

Species as in GenBank	Stage	Host	GenBank accession numbers			Country	Source
			28S	ITS1–8.5S–ITS2	*cox*1		
Diplostomum sp. 4	A	*Larus argentatus*	–	–	HM064700	Canada	Locke *et al*. (2010b)
Diplostomum sp. 4	M	*Carpiodes cyprinus*	–	–	HM064711	Canada	Locke *et al*. (2010b)
Diplostomum sp. 5	M	*Perca flavescens*	–	–	GQ292498	Canada	Locke *et al*. (2010a)
Diplostomum sp. 6	M	*Pimephales notatus*	–	–	GQ292499	Canada	Locke *et al*. (2010a)
Diplostomum sp. 6	M	*Fundulus diaphanus*	–	–	KR271392	Canada	Locke *et al*. (2015)
Diplostomum sp. 6	M	*Fundulus diaphanus*	–	–	KR271393	Canada	Locke *et al*. (2015)
Diplostomum sp. 7	M	*Pimephales notatus*	–	–	GQ292500	Canada	Locke *et al*. (2010a)
Diplostomum sp. 7	M	*Percopsis omiscomaycus*	–	–	KR271400	Canada	Locke *et al*. (2015)
Diplostomum sp. 7	M	*Salvelinus fontinalis*	–	–	KR271404	Canada	Locke *et al*. (2015)
Diplostomum sp. 8	M	*Lithobates pipiens*	–	GQ292510	–	Canada	Locke *et al*. (2010a)
Diplostomum sp. 8	M	*Lithobates pipiens*	–	–	GQ292497	Canada	Locke *et al*. (2010a)
Diplostomum sp. 9	M	*Percina caprodes*	–	GQ292504	–	Canada	Locke *et al*. (2010a)
Diplostomum sp. 9	M	*Percina caprodes*	–	–	GQ292496	Canada	Locke *et al*. (2010a)
Diplostomum sp. 9	M	*Salvelinus fontinalis*	–	–	KR271410	Canada	Locke *et al*. (2015)
Diplostomum sp. 9	M	*Cottus asper*	–	–	KR271413	Canada	Locke *et al*. (2015)
Diplostomum sp. 10	M	*Pimephales promelas*	–	KT186788	–	Canada	Locke *et al*. (2015)
Diplostomum sp. 10	M	*Ambloplites rupestris*	–	–	KR271096	Canada	Locke *et al*. (2015)
Diplostomum sp. 10	M	*Ambloplites rupestris*	–	–	KR271097	Canada	Locke *et al*. (2015)
Diplostomum sp. 10	M	*Pimephales promelas*	–	–	KR271098	Canada	Locke *et al*. (2015)
Diplostomum sp. 12	M	*Cottus cognatus*	–	–	KR271100	Canada	Locke *et al*. (2015)
Diplostomum sp. 12	M	*Cottus cognatus*	–	–	KR271101	USA	Locke *et al*. (2015)
Diplostomum sp. 12	M	*Cottus cognatus*	–	–	KR271102	USA	Locke *et al*. (2015)
Diplostomum sp. 13	M	*Gasterosteus aculeatus*	–	–	KR271104	USA	Locke *et al*. (2015)

Table F1 (continued)

Species as in GenBank	Stage	Host	GenBank accession numbers			Country	Source
			28S	ITS1–8.5S–ITS2	*cox*1		
Diplostomum sp. 14	M	*Tinca tinca*	–	KT186789	–	China	Locke *et al*. (2015)
Diplostomum sp. 14	M	*Channa argus*	–	–	KR271113	China	Locke *et al*. (2015)
Diplostomum sp. 14	M	*Tinca tinca*	–	–	KR271115	China	Locke *et al*. (2015)
Diplostomum sp. 14	M	*Cyprinion macrostomum*	–	–	KR271120	Iraq	Locke *et al*. (2015)
Diplostomum sp. 15	M	*Hypophthalmichthys nobilis*	–	KT186791	–	China	Locke *et al*. (2015)
Diplostomum sp. 15	M	*Hypophthalmichthys nobilis*	–	–	KR271124	China	Locke *et al*. (2015)
Diplostomum sp. 15	M	*Chanodichthys dabryi*	–	–	KR271126	China	Locke *et al*. (2015)
Diplostomum sp. 15	M	*Hypophthalmichthys nobilis*	–	–	KR271127	China	Locke *et al*. (2015)
Diplostomum sp. 16	M	*Alburnus caeruleus*	–	–	KR271129	Iraq	Locke *et al*. (2015)
Diplostomum sp. 17	M	*Cottus cognatus*	–	–	KR271130	Canada	Locke *et al*. (2015)
Diplostomum sp. 17	M	*Cottus cognatus*	–	–	KR271132	Canada	Locke *et al*. (2015)
Diplostomum sp. 17	M	*Cottus cognatus*	–	–	KR271134	Canada	Locke *et al*. (2015)
Diplostomum sp. 18	M	*Cottus cognatus*	–	–	KR271137	Canada	Locke *et al*. (2015)
Diplostomum sp. 18	M	*Cottus asper*	–	–	KR271138	Canada	Locke *et al*. (2015)
Diplostomum sp. 18	M	*Cottus cognatus*	–	–	KR271139	Canada	Locke *et al*. (2015)
Diplostomum sp. 19	M	*Cottus cognatus*	–	–	KR271143	USA	Locke *et al*. (2015)
Diplostomum sp. 19	M	*Cottus cognatus*	–	–	KR271144	Canada	Locke *et al*. (2015)
Diplostomum sp. 19	M	*Cottus ricei*	–	–	KR271145	Canada	Locke *et al*. (2015)
Diplostomoidea cf. Tylodelphys mashonensis = Tylodelphys mashonensis (outgroup)	M	*Tilapia sparrmanii*	KF189071	–	–	South Africa	Moema *et al*. (2013)
Tylodelphys clavata (outgroup)	M	*Coregonus lavaretus*	–	JQ665459	–	Germany	Behrmann-Godel (2013)
Tylodelphys clavata (outgroup)	M	*Perca fluviatilis*	–	–	JX986909	Germany	Georgieva *et al*. (2013)

Life cycle stages: A, adult; C, cercaria; M, metacercaria

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Appendix G – Publication: Resolution of the identity of three species of *Diplostomum* (Digenea: Diplostomidae) parasitising freshwater fishes in South Africa, combining molecular and morphological evidence

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Resolution of the identity of three species of *Diplostomum* (Digenea: Diplostomidae) parasitising freshwater fishes in South Africa, combining molecular and morphological evidence

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ABSTRACT

Reliable data on the diversity of the genus *Diplostomum* (Digenea: Diplostomidae) parasitising freshwater fishes in South Africa, as well as in Africa, is almost non-existent. Most of the morphology-based identifications of species within this genus reported from Africa require critical revision. The aim of the present study was to determine the diversity of *Diplostomum* metacercariae in South African fishes applying molecular and traditional morphological techniques. To achieve this aim, a total of 216 fishes belonging to 21 species collected in the Rivers Phongolo, Riet, Usuthu and Mooi in three provinces of South Africa were examined. Metacercariae of *Diplostomum* were recovered from the eye lenses of 38 fishes belonging to five species of the families Anguillidae, Cichilidae and Mochokidae, with an overall low prevalence of infection (18%). Metacercariae were subjected to morphological study and molecular sequencing of the partial mithochondrial *cox*1 and ribosomal 28S rDNA genes as well as of ribosomal ITS1-5.8S-ITS2 region. Morphological and phylogenetic analyses revealed the presence of three species which matched those previously reported from Nigeria, Iraq and China, therefore those from *Tilapia sparrmanii* and *Synodontis zambezensis* were named *Diplostomum* sp.; those from *Anguilla labiata*, *Oreochromis mossambicus* and *S. zambezensis* were named *Diplostomum* sp. 14; and those from *Pseudocrenilabrus philander* were named *Diplostomum* sp. 16. Geographic distribution of several species of *Diplostomum* appeared to be wider than expected. Morphological description and novel sequence data generated during this study will contribute to the elucidation of the life cycles of *Diplostomum* sp., *Diplostomum* sp. 14 and *Diplostomum* sp. 16 and advance further research of diplostomids in Africa.

1. Introduction

Trematodes from the genus *Diplostomum* von Nordmann, 1832 (Digenea: Diplostomidae) are intestinal parasites of fish-eating birds, reported from all continents, but with the majority of species described from the Nearctic and Palaearctic (Shigin, 1986, 1993). For the successful completion of their life cycles, species of *Diplostomum* utilise freshwater snails and fish as intermediate hosts (Niewiadomska, 2002). Metacercarial stages are regarded as pathogenic for their fish hosts and therefore they remain the focus of numerous ecological, behavioural and evolutionary studies (Ballabeni and Ward, 1993; Owen et al., 1993; Kalbe and Kurtz, 2006; Seppäla et al., 2004, 2011; Benesh and Kalbe, 2016; Klemme et al., 2016). A significant amount of research effort has recently been invested in developing the molecular sequence library for species within this genus (Galazzo et al., 2002; Moszczynska et al., 2009; Locke et al., 2010a, 2010b; 2015; Behrmann-Godel, 2013; Georgieva et al., 2013; Pérez-del-Olmo et al., 2014; Blasco-Costa et al., 2014; Selbach et al., 2015; Kuhn et al., 2015; Soldánová et al., 2017; Kudlai et al., 2017; Enabulele et al., 2018). The complete mitochondrial genomes of two closely related species from the Palaearctic, *D. spathaceum* (Rudolphi, 1819) and *D. pseudospathaceum* Niewiadomska, 1984 were characterised (Brabec et al., 2015). Currently, molecular data for *Diplostomum* available in GenBank includes sequences for eight species and 38 unidentified species/species-level genetic lineages from Europe (4 species and 15 unidentified species/species-level genetic lineages), North America (4 and 19, respectively), Asia (1 and 3, respectively) and Africa (1 unidentified species) (see Chibwana et al., 2013; Locke et al., 2015; Kudlai et al., 2017; Soldánová et al., 2017; Gordy and Hanington, 2019). Nevertheless, the utility of available molecular data remains limited due to the heavy bias nature towards

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