Haemoprotozoans infecting near-shore catsharks (Elasmobranchii: Scyliorhinidae) off the Western Cape

C Pretorius

orcid.org 0000-0003-1396-8312

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Supervisor:	Dr CA Cook
Co-supervisor:	Prof NJ Smit
Co-supervisor:	Dr BC Schaeffner

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“It seems to me that the natural world is the greatest source of excitement; the greatest source of visual beauty; the greatest source of intellectual interest. It is the greatest source of so much in life that makes life worth living.”

Sir David Attenborough

ABSTRACT

Haemoprotozoans infecting near-shore catsharks (Elasmobranchii: Scyliorhinidae) off the Western Cape.

Chantelle Pretorius. May 2021.

Key words: Haemoparasite, Chondrichthyan, *Trypanosoma*, Haemogregarine, Biodiversity, Marine parasitology, PCR, Morphology, Genetics, South Africa.

Little is known on blood parasites or haemoprotozoans (haemogregarines and trypanosomes) infecting elasmobranchs worldwide, and knowledge on these parasites in South African cartilaginous fishes is even more limited. Even though haemoprotozoans have been reported infecting two scyliorhinid species off the coast of the Western Cape of South Africa, only one of these has been formally described, *Trypanosoma haploblephari* Yeld and Smit, 2006 (Trypanosomatida: Trypanosomatidae). Furthermore, descriptions are based entirely on morphology of peripheral blood stages of these parasites. At microscopy level, the available characters that may be used to differentiate parasite species and, even in some cases, genera, are limited, and as such morphological characteristics alone cannot be relied upon. Additionally, some species of *Trypanosoma* Gruby, 1943 are known for pleomorphism (having more than one form), which limits the use of morphological characters for differentiation even further. Over the last decade, there has been increased effort to use molecular characteristics in the description and differentiation of haemoprotozoans to genus and species levels. However, until recently, this effort has been largely focused on the haemoprotozoans of other vertebrate groups (mammals, birds, reptiles, and amphibians). With these groups, the combination of both morphological and molecular characteristics has greatly aided in determining the phylogenetic placements, thus the identity and taxonomy of these parasites, which in turn has aided in assessing their biodiversity. Haematophagous vectors such as leeches (for both trypanosomes and haemogregarines) and gnathiid isopods (for some haemogregarines) have been implicated in the transmission of these parasites.

Within the framework of this project, we assessed the species diversity of haemoprotozoans infecting near-shore scyliorhinids off the Western Cape. This study aimed to provide both morphological descriptions and molecular characterisation of these parasites, aiding in formal descriptions, phylogenetic placement and thus taxonomic identity, which will assist in determining their biodiversity in these catsharks from this region. Four shark species were targeted including the dark shyshark *Haploblepharus pictus* (Müller and Henle), the puffadder shyshark *Haploblepharus edwardsii* (Schinz), pyjama catshark *Poroderma africanum* (Gmelin) and the leopard catshark *Poroderma pantherinum* (Müller and Henle).

Blood collected was used for both the preparation of blood smears, to study morphology (presence, identity and parasitaemia of haemoprotozoans), and a volume fixed in 70% ethanol for molecular work. A subset of samples (those with high parasitaemias) was selected for molecular and phylogenetic analysis. Of the 98 shark individuals screened, 45 (96 %) of *H. pictus*, 13 (100 %) of *H. edwardsii*, 15 (63 %) of *P. africanum* and 14 (100 %) of *P. pantherinum* were infected with trypanosomes. While the individuals of *H. pictus* and *H. edwardsii* were parasitised by a trypanosome identifying morphologically with *T. haploblephari*, individuals of *P. africanum* and *P. pantherinum* were parasitised by an unknown morphotype of *Trypanosoma*. Phylogenetic analysis placed the trypanosomes within the marine fish *Trypanosoma* clade, with a divergence of only 0.5 %, showing that *T. haploblephari* demonstrates extreme pleomorphism, particularly between the two sympatric genera of catsharks. Similarly, 44 (94 %) of *H. pictus*, 13 (100 %) of *H. edwardsii*, 21 (88 %) of *P. africanum*, and 7 (50 %) of *P. pantherinum* were parasitised by two morphotypes of haemogregarines. Interestingly, phylogenetic analysis did not place the two morphotypes within the *Haemogregarina* clade, as was anticipated based on work by Yeld (2009). Instead, these two morphotypes fell together within the Dactylosomatidae, grouping closely together with species of *Dactylosoma* described from amphibians. Due to not enough molecular evidence suggesting that these two morphotypes are two different species, they are described as a single species. As such, this represents the first report of species of *Dactylosoma* infecting sharks globally. During the present study, leeches were also collected from shark individuals in order to determine the potential of these to act as vectors for these haemoprotozoans, in turn assisting with the elucidation of life cycles. Molecular analysis of leeches suggested the presence of two leech species, both potentially new to science. To determine the presence of haemoprotozoan stages, the dissected salivary glands and crop were used in molecular analyses. Although the presence of trypanosomes was detected, no haemogregarine sequences could be generated, suggesting that further refinement of the molecular protocol may be needed.

It is anticipated that the research presented here will lay the foundation for further research on haemoprotozoans parasitising sharks off the coasts of South Africa. It also highlights the need for greater focus on elasmobranch haemoprotozoan fauna, a likely wealth of biodiversity, but a currently neglected group of parasites.

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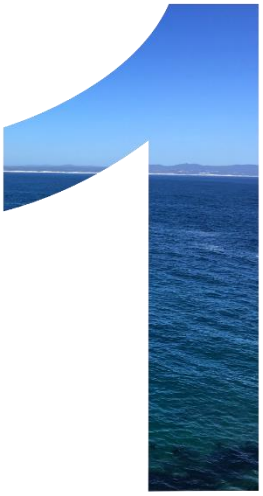
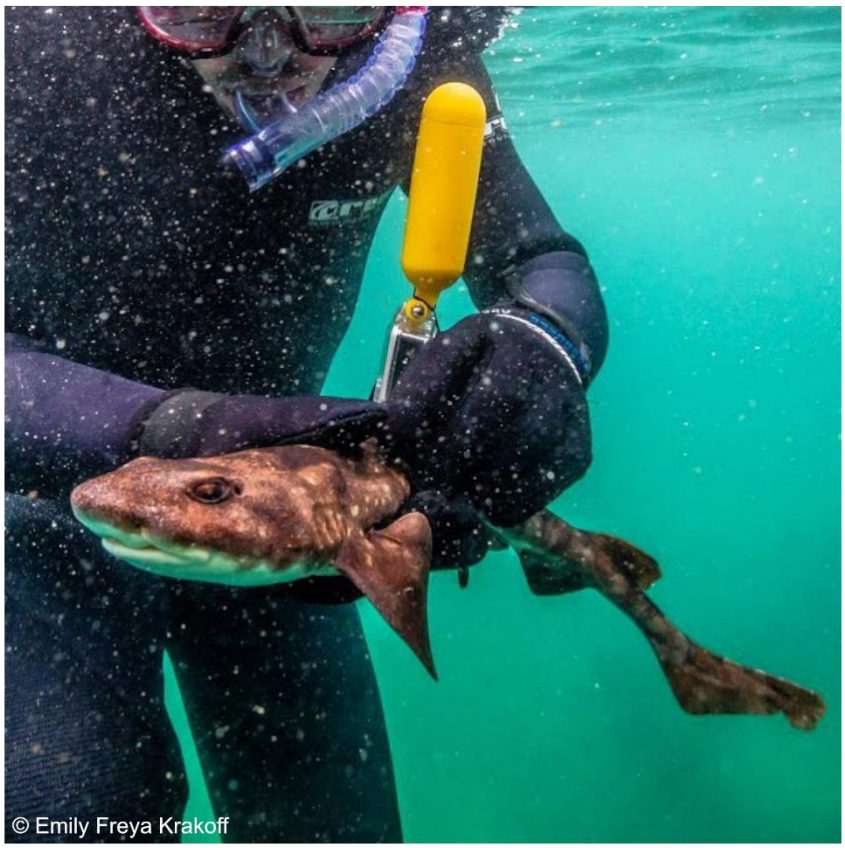


GENERAL INTRODUCTION



Chapter 1: General introduction

1.1 INTRODUCTION

South Africa is home to a high level of marine biodiversity, and even though it is classified as the third most biological diverse country in the world, the marine biodiversity is relatively understudied in comparison to countries such as Australia, China and Japan (Costello et al. 2010, Griffiths et al. 2010, Smit and Hadfield 2015). Griffiths et al. (2010) reported that the low species richness of certain taxa may be attributed to the lack of even taxonomic effort (Costello et al. 2010). South Africa's coastline is divided into nine marine bioregions, including the Namaqua-bioregion, which is classified as a cool-temperate area, the warm-temperate Agulhas-bioregion, the South-western Cape-bioregion, the Natal Bioregion, Atlantic Offshore Bioregion, West Indian Offshore Bioregion, South-west Indian Offshore Bioregion and a deep-water Indio-Pacific Offshore Bioregion. With an estimated total biodiversity of 12 915 different species (Griffiths et al. 2010), the South African coastline is home to more than 1900 species of fish, approximately 47 of which are endemic to the south coast where the warm Agulhas Current meets the cold, nutrient rich Benguela Current (Smit and Hadfield 2015). Incorporated in this biodiversity are Chondrichthyans, which amongst rays and chimaeras also includes sharks, of which 30% of these species are estimated as endemic to South Africa and its neighbouring coastal countries. Within South African coastal waters, more than 109 species of shark have been recorded to date. However, regardless of this high level of diversity and endemism, knowledge on the biology and ecology of South African sharks is scarce (Bester-van der Merwe and Gledhill 2016), and especially on the knowledge of their parasitic fauna (Schaeffner and Smit 2019). This is particularly true for the blood parasites such as haemoflagellates of the genus *Trypanosoma* Gruby, 1843 and their potential vectors. To date, only a single species of trypanosome *Trypanosoma haploblephari* Yeld and Smit, 2006, has been described infecting sharks collected off the South African coast during a multi-parasite survey of shysharks (or otherwise known as catsharks) (Yeld and Smit 2006). This trypanosome species was only morphologically characterised and as such there is no knowledge on its phylogenetic relationship to other fish trypanosomes. In the case of apicomplexan parasites, little is known on their infection rates among marine organisms in South Africa. Studies have reported *Haemogregarina* Danilewsky, 1885 spp. infecting marine teleosts (Hayes et al. 2006) but information on haemogregarines infecting elasmobranchs in South Africa is still lacking (Schaeffner and Smit 2019). No specific vectors have been identified for being responsible for the transmission of haemoprotozoans in sharks, however, suspected vectors include leeches, and parasitic isopods of the Family Gnathiidae (see Yeld and Smit 2006; Yeld 2009). Smit

and Basson (2002) has described a gnathiid parasite present on the leopard catshark that could potentially be responsible for parasite transmission, whereas Yeld and Smit (2009) collected leeches from the shark hosts that they were studying but did not do any further studies on the development or transmission of the haemoprotozoans to the sharks. The overall aim of this research therefore is to increase the biodiversity knowledge of haemoprotozoans found infecting near-shore scyliorhinids, using a combined morphological and molecular approach to characterise all species found. Furthermore, to also use both morphological and molecular approaches to identify potential vectors of any haemoprotozoans found.

1.2 AIMS OF THIS STUDY

To determine:

i. the biodiversity of haemoprotozoans infecting scyliorhinid sharks in coastal waters of the Western Cape,
ii. the phylogenetic placement and relationships of these parasites within their respective groups, subsequently determining their taxonomy and
iii. the potential vector(s) for these parasites, assisting in determining transmission routes and assisting with the elucidation of their complex life cycles.

1.3 OBJECTIVES OF THIS STUDY

In order to achieve the abovementioned research aims, the following objectives form the basis of this study:

i. To collect blood from selected scyliorhinid shark species occurring in the coastal waters of the Western Cape.
ii. To collect blood from the dark shyshark, *Haploblepharus pictus* Müller and Henle, and the puffadder shyshark*, Haploblepharus edwardsii* Schinz, both known hosts of *T. haploblephari*, from the type locality Granger Bay.
iii. Describe the morphology of the peripheral blood stages of trypanosomes, comparing these with the morphometrics of *T. haploblephari*.
iv. Molecular characterisation of trypanosomes, and determination of their phylogenetic placement and relationships to other trypanosomes, specifically those of marine hosts.
v. Describe the morphology of haemogregarines, comparing the morphometrics with previously reported and unnamed species of haemogregarines infecting scyliorhinids off the coast of the Western Cape.

vi. Molecular characterisation of species of haemogregarines, and determination of their phylogenetic placement and relationships, and subsequently their taxonomy.
vii. Collect any ectoparasites (i.e. leeches and gnathiid isopods) that can potentially act as vectors of haemoprotozoa.
viii. Identify the potential vectors using both morphological and molecular techniques
ix. Screen potential vectors for haemoprotozoa using molecular techniques

1.4 DISSERTATION OUTLINE

This general introduction (Chapter 1) will give a brief overview of the field and focus of this study as well as the aims and objectives. It will then provide an in-depth literature review (Chapter 2) on the elasmobranchs of South Africa followed by the haemoprotozoans infecting elasmobranchs worldwide as well as in South Africa. Following the literature review, methods relating to the collection of sharks and haemoprotozoans are detailed in Chapter 3 as well as detailed methods relating to the morphological descriptions, molecular characterisation, and phylogenetic analysis of the trypanosomes infecting the sharks of South Africa. This chapter will also provide the results of the trypanosomes observed in this study, including new morphological descriptions, molecular characterisation as well as the phylogenetic analysis of the trypanosomes found infecting the sharks. Following the chapter on trypanosome infections, Chapter 4 will provide the morphological descriptions and molecular analyses of two previously reported, but unnamed haemogregarine species. Life cycle information and potential vectors of the haemoparasites are given in Chapter 5, along with a brief discussion of the findings with recommendations for future research in Chapter 6. A list of references is provided according to the referencing style of *African Zoology* and appendices will provide any additional information relating to this study.

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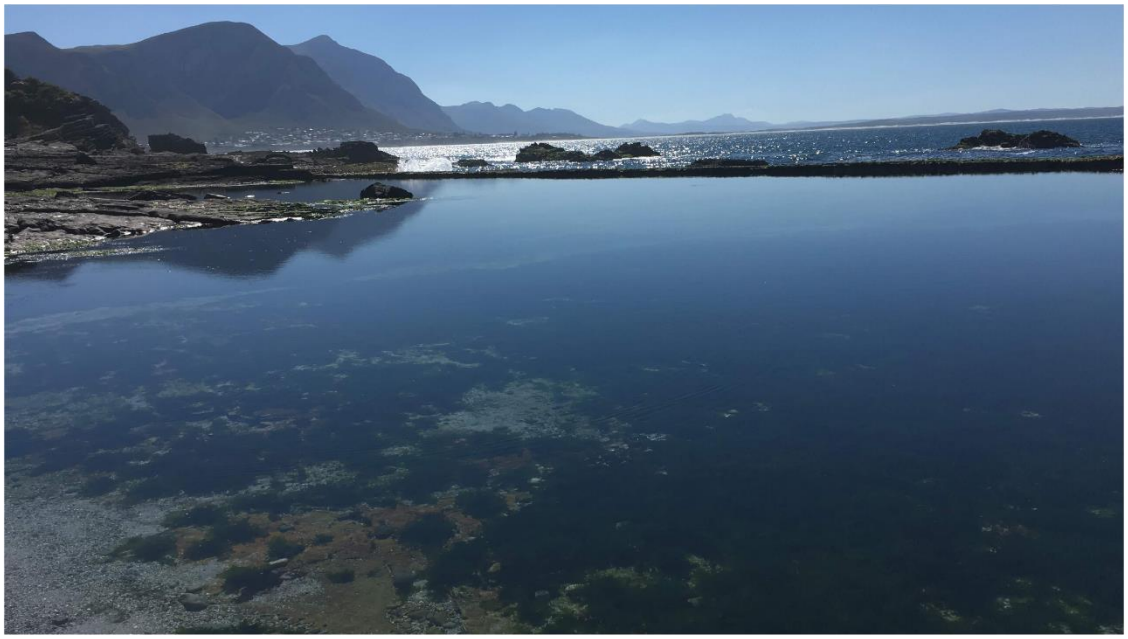
LITERATURE REVIEW



Chapter 2: Literature review

2.1 INTRODUCTION TO THE VERTEBRATE HOST

Sharks arose during the Ordovician period 485 million years ago, during which they survived five mass extinctions (O'Donoghue 2017; Sims 2015), evolving into what is now referred to as 'the perfect predators' (Shark Savers 2019). The earliest known sharks arose from a group of fish called acanthodians ("spiny sharks") which resembled the sharks we know today, but these fish had varying numbers of spiny fins in comparison to the eight fins commonly found on sharks today (Maisey et al. 2017; Davis et al. 2012; Dearden 2015; Jones 2015). The golden age of sharks came over 360 million years ago when these predators diversified into the Chondrichthyans, which are known to be some of the largest predators (Jones 2015) to roam the oceans worldwide. The class Chondrichthyes consists of cartilaginous fishes that include elasmobranchs (sharks and rays), holocephalans (chimaeras and elephant fish) and a variety of extinct cartilaginous fishes (Compagno 1999).

A possible reason why sharks were able to survive the extinction events and subsequently diversify was largely due to the ability of their bodies to adapt (Jones 2015; Shark Savers 2019) and the fact that so many other organisms were wiped out during these extinction events. Jones (2015) further states that following the extinction of dinosaurs, sharks were able to reproduce and colonise a larger variety of habitats around the world. Even after the Cretaceous period when the dissolved oxygen levels in the ocean dropped significantly (Schmidtko et al. 2017) and larger species went extinct, some smaller species moved to deeper water and developed the ability to glow or fluoresce in the dark (e.g., puffadder shyshark) (Gruber et al. 2016; Hall 2019; Jones 2015; Claes et al. 2015).

Modern-day chondrichthyans can be characterised by a variety of unique features including an endoskeleton of calcified cartilage, four to seven separate internal and external gill openings, absence of a swim bladder, but instead a liver made up of a natural oil called squalene, which regulates buoyancy. Chondrichthyans also have a dermal layer of placoid scales, paired pectoral and pelvic fins and a caudal fin which serves as additional support and balance (Compagno 1999). Males and females can be distinguished in two different ways: males tend to be smaller than females, however in the absence of both sexes for the purpose of comparison, it can become difficult to distinguish. As such, the easiest way to tell sexes apart is by the presence of modified pelvic fins in males, called claspers, used for mating. Courtship behaviour has not been extensively studied, although those that have been, show that the behaviour might be aggressive and females often end up with teeth marks, similar to 'love bites' on their bodies

(Shark Trust 2019). Sharks in general follow a variety of reproduction methods including oviparity, ovoviviparity and viviparity (Parsons et al. 2008). Oviparous species produce eggs (also known as a 'mermaid's purse') in egg cases, which then settle on the ocean floor or stick to seaweed until the embryos develop and emerge (Parsons et al. 2008). In the case of ovoviviparity, instead of females laying eggs, they carry the eggs internally for extra protection against predators. The embryos develop inside the eggs, hatch inside the mother which then gives live birth to her pups (Parsons et al. 2008). The final and most evolved method of reproduction is viviparity. Here, pups develop inside the mother's body and receive the necessary nutrients and oxygen via an umbilical cord (Hamlett and Koob 1999, Parsons et al. 2008). In sharks such as the blacktips [*Carcharhinus limbatus* (Müller and Henle, 1839)] and bonnetheads (*Sphyrna tiburo* L.), a phenomenon called parthenogenesis takes place, where females are able to reproduce without the fertilization by males (Edwards 2007; Chapman et al. 2008; Domingues et al. 2018). The closest form of parental care in sharks is when the sharks give birth to their offspring in nurseries (e.g., mangroves) for protection against larger predators (Mourier and Planes 2012).

Even though sharks are some of the oldest living organisms on the planet, they are under threat due to human activity. Some sharks have long pregnancies ranging from nine months up to 31 months, some produce very few young, they are late to mature, and some species may not even reproduce every year (Hamlett and Koob 1999; Parsons et al. 2008). Habitat degradation, climate change, commercial shark finning and the exploitation of bottom food chain prey are all additional threats sharks face today. However, there may be an improvement in this situation (Knowlton and Benchley 2014). Knowlton and Benchley (2014) claim that due to better fishery management, the falling demand for shark fins and the rising appreciation and fascination by the public for sharks have led to better conservation efforts. With the movement towards banning of shark finning in many countries, shark products and changes in fishing gear, sharks and shark sanctuaries are starting to experience a trend in the better protection of these animals. In areas where sharks are critical for the tourism industry, people have started realising that sharks are "more valuable alive than dead" which has also led to better legal protection (Knowlton and Benchley 2014). Even though many countries have implemented clear regulations on shark finning, many more countries still need to get on-board this initiative and implement clear regulations that are controlled, monitored, and enforced in order to save the sharks from extinction.

2.2 NEAR-SHORE SCYLIORHINIDS OFF THE WESTERN CAPE

Within the order Carcharhiniformes, the family Scyliorhinidae is recognized as the largest and among the oldest shark family with 15 genera and more than 100 different species (Weinheimer 2004). They can be found in warmer seas worldwide and are often endemic to certain areas. They tend to live towards the bottom of the ocean from shallow depths to deep-water areas of 2000 m. Almost 90 % of these sharks are oviparous, producing eggs all year round. Eggs will attach to a substrate and the young hatchlings emerge looking like miniature adults (Weinheimer 2004). This family of sharks are known as catsharks (Compagno 1984), however those of the genus *Haploblepharus* Garman, 1913 are often referred to as shysharks due to their behaviour of curling their tails over to cover their eyes when threatened (Human, 2007). Three different species of shysharks can be found along the western coast of South Africa including the brown shyshark *Haploblepharus fuscus* Smith, 1950, dark shyshark *Haploblepharus pictus* (Müller and Henle, 1838), and the puffadder shyshark *Haploblepharus edwardsii* (Schinz, 1822). Along with the shysharks, two different types of catsharks can also be found including the pyjama catshark *Poroderma africanum* (Gmelin, 1789) and the leopard catshark *Poroderma pantherinum* (Müller and Henle, 1838).

2.2.1 Brown shyshark *Haploblepharus fuscus* Smith, 1950

The brown shyshark, *Haploblepharus fuscus* (Fig. 2.1 A), can be found along the coast from Port Elizabeth to Durban (Pollom et al. 2020a), inhabiting sandy areas near the continental shelf (Shark Research Institute 2018). This small shark reaches approximately 65 cm in length and can be identified by the brown colour with darker spots on the dorsal side. The diet of this species consists primarily of bony fishes and lobsters. When threatened it will curl up with its tail over its eyes, hence the name shyshark as mentioned above (Human 2007, Shark Research Institute 2018). Listed as Vulnerable by the IUCN due to their endemic distribution close to the shore, threats include recreational fishing (da Silva et al. 2015), habitat degradation and human utilization (coastal housing development and boating) of the habitat (Pollom et al. 2020a). Little information is available on their population structure as these organisms are rarely caught and studied for their behaviour.

2.2.2 Dark shyshark *Haploblepharus pictus* (Müller and Henle, 1838).

Haploblepharus pictus, or commonly known as the dark shyshark (Fig. 2.1 B), is endemic to the coast of Namibia through to the cooler west coast of South Africa up until East London (Pollom et al. 2019). This shark prefers kelp forest and rocky reef habitat where it preys upon

crustaceans, molluscs, and bottom-dwelling fishes (Pollom et al. 2019). Dark shysharks can be distinguished from the morphologically similar, but not easily distinguishable puffadder shysharks (see 2.2.3) by their more rounded snouts, depressed heads and large light spots on their dark body. Due to their highly variable colour patterns, they are often misidentified as other members within the *Haploblepharus* genus, most commonly they get misidentified with the brown shyshark,

2.2.3 Puffadder shyshark *Haploblepharus edwardsii* (Schinz, 1822).

The puffadder shyshark, sometimes referred to as Happy Eddie (Fig. 2.1 C), is found from the northern Western Cape to Port Alfred (Pollom et al. 2020b). These endemic sharks prefer both the inshore and offshore waters of the continental shelf where they can feed on bony fishes, crustaceans and cephalopods (Carpenter 2018; Pollom et al. 2020b). They can be identified by their bright orange markings on a relatively pale body, more narrowly pointed head and claspers of the male being more slender than other species within this genus (Human 2007). Considered Endangered by the IUCN, the number of individuals is declining due to fishing pressure and habitat destruction caused by pollution and the inshore waters being disturbed by recreational diving and coastal housing development (da Silva et al. 2015; Ellis et al. 2017). In recent years, the distribution of this shark species has shifted more southward, possibly due to loss of habitat and climate change (Currie et al. 2019; Rouault et al. 2010; Blamey et al. 2015).

2.2.4 Pyjama catshark *Poroderma africanum* (Gmelin, 1789).

This shark, also known as the striped catshark (Fig. 2.1 D), is endemic to South Africa and is found commonly in the temperate waters between the northern parts of the Western Cape to Durban, KwaZulu-Natal; however, it is most commonly found in the Western Cape (Pollom et al. 2020c). These sharks are listed as Least Concern by the IUCN and are characterised by the five to seven stripes on their dorsal side. They live in the intertidal and subtidal zones and favour kelp beds during daytime and caves when resting. Some of their prey items include small marine organisms such as cephalopods, crustaceans and bony fishes (Pollom et al. 2020c). Due to unregulated near-shore fisheries, these sharks are often caught as bycatch causing their numbers to decline (da Silva et al. 2015).

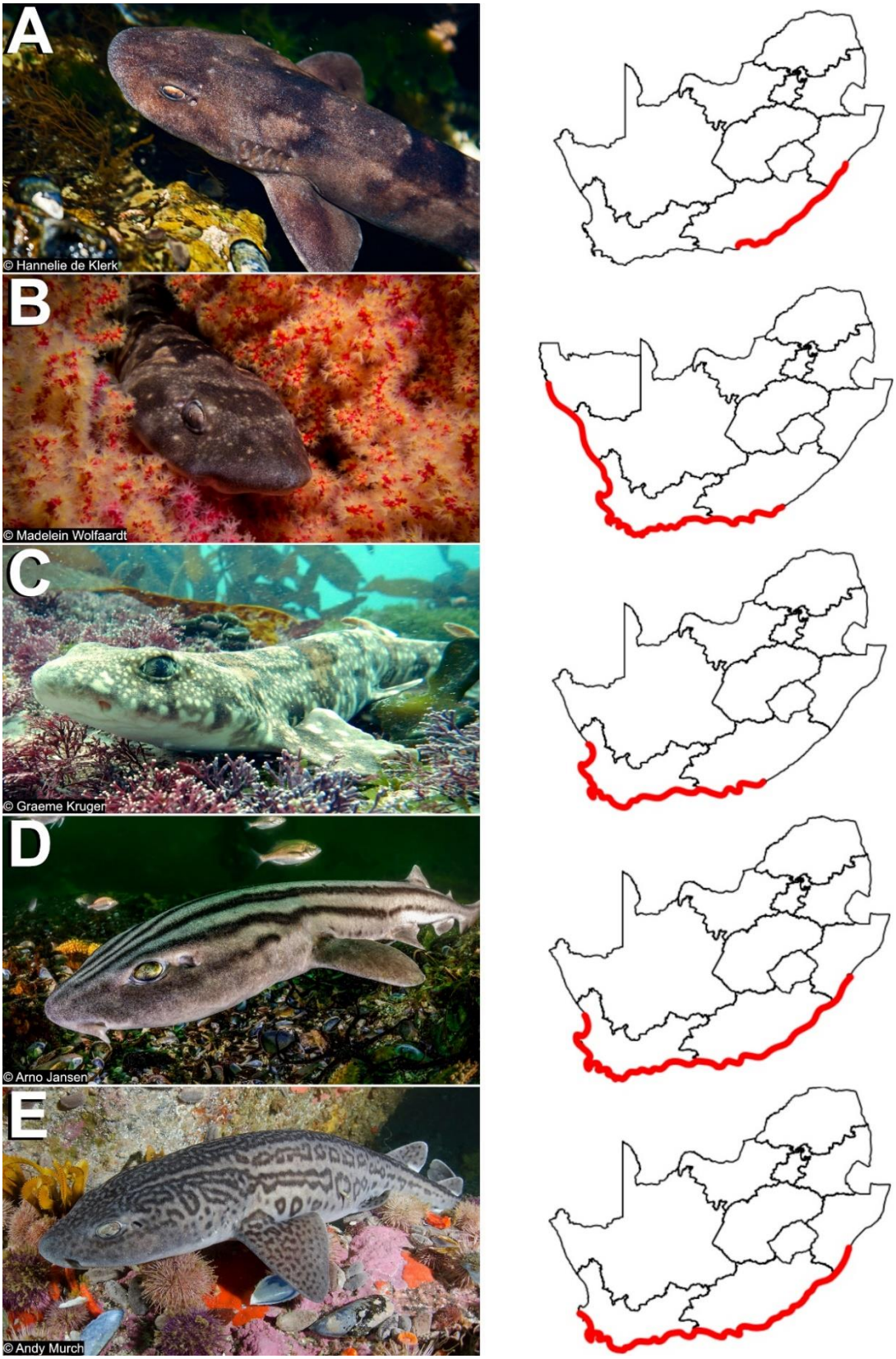


Figure 2.1 Shysharks and catsharks found along the southern coast of Africa, including their distribution maps. A, brown shyshark *Haploblepharus fuscus*; B, dark shyshark *Haploblepharus pictus*; C, puffadder shyshark *Haploblepharus edwardsii*; D, pyjama catshark *Poroderma africanum*; E, leopard catshark *Poroderma pantherinum*.

2.2.5 Leopard catshark *Poroderma pantherinum* (Müller and Henle, 1838).

The leopard catshark (Fig. 2.1 E) is found along most of the South African coast, ranging from Saldanha Bay to Durban (Pollom et al. 2020d). This shark can be found up to a depth of 274 m along rocky reefs, sandy areas and kelp forests (Ebert et al. 2013; Weigmann 2016). These sharks can be distinguished from the pyjama catsharks based on the markings present on their bodies. Leopard catsharks lacks the seven dorsal stripes that the pyjama catsharks have, instead they have small and large black spots and whole or broken rosette-patterns (Human 2007). Even though it is listed as Least Concern by the IUCN, some of the threats these sharks face include being caught as bycatch, not being released or being mistreated, often leading to post-release mortality (Ellis et al. 2017).

2.3 HAEMOPROTOZOANS INFECTING ELASMOBRANCHS

A parasite can be defined as an organism that lives in or on a host, often causing harm to the host by feeding on it or by obtaining food at the host's expense (CDC 2016; Nordqvist 2016; Solomon et al. 2015). Blood parasites can be found infecting almost all vertebrate and invertebrate classes in both the aquatic as well as the terrestrial environment (Barta et al. 2012). Studies have mainly focused on the blood parasites infecting mammals, reptiles and amphibians, however, in recent years interest in parasites infecting fishes has increased. Five groups of haemoprotozoans are recognized as fish blood parasites including euglenozoan flagellates, such as trypanosomes, and four apicomplexan groups including haemogregarines, haemococcidia, haemosporidia and piroplasms (Magro et al. 2016).

The phylum Euglenozoa Cavalier-Smith, 1981 contains organisms known as flagellates, and members within the class Kinetoplastida are known to be both of medical and veterinary importance (Wellehan and Walden 2019). This order includes trypanosomes and the genus *Leishmania* Borovsky 1898 (Ross 1903). Belonging to an ancient lineage of mitochondriate eukaryotes, the trypanosomatids can most easily be identified by the presence of a kinetoplast, an organelle which has mitochondrial DNA (kDNA) which is separate from nuclear DNA. Trypanosomes are blood parasites found in both vertebrates and invertebrates (O'Donoghue 2017). These parasites are believed to have become parasitic almost 150 million years ago in terrestrial insects where they had monoxenous life cycles (O'Donoghue 2017). After they evolved into blood-feeding parasites and were transmittable to terrestrial vertebrates, they developed two different transmission methods; firstly, the stercorarian (posterior station) transmission method, which is most effective when transmitted from insects to nesting vertebrates and secondly the salivarian (inoculative or anterior station) transmission method,

which is most effective when transmitted from dipteran vectors to sociable vertebrates (O'Donoghue 2017).

Within the phylum Apicomplexa Levine, 1970, two classes are recognized – Coccidea Leuckart, 1879 and Haematozoa Vivier 1982, the former containing the orders Adeleina Lèger, 1911 and Eimeriina Lèger, 1911 and the latter the orders Haemosporidia (or Haemosporida) Danilewsky, 1885 and Piroplasmida Wenyon, 1926 (Davies and Johnston 2000; O'Donoghue 2017). This phylum gets its name from the trophic stages which have a unique complex of anterior organelles, together forming what is known as the apical complex, which assists in the invasion of host cells (O'Donoghue 2017). It was recently discovered that apicomplexans have a unique organelle which shares sequence similarities with plastids, which are found in photosynthesizing plants and algae (Vargas Parada 2010). Köhler et al. (1997) proposed that this organelle be given a name with the combination of the words "apicomplexan" and "plastid" after it was discovered that the apicoplast is a vestigial plastid (Köhler et al. 1997; Vargas Parada 2010). Later, the question arose to why apicomplexans retained a vestigial plastid despite losing the capability for photosynthesis. It was determined that this was because the apicoplast participates in lipid biosynthesis and iron metabolism. This organelle laid the foundation for a variety of antibiotics and herbicide tests due to their evolutionary similarities with chloroplasts and prokaryotes (Vargas Parada 2010).

This study focuses on the trypanosomes and haemogregarines infecting elasmobranchs.

2.3.1 *Trypanosoma* Gruby, 1843

Species of *Trypanosoma* (Trypanosomatida: Trypanosomatidae) (Fig. 2.2) are haemoflagellate protozoan parasites that are found infecting almost all vertebrate classes (Kaufer et al. 2017). Species of trypanosomes belong to the class Kinetoplastea within the phylum Euglenozoa and are transmitted by haematophagous vectors such as leeches, biting flies and bugs. Species of *Trypanosoma* are best known for the disease Trypanosomiasis, a disease that some of these species cause in mammalian hosts. In Africa, the tsetse fly (*Glossina* spp.) transmits species of *Trypanosoma* that are responsible for the parasitic disease Human African Trypanosomiasis (HAT) or commonly known as sleeping sickness (Franco et al. 2014; Hayes et al. 2014; Kaufer et al. 2017).

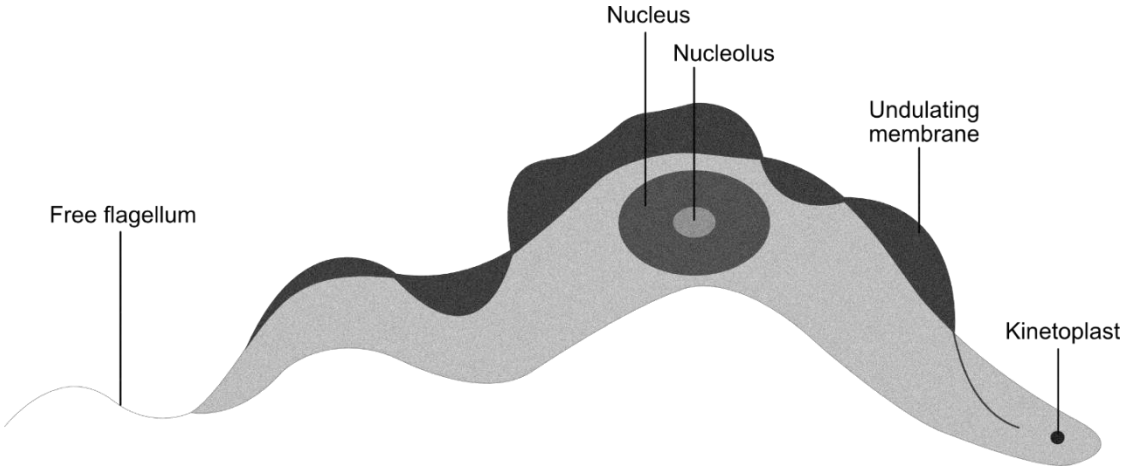


Figure 2.2 Schematic representation of the general body morphology of *Trypanosoma.* Adapted from Uilenberg (1998).

Trypanosomes can be found infecting organisms of both terrestrial and aquatic habitats. Within the terrestrial clade, trypanosomes infect mammals, snakes, lizards, crocodilians and birds (Fermino et al. 2015); and within the aquatic clade they are reported infecting fishes and semi-aquatic organisms such as turtles, frogs and the platypus (Fermino et al. 2015). Within recent years, the combination of trypanosome morphology and host-association with molecular data has revealed discrete phylogenetic relationships within the genus *Trypanosoma*, supporting the separation of these species into aquatic and terrestrial clades (see O'Donoghue 2017). Furthermore, molecular studies have also revealed a further division within the aquatic clade, separating species into either freshwater or marine clades (Hayes et al. 2014, Lemos et al. 2015, O'Donoghue 2017). Interestingly, previous studies on marine trypanosomes (Hayes et al. 2014; Pretorius et al. 2021) have found that the marine trypanosome clade is separated by the trypanosomes infecting aquatic tetrapods. It is important to note that there is a large degree of polyphyly within the trypanosomes and that those infecting aquatic tetrapods have likely evolved many times. Even though species of *Trypanosoma* have been well studied in mammals due to their veterinary and medical importance, in fishes both the biodiversity and the pathogenicity of this genus remains poorly researched (Ferreira and Avenant-Oldewage 2013; Su et al. 2014; Smit et al. 2020).

2.3.1.1 Life cycle of trypanosomes

The general life cycle of species of *Trypanosoma* (Fig. 2.3) follows one of two developmental patterns, salivarian or stercorarian (see O'Donoghue 2017). Both forms are characterised by trypomastigote stages in the vertebrate host's peripheral blood. A vector takes up these stages during a blood meal, after which they transform into stages including promastigotes,

amastigotes, sphaeromastigotes, epimastigotes or metacyclic trypanosomes (Telford 2009). Transmission of infective parasite stages from the vector to a novel host differs for salivarian and stercorarian species. Salivarian trypanosomes undergo ‘anterior station’ development in the vector and are transmitted through inoculation from the salivary glands during the blood meal. Stercorarian trypanosomes undergo ‘posterior station’ development in the vector and are transmitted to the vertebrate through contamination as the vector defecates whilst it is feeding (see O’Donoghue 2017). Even though trypanosomes are frequently reported infecting fishes, they remain relatively understudied and as such only a few species have had their life cycles elucidated. However, for those species for which the life cycle is known, all have been shown to follow salivarian development for which transmission is through a leech vector (Hayes et al. 2014; Lemos et al. 2015; O’ Donoghue 2017).

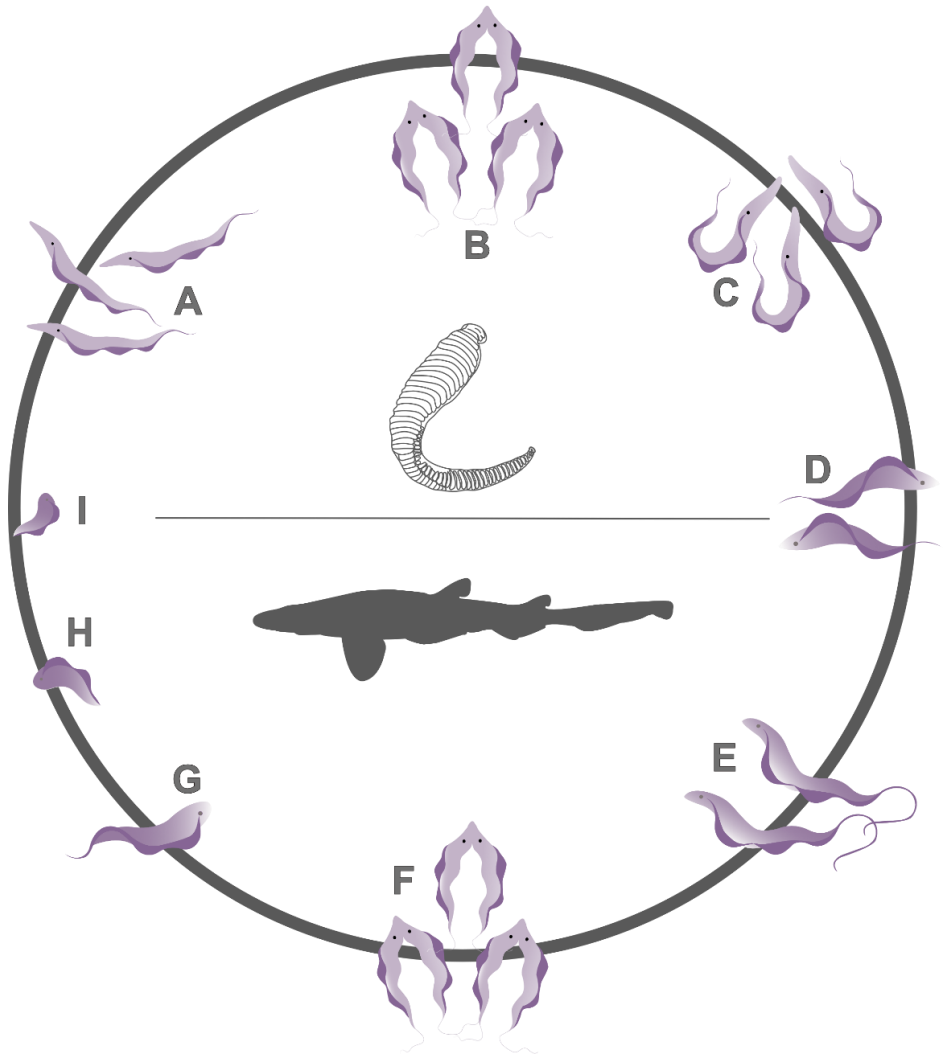


Figure 2.3 General life cycle of *Trypanosoma* spp. in a leech vector and marine vertebrate host based on Hayes et al. (2014). A, procyclic trypomastigotes; B, binary fission; C, epimastigotes; D, metacyclic trypomastigotes; E, trypomastigotes; F, binary fission; G, H, I, developing procyclic trypomastigotes.

Metacyclic trypomastigotes are injected into the host via the vector, in sharks this is most likely performed through a leech vector (Yeld and Smit 2006; Morillas et al. 1987; Neumann, 1909). The metacyclic trypomastigotes will then transform into bloodstream trypomastigotes and then be transferred to other areas in the organism. These parasites are capable of multiplying by binary fission in body fluids such as blood, lymph and spinal fluid. Trypomastigotes in the blood will then be passed on when the vector (leech) takes a blood meal, these in turn developing into procyclic trypomastigotes in the midgut of the vector before multiplying by binary fission. When these stages leave the midgut, they will form into epimastigotes, which multiply in the salivary gland of the vector. Epimastigotes will then form to produce metacyclic trypomastigotes ready to infect a new host (Negm-Eldin 1997).

2.3.1.2 Identification of species of *Trypanosoma*

Species of *Trypanosoma* are described mainly on the trypomastigote forms observed in the peripheral blood of the vertebrate (Smit et al. 2000; Smit et al. 2004; Davies et al. 2005; Hayes et al. 2014; Smit et al, 2020). Species descriptions and identification are based on morphometric data, including measurements of midnucleus to anterior, midnucleus to posterior, midnucleus to kinetoplast, posterior to kinetoplast, nuclear length, body width, total body length, and the nuclear index (Hayes et al. 2014). The presence or absence of a free flagellum is also noted in morphometric descriptions. Morphology alone however has proven inadequate in accurately identifying and differentiating trypanosome species as morphometrically variable haemoflagellates can exist within a single host. This usually raises the question of whether these are morphotypes or different life stages of the same species or co-infections as trypanosomes are well known for their pleomorphism. This term is used to describe where an organism expresses more than one phenotype, or morphological shape and is expressed in ways including size changes (length and width), differences in the width and depth of the undulating membrane, the length of the free flagellum as well as differences in the position of the nucleus (Gupta 2016). For example, in freshwater fishes of the Okavango Delta, Botswana, various morphotypes of what was morphometrically suggestive of being *Trypanosoma mukasai* Hoare, 1932 were found to represent two genotypes (Davies et al. 2005; Smit et al. 2004). Additionally, a recent Smit et al. (2020) article experienced the same difficulties when the freshwater fish trypanosomes they observed during their study, fell within the small range of measurements for *T. mukasai*. They noted that the size variation may be related to the age of the trypanosomes, which would ultimately cause difficulties when identifying these trypanosomes, especially in the case where some of these species have records of known

pleomorphism. Furthermore, their sequence data showed that the trypanosome isolated from the leech host *Batracobdelloides tricarinata* (Blanchard, 1897) had only a 0.7 % divergence with the trypanosome isolated from the fish host *Synodontis zambezensis* Peters, 1852. Interestingly, the sequences generated from the South African samples, also showed a low divergence with other trypanosomes infecting European fish hosts (Smit et al. 2020), well below the standard 3 % divergent threshold used to differentiate between different species. This further emphasizes that morphology alone is not the most accurate way to identify and differentiate species of trypanosomes.

2.3.1.3 *Trypanosoma* of fish, with a focus on those of Africa

In 1841, Valentine discovered the first fish trypanosome from the brown trout, *Salmo fario* Linnaeus, 1785 (Ferreira and Avenant-Oldewage 2013). To date, over 190 species have been recorded from fishes worldwide, however few have been described from African fish, both marine and freshwater (Table 2.1). Studies on fish trypanosomes of Africa are predominantly focused on those of freshwater fishes (Scholz et al. 2018) and the marine trypanosome diversity remains poorly explored. Only two species have been described from marine fishes off the coasts of South Africa, the first *Trypanosoma nudigobii* Fantham, 1919 from fishes belonging to the superclass Osteichthyes (see Hayes et al. 2014), the other *T. haploblephari* to fishes of the class Chondrichthyes (Yeld and Smit, 2006; Hayes et al. 2014).

Table 2.1 Species of *Trypanosoma* Gruby, 1843 infecting marine and freshwater fishes in Africa. (adapted from Scholz et al. (2018)).

***Trypanosoma* species**	**Host species**	**Reference**
	Freshwater trypanosomes	
T. alhussaini	*Clarias gariepinus*	Mohamed (1978)
T. cyanophilum	*Coptodon zillii*	Mohamed (1978)
T. mansouri	*Coptodon zillii*	Mohamed (1978)
T. cf. *mugilicola*	*Mugil cephalus*	Becker and Overstreet (1979)
T. mukasai	*Astatoreochromis alluandi*	Scholz et al. (2018)
	Bagrus docmak	
	Clarias gariepinus	Smit et al. (2004)
	C. theodorae	Smit et al. (2004)
	Haplochromis cinereus	
	H. humilior	
	H. nubilus	
	H. serranus	
	Haplochromis spp.	
	Mormyrus kannume	
	Oreochromis andersonii	Smit et al. (2004)
	O. esculentus	
	O. mossambicus	
	O. niloticus	
	O. variabilis	
	Parauchenoglanis ngamensis	Smit et al. (2004)
	Schilbe intermedius	Smit et al. (2004)
	Serranochromis angusticeps	Smit et al. (2004)
	Se. macrocephalus	Smit et al. (2004)
	Se. robustus	Smit et al. (2004)
	Synodontis nigromaculatus	Smit et al. (2004)
	Sy. vanderwaali	Smit et al. (2004)

Table 2.1 Continued.

***Trypanosoma* species**	**Host species**	**Reference**
	Tilapia sparrmanii	
T. tobeyi	*Clarias angolensis*	Dias (1952)
T. toddi	*Clarias angolensis*	
	C. anguillaris	Bouet (1909)
Trypanosoma sp.	*Coptodon zillii*	Scholz et al. (2018)
Trypanosoma sp.	*Sy. zambezensis*	Smit et al. (2020)
	Batracobdelloides tricarinata	
	Marine trypanosomes	
T. nudigobii		Fantham (1919); Hayes et al. (2014)
T. haploblephari	*Haploblepharus pictus*	Yeld and Smit (2006)
	Ha. edwardsii	

2.3.1.4 Elasmobranch trypanosomes

More than 10 species of *Trypanosoma* have been described infecting elasmobranchs worldwide (Table 3.1, Chapter 3), however most are described from skates and rays (Yeld and Smit 2006). In 1902, Laveran and Mesnil described the first two trypanosomes infecting elasmobranchs, *Trypanosoma rajae* Laveran and Mesnil, 1902, from *Raja* spp. and *Trypanosoma scylli* Laveran and Mesnil, 1902, from *Scyliorhinus stellaris* (L.) from the Northern Atlantic (specific localities unknown) and Roscoff, France respectively (Yeld and Smit 2006). Since then, *Trypanosoma carchariasi* Laveran, 1908*, Trypanosoma torpedinis* Sabrazes and Muratet, 1908, and *Trypanosoma giganteum* Neumann, 1909, have been described from *Odontaspis* sp., *Torpedo marmorata* Risso, 1810, and *Raja oxyrhynchus* (L.) respectively. In 1951, Laird described *Trypanosoma gargantua* Laird, 1951, from *Zearaja nasuta* (Müller and Henle, 1841) (see Yeld and Smit 2006). The total length of the parasite ranges between 66.7 to 131.1 µm and is found in the rough skate which occurs in the waters of the Southern Pacific but more specifically New Zealand. Almost 40 years later, *Trypanosoma taeniurae* Burreson, 1989 was described from *Taeniura lymma* (Forsskål, 1775) off Heron Island, Australia (Yeld and Smit 2006). That same year, Burreson described an additional trypanosome, *Trypanosoma mackerrasi* Burreson, 1989, from *Hemiscyllium ocellatum* (Bonnaterre, 1788), adding yet another species to the list of trypanosomes infecting elasmobranchs worldwide. Between 1948 and 1989, three additional trypanosomes, *Trypanosoma marplatensis* Bacigalupo and de la Plaza, 1948*, Trypanosoma boissoni* Ranque, 1973*,* and *Trypanosoma humboldti* Morillas et al., 1987, had been described from two species of ray, *Sympterygia microps* (Günther, 1880) and *Zanobatus schoenleinii* (Müller and Henle, 1841), and a catshark *Schroederichthys chilensis* (Guichenot, 1848) respectively.

Although the first reports of trypanosomes from marine fishes off the coasts of South Africa were from elasmobranchs, skates in particular, in 1918, the first and only trypanosome that has been formally described to date from this group is *T. haploblephari* (Smit and Hadfield 2015; Yeld and Smit 2006). This trypanosome was found infecting two species of shysharks collected off the west and south coasts of South Africa. As South African waters are home to such a high diversity of elasmobranchs, the potential of finding additional haemoprotozoan species is high, particularly with increased survey efforts. In some freshwater and marine fishes, leeches have been identified as vectors of *Trypanosoma* (Hayes et al. 2006, Hayes et al. 2014). Leeches are known to transmit haematozoans to other organisms, therefore it is very likely that hirudineans will also be the vector of these parasites in marine fishes, yet no information is available on parasite-host relationships (Hayes et al. 2014; Jones and Woo 1991; Yeld and Smit 2006). Yeld

and Smit (2006) proposed that an unidentified leech found on the shysharks examined might be the possible vector. Since these sharks are mostly sedentary, leeches and possibly other parasitic organisms will have easier access to them, suggesting why such high parasitaemias of these parasites are observed in these sharks (Schaeffner and Smit 2019). It is also noted that sharks living in colder waters are very likely to be infected with these parasites (Khan et al. 1980; Yeld and Smit 2006).

In terms of molecular data, there are only two sequences available for trypanosomes infecting elasmobranchs to date. These are *T. boissoni* described from the skate *Zanobatus schoenleinii* (Müller and Henle, 1841) (Maslov et al. 1996) and *Trypanosoma rajae* described from various species of *Raja* in France. However, the genetic sequence available on GenBank for the latter is from an unpublished paper.

2.3.2 Intracellular haemogregarine blood parasites

Haemogregarines (Fig. 2.4) are intracellular protozoans belonging to the phylum Apicomplexa that infect almost every vertebrate and invertebrate class in both the aquatic as well as the terrestrial environments. Even though haemogregarines are well studied in most vertebrate groups, there is a lack of information on the biology and phylogenies of haemogregarines infecting fish.

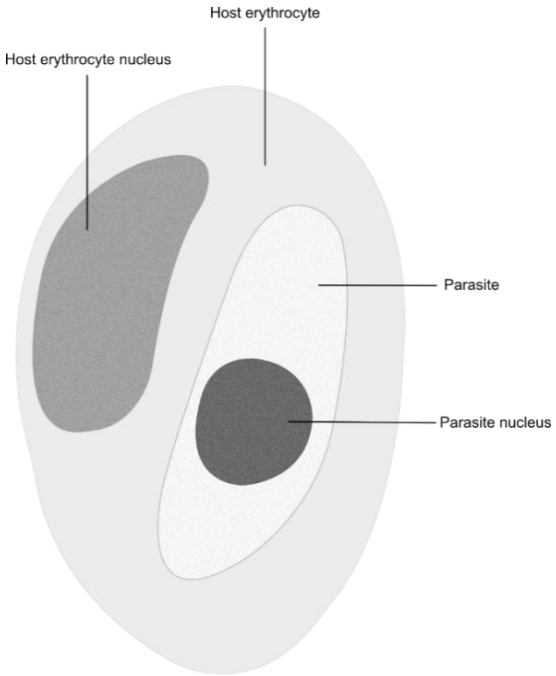


Figure 2.4 Schematic representation of the general body morphology of a haemogregarine gamont. Adapted from Baker and Lainson (1972).

The first species of haemogregarines of fish was described by Laveran and Mesnil in 1901 when they recorded *Haemogregarina simondi* in Dover sole *Solea solea* L. These parasites are cosmopolitan in ectothermic vertebrates with descriptions and reports from tortoises (Cook 2009), frogs (Netherlands et al. 2015) and various freshwater and marine fishes (Davies 1995; Davies and Johnston 2000) to name a few. A previous study of Puerto Rican fishes found that hosts who were parasitized by haemogregarines showed an abnormally high number of erythrocytes, indicating that organisms with haemogregarine infections may increase the number of their erythrocytes to compensate for the parasite load (Saunders 1966). These parasites are ecologically important because they can cause negative physiological effects to the host, such as mentioned above, and furthermore may modify the size and shape of blood cells, as well as lead to a decrease in oxygen carrying capacity (Smit et al. 2006).

2.3.2.1 Life cycle of haemogregarines

Haemogregarines have an obligate heteroxenous life cycle and are transmitted by haematophagous invertebrates such as acarines (ticks and mites), biting flies, leeches and gnathiids; these acting as vectors (Davies and Smit 2001; O'Donoghue 2017; Siddall 1995). It is in these vectors where sexual reproduction of the parasite occurs, including fertilization and sporogony, before being transmitted, either through inoculation during a blood meal by the vector or ingestion of an infected vector, to the vertebrate host where cyclic merogony and gamogony occurs (O'Donoghue 2017).

Generally, in the life cycle of a haemogregarine (Fig. 2.5), an infected invertebrate vector either injects infective sporozoites into the bloodstream of the vertebrate or is ingested, containing the infective sporozoites, by the vertebrate. The sporozoites then enter either the blood cells (erythrocytes, sometimes leucocytes) and/or other fixed tissues of the internal organs and begin multiplying by a process known as cyclic merogony, often producing macro- and then micro-merozoites (Davies and Johnston 2000; O'Donoghue 2017). This process is a form of asexual reproduction. Merozoites will then either infect new host blood cells or fixed tissues. Some may undergo gametogony to produce sexually reproductive stages namely microgamonts (male) and macrogamonts (female). Gametogony is completed when the invertebrate host takes up these stages during a blood meal where they transform into micro- and macrogametes. Fertilization takes place to form a zygote. This stage then undergoes sporogony to form oocysts, which will subsequently contain either free sporozoites or sporozoite-containg sporocysts. These stages are formed either in the gut or haemocoel (depending on the genus of the haemogregarine) of the definitive vector (see Davies and Johnston 2000; O'Donoghue 2017). Different vectors have been associated with different genera of the group. For example, *Hepatozoon* Miller, 1908, is

associated with ticks, mites and biting insects, unless one takes into consideration *Bartazoon*, which then *Hepatozoon* is associated with ticks and mites only; *Hemolivia* Petit, Landau, Baccam and Lainson, 1990 is associated with tick vectors; *Karyolysus* Labbé, 1894, with mites; while *Haemogregarina, Desseria* Siddall, 1995 and *Dactylosoma* Labbé, 1894 are associated with leech vectors (Davies and Johnston 2000; Karadjian et al. 2015).

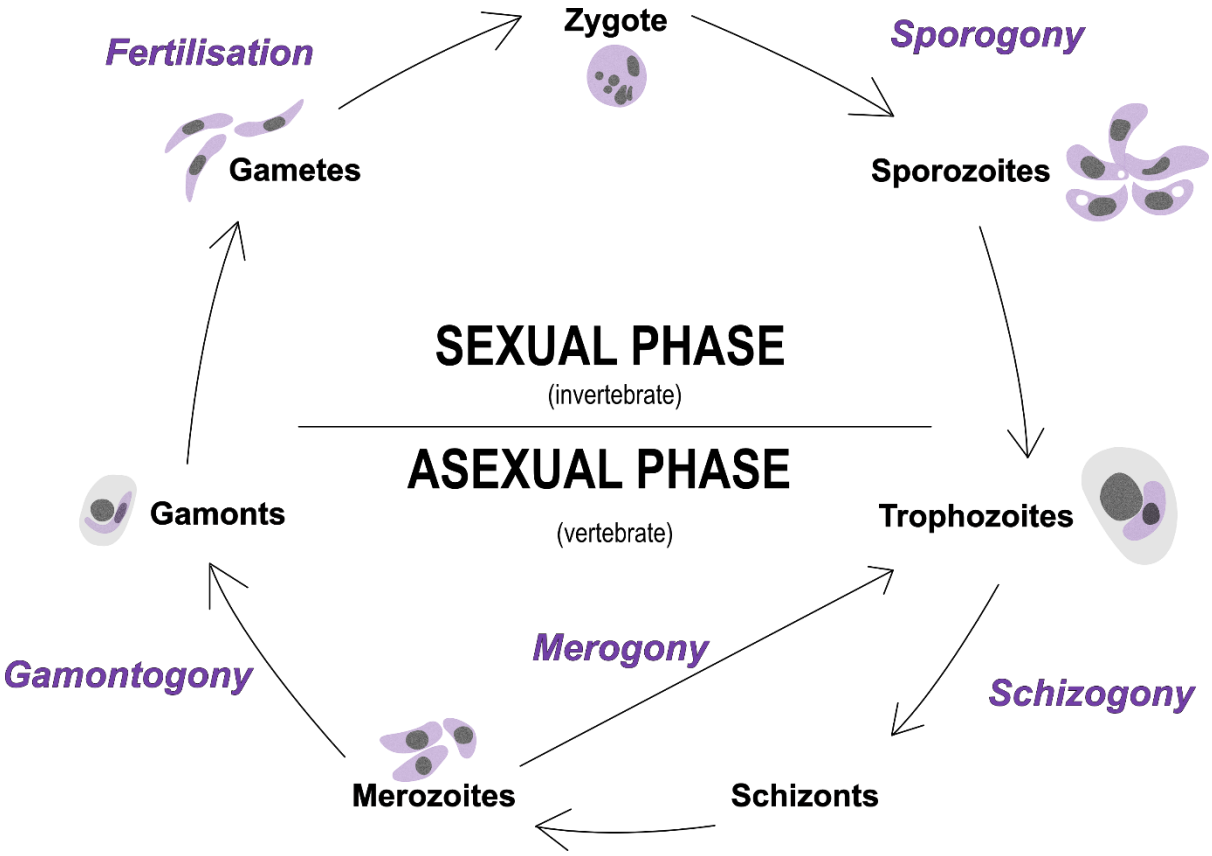


Figure 2.5 General life cycle of haemogregarines. Adapted from Davies and Smit (2001).

2.3.2.2 Identification of haemogregarines

Within the group of haemogregarines, four families are recognized, the Dactylosomatidae, containing the genera *Babesiosoma* Jakowska et Nigrelli, 1956 and *Dactylosoma* Labbé, 1894, the Haemogregarinidae, containing genera *Haemogregarina* Danilewsky, 1885, *Cyrilia* Lainson, 1981 and *Desseria* Siddall, 1995, the Hepatozoidae, containg the genus *Hepatozoon* Miller, 1908, the Karyolysidae, containing genera *Hemolivia* Petit, Landau, Baccam and Lainson, 1990 and *Karyolysus* Labbé, 1894 (Davies and Johnston 2000; O'Donoghue 2017). During a systematic revision of the haemogregarines by Karadjian et al. (2015), a new genus was erected *Bartazoon* Karadjian, Chavatte and Landau, 2015. The aim of this was to aid in the resolution of the currently paraphyletic genus *Hepatozoon*. However, the monophyly of the newly erected genus is not well supported and furthermore is based on phylogenetic estimates

of a single gene – the 18S rRNA gene. As such, this genus has not been widely accepted and has been considered premature in its establishment (Maia et al. 2016, Borges-Nojosa et al. 2017; Cook et al. 2018). Even though the 18S rRNA gene is widely accepted as the optimal gene region for studying Apicomplexan genetic variability, some studies have shown discrepancies of unusually high levels of diversity (Maia et al. 2016). A study by Harris et al. (2015) mentioned that faster evolving genes must also be included in molecular analyses in order to provide a more holistic picture of the true taxonomy of haemogregarines (Harris et al. 2015).

The majority of descriptions of these parasites are primarily based on the stages within host erythrocytes and sometimes leucocytes in the peripheral blood as well as their specificity to a certain host (Davies 1995; Davies and Johnston 2000; Cook et al. 2014; Siddall 1995). Based on the early descriptions of the gametocyte morphology, most species were first placed in the *Haemogregarina* and *Hepatozoon* genera, however recent studies support that the stages that are observed in the vector are indicative of the genus position (Barta et al. 2012; Karadjian et al. 2015; Siddall 1995). With the above in mind, it is, as such, important to describe or identify these parasites on as many life stages as possible, the accuracy of identifying and assigning the parasite to the correct genus increasing with the additional observations of stages found within the vector.

The genus *Haemogregarina* hosts several species, however recently it has been suggested that those species which use leeches as definitive hosts are the only parasites that truly belong in this genus (Davies 1995, Siddall 1995; Davies and Johnston 2000; Davies et al. 2004; Karadjian et al. 2015). Species in this genus are described as having an oocyst with eight sporozoites and are often found infecting fish and reptiles (Davies 1995, Davies and Johnston 2000). Parasites in the genus *Hepatozoon* infect vertebrates including amphibians, reptiles, birds, and mammals, where merogony takes place outside of the erythrocytes, the vertebrate becoming infected when ingesting an invertebrate host such as mites, ticks, insects and perhaps leeches in which sporogony takes place within the lumen of the wall of the gut (Davies 1995, Davies and Johnston 2000). Transmission can also occur when one vertebrate preys on another which is infected with tissue cysts (Davies and Johnston 2000). Davies (1995) stated that the oocysts of these parasites are very large in size with each having four to 16 sporozoites present. Species placed in the genus *Cyrilia* are considered haemogregarines of only freshwater fishes with the intraerythrocytic meronts and gamonts taken up via the bite of an invertebrate host such as a leech (Davies 1995, Davies and Johnston 2000, Steinhagen and Davies 2008). The oocysts of members in the genus *Cyrilia* produce 20 sporozoites, which in turn get transferred to the fish when a leech takes a blood meal (Davies 1995, Davies and

Johnston 2000, Hayes et al. 2006, Steinhagen and Davies 2008). Like the genus *Cyrilia*, members of the genus *Desseria* infect fishes, and are known to have the highest diversity of fish haemogregarine species (Steinhagen and Davies 2008). These members differ from parasites in the *Haemogregarina* and *Cyrilia* genera by not undergoing merogony in the erythrocytes, while the gamonts in the erythrocytes appear to be dimorphic (having two distinct forms) (Siddall 1995). Within the invertebrate host (leeches), gametogenesis and sporogony take place in the intestinal epithelial cells (Steinhagen and Davies 2008). Transmission, like in the *Haemogregarina* and *Cyrilia* genera, occurs when merozoites are transferred to the fish whilst the leech is feeding on it (Davies and Johnston 2000).

Dactylosomes infect fishes (Saunders 1960; Fantham, Porter and Richardson 1942) and anurans (Barta 1991; Fantham, Porter and Richardson 1942; Manwell 1964; Netherlands et al. 2020). Species of this family can be differentiated from other haemogregarines based on a number of differences in the peripheral blood stages, including the presence of elongated and amoeboid body forms, a different areolar structure, vesicular nucleus, and the hyaline appearance of the protoplasm (Netherlands et al. 2020; Labbé 1894). The blood stages lack pigments but do have retractile granules present. These parasites also have minimal effects on the host cell and nucleus and when they undergo merogony, five to 12 merozoites form, which are grouped in a rosette-like appearance (Netherlands et al. 2020). Life cycles for this genus have not yet been elucidated despite several attempts of transmission studies (Barta 1991; Nöller 1913; Boulard et al. 1982).

The genus *Babesiosoma* can be distinguished from dactylosomes based on a number of morphological characteristics, including a less granulated but more vacuolated cytoplasm, with a nucleus similar to those parasites in the genus *Babesia,* without a definite karyosome. Additionally, they reproduce by schizogony or binary fission and do not produce more than four merozoites (Netherlands et al. 2020). Babesiosomes have been found infecting fishes, frogs and lizards and are transmitted when merozoites are transferred from the bite of an invertebrate (Davies and Johnston 2000). Merogony and gamogony occurs in the vertebrate host. Within the invertebrate, gametogenesis and syngamy takes place leading to ookinete formation and oocysts with eight sporozoites infecting the epithelium cells. These undergo sporogony and produce merozoites within salivary cells, ready to infect the next vertebrate host via inoculation during the next blood meal (Davies and Johnston 2000).

Vertebrates such as anurans, chelonians and lizards are parasitised by members of the genus *Hemolivia*. They are transmitted when the vertebrate host ingests an invertebrate host – a tick (Davies and Johnston 2000; Karadjian et al. 2015). Within the vertebrate, merogony occurs

within the erythrocytes, the process producing gamonts. In the invertebrate, sporogony takes place within the intestinal cells producing oocysts, which are typically found in a star-form and produce sporocysts, which in turn invade gut cells (Davies and Johnston 2000). The genus *Karyolysus* infects mainly lizards when the infected invertebrate host (mites) are ingested. Merogony takes place in vascular endothelial cells and gamonts are found in the erythrocytes. Within the mite, sporogony takes place and oocysts produce many sporokinetes, which in turn enter the mite's eggs to form sporocysts with 20–30 sporozoites each (Davies and Johnston 2000).

Molecular data is more readily available for haemogregarines infecting reptiles, amphibians, and mammals (O'Donoghue 2017). The five major clades in phylogenies include two *Hepatozoon* clades, a *Hemolivia* clade, a clade which includes *Haemogregarina* parasites infecting freshwater turtles and another *Dactylosoma* and *Babesiosoma* clade, which includes parasites of fishes and frogs (Kvicerova et al. 2014; O'Donoghue 2017). Haemogregarines infecting frogs (Netherlands et al. 2018), freshwater turtles (Úngari et al. 2018), tortoises and snakes (Cook et al. 2014, 2018, Cook et al. 2015, 2016) were primarily described using both morphological and molecular approaches, in comparison with haemogregarines of fishes (Hayes et al. 2006; Smit and Davies 2006), where descriptions are based on morphological and morphometric information alone (Hayes and Smit 2019). When looking at the phylogeny of haemogregarines as a whole, it is clear that most research done on these parasites is primarily from reptiles, amphibians and mammals (Barta et al. 2012). Thus, more research is needed into apicomplexans, particularly haemogregarines, parasitising fishes, both marine and freshwater.

2.3.2.3 Haemogregarines infecting fish

Most research conducted into fish diseases are primarily focused on diseases infecting economic or recreationally important fish species, as well as the aquaculture industry (Sikkel et al. 2018), with these not necessarily focusing on the diseases caused by parasites. Even though haemogregarines are well studied in other vertebrate groups such as reptiles, amphibians and especially in mammals (particularly in the case of those of medical and veterinary importance), there is a lack of information on the biology of haemogregarines infecting fishes (Hayes and Smit 2019). During a taxonomic revision of the haemogregarines, Siddall (1995) placed fish haemogregarines in three different genera, including *Cyrilia*, *Desseria*, and *Haemogregarina* (*sensu lato*), whereas *Haemogregarina* (*sensu stricto*) are specifically for those infecting chelonians (Siddall 1995; Davies et al. 2004). From literature haemogregarines belonging to the genera *Haemogregarina* (*s.l.*) and *Desseria* are typically described infecting marine hosts, while *Cyrilia, Dactylosoma* and *Babesiosoma* are typically

described infecting freshwater fishes (Smit and Davies 2006; Magro et al. 2016; Saunders 1960; Netherlands et al. 2020; Smit et al. 2003). Life-cycle data have been elucidated for only a few haemogregarines of fishes, this comprising one species of *Babesiosoma* (Negm-Eldin 1998), *Cyrilia* (Negm-Eldin 1999) and *Desseria* (Siddall and Desser 1992; Davies and Johnston 2000), and for two species of *Haemogregarina* (*s.l*.) (Davies and Smit 2001; Curtis et al. 2013). Off the coast of South Africa one haemogregarine of fishes *Haemogregarina* (*s.l*) *bigemina* has life stage data provided, with the proposed life cycle diagrammatically presented in Davies et al. (2001). These authors suggested the strong possibility of this haemogregarine being transmitted via ingestion of an infected gnathiid isopod. Briefly, after ingestion by a potential fish host, merozoites enter into the bloodstream of the fish forming intraerythrocytic trophozoite and meront stages in the peripheral blood. Following this, binary fission takes place, which results in the formation of mature gamonts. Paired gamonts are then released from the host nucleus where some will invade other host erythrocytes, while others get taken up when the invertebrate takes a blood meal (Davies and Smit 2001). From here, they undergo syzygy and form oocysts, sporogony takes place and sporozoites are formed. These sporozoites divide and form meronts in the gut, the subsequent merozoites being the infective stages taken up when the vertebrate feeds on an infected gnathiid isopod. This is different from other haemogregarine genera including *Cyrilia* where sporogony takes place in epithelial cells and oocysts produce more than 20 sporozoites which migrate to the invertebrate salivary glands which will in turn infect another host. Also, it differs from members of *Desseria*, where the zygote, which is produced by the leech vector, produces 16 – 32 sporozoites instead of the 4 – 8 sporozoites produced by *H. bigemina.* Lastly, members of *Dactylosoma* undergoes merogony and produces 5 – 12 sporozoites which are arranged in a rosette-like appearance (Netherlands et al. 2020). Interestingly, even though *H. bigemina* was grouped with *Haemogregarina* (*s.l.*) by Siddall (1995) due to its development in a fish host, the elucidated life cycle is more similar to the life cycle information that is known from members of *Haemogregarina* (*s.s.*) infecting turtles (Davies and Smit 2001). Before 2001, the accepted hypothesis was that species of *Haemogregarina* were solely transmitted by leeches (Siddall 1995), a finding that Davies and Smit (2001) later questioned when stages of development representing the likely life cycle of *H. bigemina* from South African fish hosts was discovered. The strong support that *H. bigemina* is likely transmitted by haematophagous isopods such as those of the genus *Gnathiia* Leach, 1814 (Davies and Smit 2001; Davies et al. 2004; Hayes and Smit, 2019) caused authors to question whether *H. bigemina* is in fact a member of *Haemogregarina* or warrants its own genus (Davies and Smit 2001). However, the authors felt at the time, that the finding of developmental stages in the gnathiid isopod, instead of the leech, was not enough to place it in a genus of its own. To date, no life cycle is known for the haemogregarines infecting elasmobranchs. However, from

the research by Yeld (2009), two potential vector groups were identified – leeches and gnathiid isopods. Thus, in order to identify the genus to which these haemogregarines belong, besides morphological analysis of these parasites' stages within the peripheral blood, stages will need to be identified in either group of invertebrates and/or molecular analysis will need to be done.

Haemogregarines of the same species have been found infecting several freshwater fishes (Table 2.2), while in the marine environment, they are more diverse with more species being described from several different hosts. Even though more species of haemogregarines have been described from the marine environment in South Africa, more work is needed for a better understanding of which marine hosts are the most valuable and most likely to be infected (Janouškovec et al. 2015).

Table 2.2 Species of haemogregarines infecting marine and freshwater species in Africa. Adapted from Scholz et al. (2018).

Haemogregarine species	Host species	Locality	Reference
	Freshwater haemogregarines		
Babesiosoma hannesi	*Chelon dumerili* *C. richardsonii* *Mugil cephalus*	South Africa	Paperna (1981); Scholz et al. (2018)
Babesiosoma mariae	*Astatoreochromis alluandi* *Haplochromis cinereus* *H. nubilus* *H. serranus* *Haplochromis* spp. *Labeo victorianus* *Oreochromis esculentus* *O. niloticus* *O. variabilis* *Serranochromis angusticeps*	Uganda; Botswana	Hoare (1930); Scholz et al. (2018); Smit et al. (2003)
Cyrilia nili	*Parachanna obscura*	Sudan	Wenyon (1909); Scholz et al. (2018)
Haemogregarine gen. sp.	*Synodontis schall*	Not provided	Scholz et al. (2018)
	Marine and brackish haemogregarines		
Haemogregarina bigemina		Europe South America Red Sea South Pacific South Africa	Davies et al. (2004)
H. curvata	*Clinus cottoides*	South Africa	Hayes et al. (2006)

Table 2.2 Continued.

Haemogregarine species	Host species	Locality	Reference
H. koppienses	*Amblyrhynchotes honckenii*	South Africa	Smit and Davies (2001)
H. kunegemina	*Helcogramma obtusirostre*	South Africa	Ferreira et al. (2012)
Desseria zei	*Zeus capensis*	South Africa	Smit and Davies (2006)
Desseria mugili	Mullet species	South Africa	Carini (1932); Smit et al. (2002)
Desseria sp.*	*Mugil cephalus*	South Africa	Smit et al. (2002)
Haemogregarina sp. A**	*Haploblepharus pictus* *Haploblepharus edwardsii* *Poroderma africanum*	South Africa	Yeld (2009)
Haemogregarina sp. B**	*Haploblepharus pictus* *Haploblepharus edwardsii* *Poroderma africanum*	South Africa	Yeld (2009)

*described but not named. **reported, but not formally described.

In South Africa, the first haemogregarine, *Haemogregarina fragilis* Fantham, 1930, was described from a blenny, *Parablennius cornutus* L. Later, during a taxonomic revision of the Haemogregarinidae, Siddall (1995), transferred this species to the genus *Desseria* and thereafter, during a taxonomic re-evaluation of *D. fragilis*, Smit et al. (2003) synonymised this species with *Haemogregarina* (*s.l.*) *bigemina* Laveran and Mesnil, 1901, first reported infecting marine fishes of South Africa by Smit and Davies (1999). As such, and as stated by Smit and Hadfield (2015), *Haemogregarina koppiensis* Smit and Davies, 2001, is now considered to be the first haemogregarine to be described from South African marine fishes. *Haemogregarina koppiensis* was described in 2001 infecting the evileye-pufferfish *Amblyrhynchotes honckenii* (Bloch, 1785) and ever since this haemogregarine was described, this group of parasites has been described more frequently through increased survey efforts of marine fishes off the coasts of South Africa. Both *Desseria zei* Smit and Davies, 2006 from the Cape dory *Zeus capensis* Valenciennes and *Haemogregarina curvata* Hayes, Smit, Seddon, Wertheim and Davies, 2006 from intertidal fishew were described in 2006 (Hayes et al. 2006; Smit and Davies 2006). The first description from a haemogregarine infecting teleost fishes in the sub-tropical region of South Africa was in 2012 when Ferreira et al. (2012) described *Haemogregarina kunegemina* Ferreira, Smit and Davies, 2012 from fishes in the Blenniidae and Triptrygiidae families.

2.3.2.4 Elasmobranch haemogregarines

Little is known on haemogregarines infecting elasmobranchs worldwide (Table 4.1, Chapter 4), and knowledge on these parasites from South African elasmobranchs is entirely absent, except for work done by Yeld (2009). A species of *Cyrilia* has been reported infecting freshwater elasmobranchs in the Amazon River in Brazil, including the cururu stingray, *Potamotrygon motoro* (Müller and Henle), the porcupine river stingray, *Potamotrygon hystrix* (Müller and Henle), as well as the Reo Negro hystrix ray *Potamotrygon wallacei* Carvalho, Rosa and Araújo, 2016 (Magro et al. 2016; Oliveira et al. 2017). Several species of *Desseria* have been described from elasmobranchs worldwide including *Desseria dasyatis* (Saunders, 1985) Siddall, 1995, *Desseria heterodonti* (von Prowazek, 1910) and *Desseria torpedinis* (Neumann, 1909) Siddall, 1995. Another study done in Galicia, Spain by Aragort et al. (2005) described *Haemogregarina delagei* Laveran and Mesnil, 1901 infecting both the blonde skate, *Raja brachyura* Lafont, and small-eyed ray, *Raja microocellata* Montagu. In Australia, *Haemogregarina carchariasi* Laveran, 1908, was described infecting various species of sand tiger sharks *Carcharias* Rafinesque as well as the epaulette shark *Hemiscyllium ocellatum* Bonnaterre. In South Africa, two species of haemogregarines have been reported in an unpublished Ph.D. thesis by Yeld (2009), however, no formal descriptions or molecular information are available to date.

2.4 PARASITIC LEECHES OF ELASMOBRANCHS

Leeches make up 30 % of the phylum Annelida Blanchard, 1894 (Aloto and Eticha 2018). Even though a small number of leeches are predators of small invertebrates, a large majority of leeches have adapted to a parasitic lifestyle, living on the blood of vertebrate hosts (Aloto and Eticha 2018). The latter can be found as ectoparasites on a large variety of animals, both terrestrial and aquatic (Aloto and Eticha 2018), including humans, terrestrial animals, and fish (Aloto and Eticha 2018; Kuo and Lai 2018; Westergren and Siddall 2004). Parasitic leeches are most well known for their important role in the early 1800s European medicinal field (Martucci 2020).

2.4.1 Life cycle and morphology of leeches

Leeches are hermaphroditic organisms, meaning that they possess both male and female reproductive organs (Aloto and Eticha 2018; Kuo and Lai 2018), however, they still need cross fertilisation to reproduce. Having an average lifespan of 18 to 27 years, leeches have an annual life cycle where they typically mate during springtime (Aloto and Eticha 2018). Following copulation and fertilisation, eggs are laid in cocoons produced by the clitellum (Aloto and Eticha 2018). Each species of leech has their own characteristic cocoon which will attach to a surface depending on the environment, for example terrestrial leech cocoons will attach to the body of the adult leech while cocoons of aquatic leeches will attach to a solid substrate (Aloto and Eticha 2018). When the young leeches hatch, they will feed on the yolk in the egg before they develop into adults (Aloto and Eticha 2018).

Once adults, parasitic leeches will feed on the blood of their hosts by using their jaws fitted with teeth to attach to the host, subsequently releasing a salivary secretion into the host's bloodstream which prevents the blood from clotting and letting the blood flow continuously (Aloto and Eticha 2018). With the use of side pouches of their intestines, leeches have the ability to ingest up to ten times their own body weight in host-blood allowing them to only feed around twice a year (Aloto and Eticha 2018).

Similar to other members of the phylum Annelida, the body of leeches are segmented, and they have no exoskeleton but instead a flexible cuticle, which prevents them from drying out (Aloto and Eticha 2018). Leeches are unique in the sense that their body segments are not divided into compartments and the coelomic space is filled with mesenchyme tissue (Aloto and Eticha 2018). The posterior sucker is formed by a group of segments at the end of the leech which allows them to temporarily anchor themselves onto a surface or substrate (Aloto and Eticha

2018). Leeches move by either swimming in an undulating motion in open water or contracting and expanding their longitudinal muscles while attaching to a substrate with either their anterior or posterior suckers (Aloto and Eticha 2018).

Within the phylum Annelida, leeches belong to the subclass Hirudinea Savigny, 1822 and are distinguished according to their feeding strategies into two orders including Rhynchobdellida Blanchard, 1894, and Arhynchobdellida Blanchard, 1894. Aquatic leeches that possess a protrusible proboscis and feed on the blood of amphibians, reptiles, birds, and fish fall within the order Rhynchobdellida, where three families are recognised, including Glossiphoniidae Vaillant, 1890, Piscicolidae Johnston, 1865 and Ozobranchidae Pinto, 1921 (Light and Siddall 1999). The order Arhynchobdellida consists of both aquatic and terrestrial leeches without a proboscis and is divided in various families including Erpobdellidae Moore, 1908, Salifidae Johansston, 1910, Haemadipsidae Blanchard, 1893, Haemopidae Richardson, 1969, Praobdellidae Sawyer, 1986 and Hirudinidae Whitman, 1886.

2.4.2 Leeches present on fish hosts

Leeches found infesting fish hosts are classified primarily in the families Glossiphondiidae, consisting of freshwater species (FAO 1996; Light and Siddall 1999), and Piscicolidae which contain both marine and freshwater species (FAO 1996; WoRMS 2020a). To differentiate between these two families, characteristics such as the body at rest, division between anterior and posterior regions, the shape and size of the anterior sucker and the position of the eyes must be considered (Table 2.3).

In Africa, leeches have been reported infecting various fresh/brackish water fish families including Clariidae Bonaparte, 1846, Synodontidae Gill, 1862, Mormyridae Bonaparte, 1831, Cichlidae Bonaparte, 1835 and Mugilidae Jarocki, 1822 (Iyaji and Eyo 2008; Oosthuizen 1989). In terms of marine blood-feeding leeches, relatively little is known as this is an understudied area in South Africa (Schaeffner and Smit 2019; Smit and Hadfield 2015). From the 18 species Moore (1958) reported on off the coasts of Africa, only one, *Malmiana stellata* Moore, 1958 (now *Ottoniobdella stellata*) from the toby fish, was recorded along the coast of South Africa (Smit and Hadfield 2015).

Table 2.3 Differentiation between the leech vector families Glossiphonidae and Piscicolidae (adapted from FAO, 1996).

Characteristics	**Glossiphoniidae**	**Piscicolidae**
Body at rest	Depressed	Cylindrical
Division between anterior and posterior regions	Head narrower than the body	Divided at segment XIII into distinct regions
Shape and size of anterior sucker	Almost indistinguishable from the body, or very slightly distinct	Distinctly marked off the body
Position of the eyes	On the head region	May be found on either the head region, neck, or posterior sucker

Since then, only three leech species have been recorded off the coast of South Africa, including *Austrobdella oosthuizeni* Utevsky, 2004 from *Jasus lalandii* H. Milne Edwards, *Lizabdella africana* Utevsky, 2007 from three species of mullets from the genus *Liza* Jordan and Swain, 1884 (Utevsky 2004; Utevsky 2007) and *Zeylanicobdella arugamensis* De Silva, 1963 from three intertidal species of *Clinus* Cuvier, 1816 (Hayes et al 2006; 2014). In an unpublished thesis of Dr Eleanor Yeld (University of Cape Town) she reported the leech *Pontobdella macrothela* (Schmarda, 1861) from three catshark species, *H. pictus, H. edwardsii* and *P. africanum,* however no formal report has been published to date (Schaeffner and Smit 2019, Yeld 2009).

Even though leeches are not considered important fish pathogens, the presence of leeches can often lead to damage on the skin, fins, gills or mouth (Woo 2006). The posterior suckers of leeches usually cause relatively minor damage to the skin when the leeches are trying to attach, however the anterior sucker will cause more damage when they use their protrusible proboscis to feed on the blood of the fish host (Woo 2006). This proboscis can cause localised haemorrhage, and when large numbers of leeches are present on one host, it can result in blood loss or secondary infections of other pathogens including trypanosomes, bacteria, and fungi (Bauer et al. 1973; Woo 2006; Hayes et al. 2014; Markevich 1963).

2.4.3 Elasmobranch leeches

A variety of piscolid leeches are known for parasitising elasmobranchs worldwide, including members of the genera *Branchellion* Savigny, 1922 and *Pontobdella* Leach, 1815. Approximately 23 species of marine leeches have been reported being parasitic on elasmobranchs, with the piscolid leech *Pontobdella macrothela* (Schmarda, 1861) (*syn. Stibarobdella macrothela* Burreson and Passarelli, 2015) being the most abundant with over 20 species of elasmobranch hosts (Keating-Daly et al. 2019; Daly et al. 2019). Found throughout the tropics and subtropics, these leeches are generally found on exterior sites of the host (fins and claspers) and have adapted to feeding on the high urea content of elasmobranch blood (Keating-Daly et al., 2019, Daly et al., 2019). Studies on distribution and host association have predominantly focused on the Atlantic and Pacific Oceans, with very few research efforts in the Indian Ocean. In Southern Africa, the only reports of this leech being parasitic on elasmobranchs, are from the coast of Kenya (*Pontobdella macrothela*; Llewellyn 1966), Mozambique (*Pontobdella macrothela*; Keating-Daly 2019), Seychelles (*Pontobdella macrothela*; Daly et al. 2019), Durban (*Branchellion angeli* Sigalas, 1921; *Pontobdella macrothela*; Moore 1958) and in the Western Cape in an unpublished thesis (Yeld, 2009). Some of the host species include the hammer-head shark (Moore 1958) (species not specified),

sicklefin lemon sharks, *Negaprion acutidens* Rüppel (Keating-Daly et al. 2019), grey reef shark, *Carcharhinus amblyrhynchos* Bleeker (Daly et al. 2019), dark shyshark, *H. pictus*, puffadder shyshark, *H. edwardsii* and pyjama catshark, *P. africanum* (Yeld 2009).

2.5 CONCLUSION

In summary, both trypanosomes and haemogregarines of elasmobranchs remain understudied globally, but in particular in South Africa. Several studies have been conducted into trypanosomiasis of farmed fishes, both freshwater and marine (de Jesus et al. 2018; Su et al. 2013; Khan 1985) in areas such as Canada, China and South America, where the effects of trypanosomes could be determined. In these studies (de Jesus et al. 2018; Su et al. 2013; Khan 1985), the trypanosomes caused several ailments including anaemia, lethargy and loss of appetite. Due to elasmobranchs not being farmed for commercial purposes, no studies have been able to show the effect of trypanosomiasis in these animals, or even if trypanosomiasis occurs. The abovementioned studies also found that juveniles that had high trypanosome infections showed a high mortality rate, again an outcome that cannot be measured in sharks as of yet. Studies similar to that of Conradie et al. (2016) needs to be conducted where the effect of haemogregarines are determined on the condition of the infected blood cells, to see if there would be a possible negative effect on the hosts. Effort needs to be placed in acquiring more data on these parasites infecting elasmobranchs worldwide, as these relationships might show not to be parasitic or harmful. As elasmobranchs are some of the oldest animals alive today, as well the parasites that diverged with them, these relationships might prove to be different than the way that parasitic relationships are viewed today. In-depth studies are needed to determine if haemoprotozoans are truly parasites in the original context of parasitism, or if there lies a connection between parasitism, commensalism and even mutualism. Additionally, further research should aim at expanding the knowledge of hirudinids, especially marine leeches of South Africa. This should include investigating the diversity of South African leeches, but also unravelling whether leeches transmit haemoprotozoans to elasmobranchs, particularly trypanosomes and haemogregarines, or if there is another or additional vectors able or responsible for the transmission of these parasites. In order for all of the abovementioned types of studies to be successful, studies such as the present one needs to be conducted to provide a basis in the form of diversity knowledge. Within the scope of this dissertation, the primary focus is on providing this diversity knowledge by expanding on the existing information that is already available for haemoprotozoans of elasmobranchs in South Africa.

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3

TRYPANOSOMES FOUND INFECTING SHARKS

Chapter 3: Trypanosomes infecting elasmobranchs

3.1 INTRODUCTION

Southern Africa is known as one of the most biodiverse regions for chondrichthyans worldwide with over 200 reported species (Ebert and van Hees 2015). The chondrichthyan diversity also encompasses catsharks of the family Scyliorhinidae, most of which are endemic to southern Africa. With such a high diversity and endemicity (i.e. 13 %) of shark species, the potential for discovering new parasites is large (Schaeffner and Smit 2019). If each fish species from cartilaginous to bony fish harbours at least one unique parasite taxon (Adlard et al. 2015; Smit and Hadfield 2015), there is a large number of parasites still awaiting discovery (Schaeffner and Smit 2019; Smit and Hadfield 2015). This is especially true for blood parasites, as at present, only two species of trypanosomes have been recorded from marine hosts in South Africa.

Trypanosomes (Kinetoplastida: Trypanosomatidae) are obligate, endoparasitic protozoans that can be found in almost every vertebrate and invertebrate class in both aquatic and terrestrial environments (Barta et al. 2012). They belong to the class Mastigophora within the phylum Euglenozoa and are transmitted by haematophagous vectors such as leeches and biting flies. Even though species of *Trypanosoma* Gruby, 1843 have been well studied in mammals, especially the causative agents of African trypanosomiasis (sleeping sickness), due to their veterinary and medical importance, the biodiversity and pathogenicity of this genus in fish and elasmobranch hosts remains poorly explored (Ferreira and Avenant-Oldewage 2013; Smit et al. 2020). The only two species currently known from marine hosts in South Africa are *Trypanosoma nudigobii* Fantham, 1919 from various intertidal teleost fishes (Hayes et al. 2014) and *Trypanosoma haploblephari* Yeld and Smit 2006 infecting elasmobranchs (Yeld and Smit 2006). To date, only 12 species of elasmobranch trypanosomes have been described worldwide (see Table 3.1) and of these, the majority have been described from skates and rays (Bacigalupo and de la Plaza 1948; Burreson 1989; Laird 1951; Neumann 1909; Yeld and Smit 2006). In South Africa, *T. haploblephari* was described from the dark shyshark, *Haploblepharus pictus* (Müller and Henle), and the puffadder shyshark, *Haploblepharus edwardsii* (Schinz). Only two other trypanosomes have been described infecting sharks in the family Scyliorhinidae, namely *Trypanosoma humboldti* Morillas, George-Nascimento, Valeria and Khan, 1987 from the Chilean catshark *Schroederichthys chilensis* Guichenot and *Trypanosoma scylliumi* Laveran and Mesnil, 1902 from the small spotted catshark *Scyliorhinus stellaris* (L.). The present study reports on the

findings of a survey on trypanosome infections in near-shore scyliorhinids off South Africa, adding to the known description of *T. haploblephari* by providing molecular data for this species, as well as a new host and locality record. Furthermore, novel information is provided on this species morphological plasticity, and its close genetic relationship with another elasmobranch trypanosome of European origin, *Trypanosoma rajae* Laveran and Mesnil, 1902 is discussed. The results presented here in this chapter have recently been published in a special issue of the International Journal for Parasitology: Parasites and Wildlife on African Wildlife Parasites (Pretorius et al. 2021).

Table 3.1 Trypanosome species described from elasmobranchs worldwide. Adapted from Yeld and Smit (2006).

***Trypanosoma* spp.**	**Host spp.**	**Country**	**Study**
T. gargantua	*Raja nasuta*	New Zealand	Laird (1951)
T. taeniurae	*Taeniura lymma*	Australia	Burreson (1989)
T. torpedinis	*Torpedo marmorata*	France	Sabrazes and Muratet (1908)
T. rajae	*Raja* spp.	France	Laveran and Mesnil (1902)
T. marplatensis	*Psammotics microps*	Argentina	Bacigalupo and De la Plaza (1948)
T. giganteum	*Dipturus oxyrinchus*	Italy	Neumann (1909)
T. carchariasi	*Odontaspis* sp.	Australia	Laveran (1908)
T. scyllii	*Scyliorhinus stellaris*	France	Laveran and Mesnil (1902)
T. mackerrasi	*Hemiscyllium ocellatum*	Australia	Burreson (1989)
T. boissoni	*Zanobatus schoenleinii*	Senegal	Ranque (1973)
T. humboldti	*Schroederichthys schilensis*	Chile	Morillas et al. (1987)
T. haploblephari	*Haploblepharus pictus*	South Africa	Yeld and Smit (2006)

3.2 MATERIALS AND METHODS

3.2.1 Sampling sites

During the period of May 2018 to February 2019 three sampling trips were undertaken, where blood was collected from shysharks and catsharks from 11 different sites in Hermanus, Western Cape (Fig. 3.1) as well as Granger Bay in Cape Town. Some of the blood samples were collected in Granger Bay in Cape Town with the help of the Save our Seas Shark Education Centre while the majority of the samples were collected from Hermanus at the South African Shark Conservancy (SASC). Situated in Old Harbour, Hermanus, the South African Shark Conservancy (SASC) is a non-governmental research organisation focused on studying sharks and their surrounding environments (South African

Shark Conservancy 2018). Hermanus (34.4092° S, 19.2504° E) (Fig. 3.2 A–C) is a seaside town situated 120 km from Cape Town. This town is famous for its southern right whales, which come to calve off its shores, and attracts numerous tourists wanting to see the whales. It has a warm-summer Mediterranean climate and receives around 518 mm rain per annum, while the majority falls during the winter (SA Explorer, 2017). The Save our Seas Shark Education Centre is situated in Kalk Bay, Cape Town is an education-based organisation forming part of the larger Save our Seas Foundation where school groups get the opportunity to learn more about the marine environment. Granger Bay (33°54'2.31"S, 18°24'56.38"E) (Fig. 3.2 D–F) is a small suburb in Cape Town, with views of Table Mountain and Robben Island. The sampling site is located right next to the Metropolitan Golf Club and is a popular launching site for boats and kayaks. South Africa is considered one of the most biodiverse regions of the world, and the presence of chondrichthyans is no exception to this as chondrichthyans found here accounts to 17 % of all known species worldwide (Compagno et al. 1999; Ebert and van Hees 2015). In the areas where the Agulhas current runs, the level of endemicity is slightly higher (175 species found in tropical and warm-temperate areas) than areas along the Benguela current where only 96 species can be found (Ebert and van Hees 2015).

Figure 3.1 Map of sampling sites.

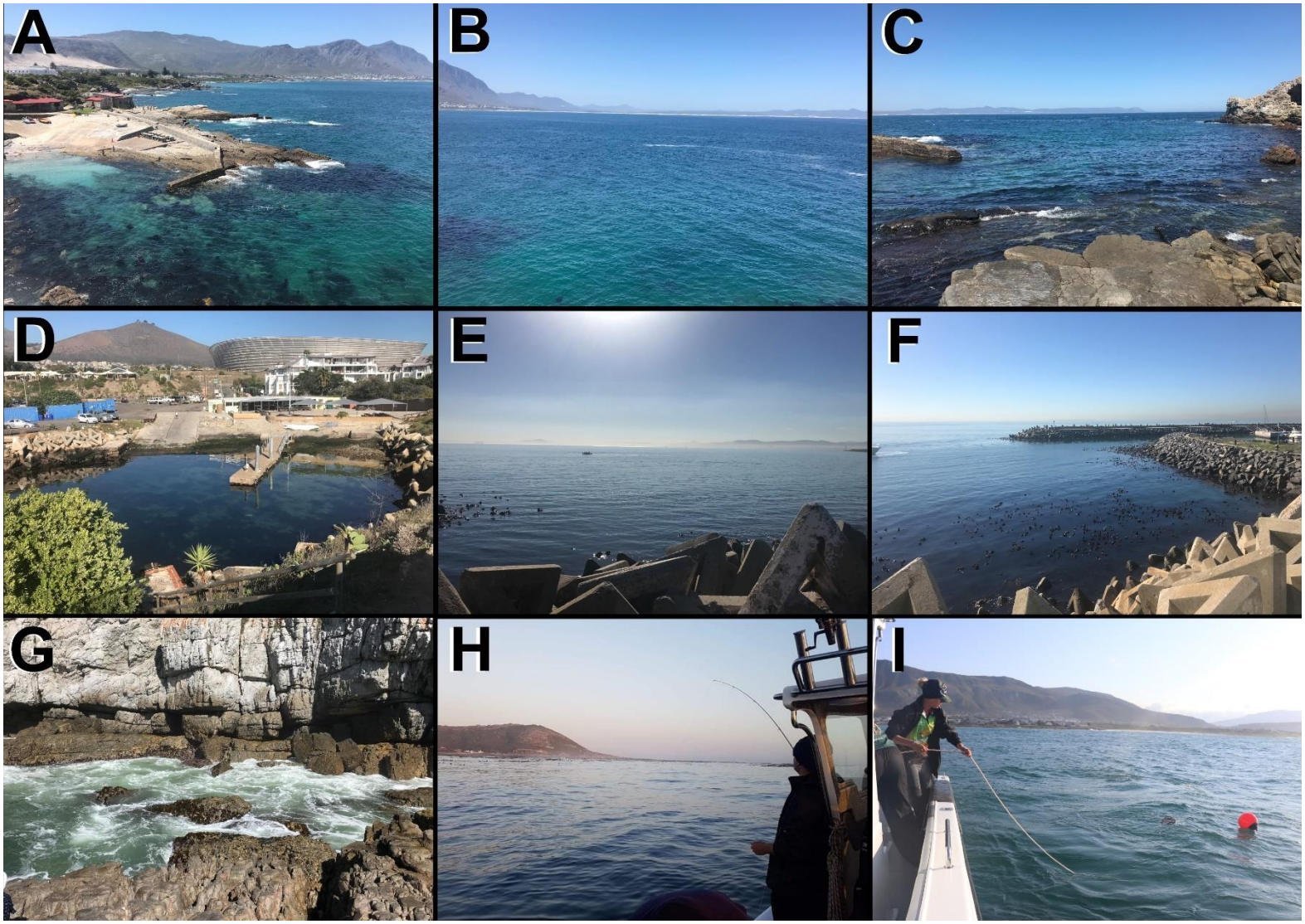

Figure 3.2 Photos of sampling sites in Hermanus (A–C), Granger Bay, Cape Town (D–F), Kraal Rock (G), and various sites when sampling on the boat (H–I).

3.2.2 Ethical considerations

The present study received the relevant ethical approval from the North-West University's AnimCare Research Ethics Committee (ethics approval nos.: NWU-00065-19-A5 and NWU-00372-16-A5) and research permits from the South African Department of Environmental Affairs (permit nos. RES2018-58 and RES2019-61 issued to Mrs. M. McCord, South African Shark Conservancy; and RES2019-105 issued to BCS).

3.2.3 Collection and identification of sharks

Various methods were used to collect the sharks, including longlines, handlines, rod and reel as well as handpicking sharks from bait containers while snorkelling (Fig. 3.3). Longlines contained 25 to 30 hooks, placed 10 meters apart and baited with sardines or squid. These were deployed and left for a maximum time of three hours before being collected again. Sharks collected via long- or handlines were measured, observed for ectoparasites and blood was taken from the caudal vein before being released again. Sharks were also collected by snorkelling in the kelp forest with a bait bag/chum and placed in a mesh bag to bring back to the holding tanks at the SASC facility. As part of a larger parasitological study, one individual of *Haploblepharus edwardsii* (Schinz), 29 of *Haploblepharus pictus* (Müller and Henle), three of *Poroderma africanum* (Gmelin) and nine of *Poroderma pantherinum* (Müller and Henle) were euthanised, while 12 individuals of *H. edwardsii,* 18 of *H. pictus*, 21 of *P. africanum* and five of *P. pantherinum* were released again following bloodletting. Sharks identified to be dissected were placed in cooler boxes with water and transported back to the SASC lab where dissections were performed following standard operating procedures. All sharks were identified with the help of members from SASC. Measurements taken consisted of the total length (TL), which was measured from the tip of the snout to the tip of the caudal fin and the sex of each individual was determined (Fig. 3.4).

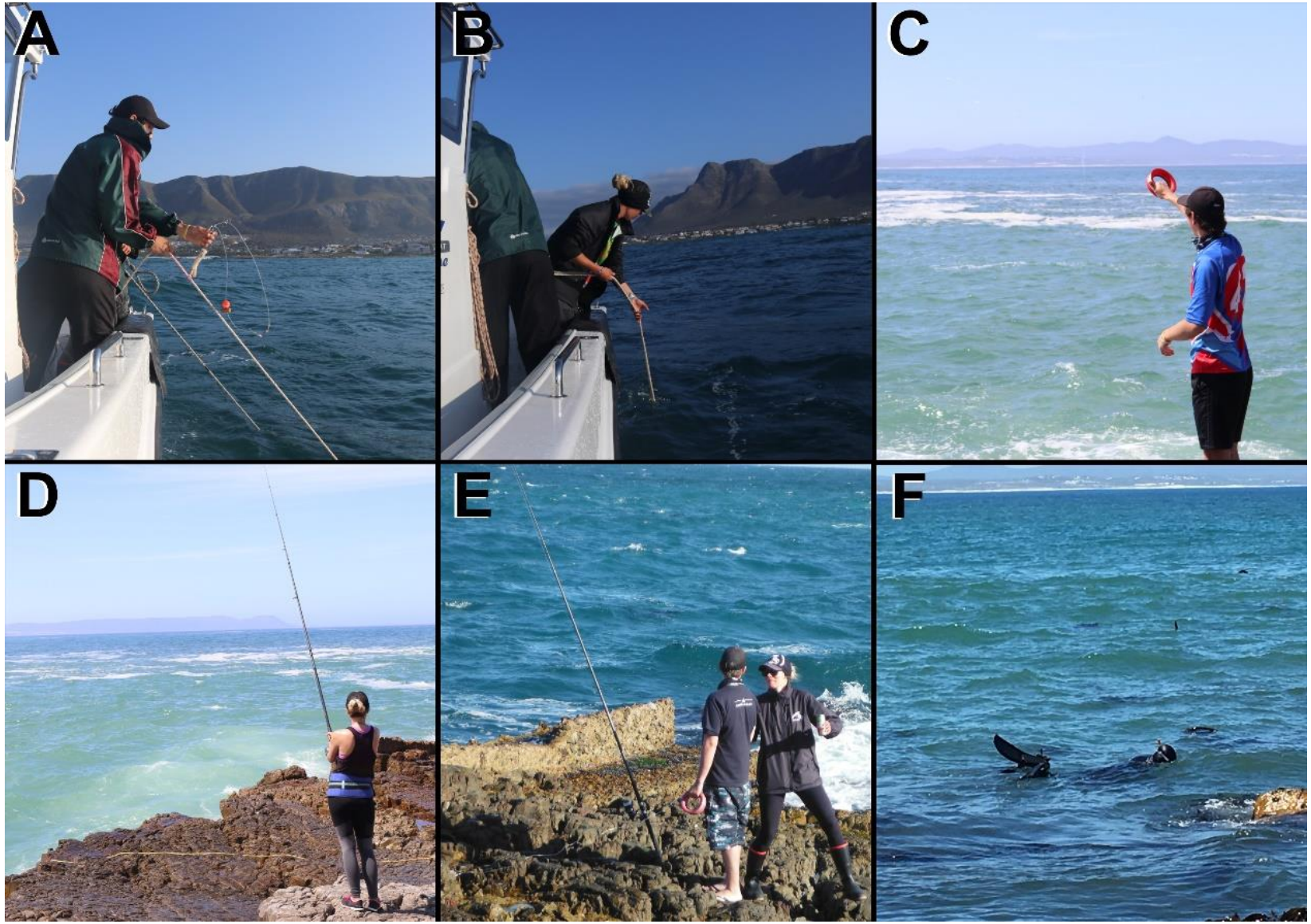

Figure 3.3 Photos showing sampling methods of longlining (A, B), handlining (C), fishing with a rod (D, E) and snorkelling (F).

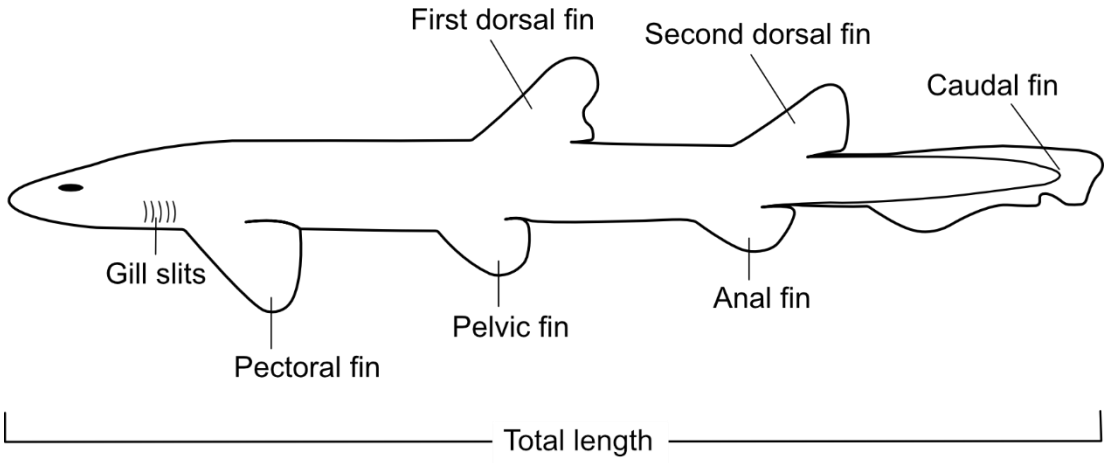

Figure 3.4 Basic diagram of a shark indicating external features as well as the measurement taken for analysis.

3.2.4 Host blood collection, blood smear preparation and screening for blood protozoans

A total of 98 sharks of four different species, *H. pictus* (47 individuals), *H. edwardsii* (13 individuals), *P. africanum* (24 individuals) and *P. pantherinum* (14 individuals) were collected at both Granger Bay and Hermanus. As such a total of 22 individuals were collected from Granger Bay (19 *H. pictus* and 3 *P. africanum*) and a total of 74 individuals were collected from Hermanus (13 *H. edwardsii*; 26 *H. pictus*; 21 *P. africanum* and 14 *P. pantherinum*). Ectoparasites (e.g., leeches) present on sharks were removed and placed in either 70 % ethanol or formalin for identification and further life-cycle evaluations. A maximum of 0.1 ml of blood was drawn from the caudal vein between the pelvic and caudal fin (Fig. 3.5 A) with a 21-gauge sterile, hypodermic needle fitted to a 1 ml syringe. Thin blood smears were prepared and fixed with absolute methanol upon being air-dried completely. Remaining blood was placed in a tube containing 70 % molecular-grade ethanol for subsequent molecular analysis. Microscope slides were stained with a dilution of 10 % Giemsa-stain (Sigma-Aldrich, Steinheim, Germany) for 20 min (Fig. 3.5 B) and screened for parasites (Fig. 3.5 C) using a Nikon Eclipse N*i* (Nikon, Amsterdam, Netherlands) at 1000× magnification, and images captured using the accompanying NIS-Elements BR Ver. 4.60 camera analysis software (Nikon, Tokyo, Japan). Trypanosome stages were measured according to Hayes et al. (2014). Measurements are given in µm, unless otherwise indicated, and include midnucleus to anterior region (MA); midnucleus to posterior region (MP); midnucleus to kinetoplast (MK); posterior region to kinetoplast (PK); nuclear length (NL); nuclear index (NI) which is calculated MP/MA; body width at nucleus [BW(N)]; body width with undulating membrane [BW(UM)]; total body length (TBL) and flagellum length (FL) (Fig. 3.6).

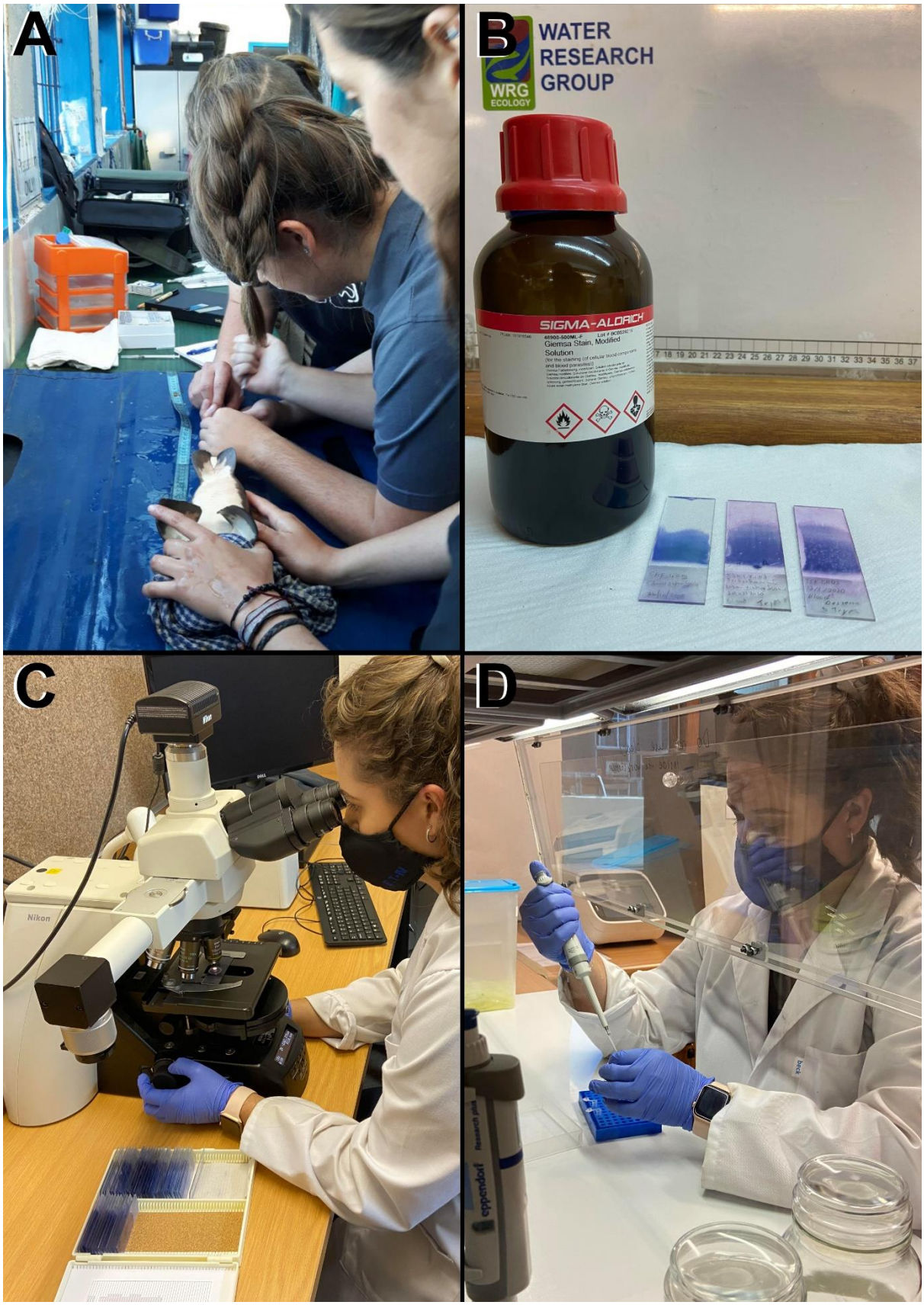

Figure 3.5 Photographs of bloodletting (A), Giemsa used for staining (B), microscopy (C) and DNA extraction and PCR preparation (D).

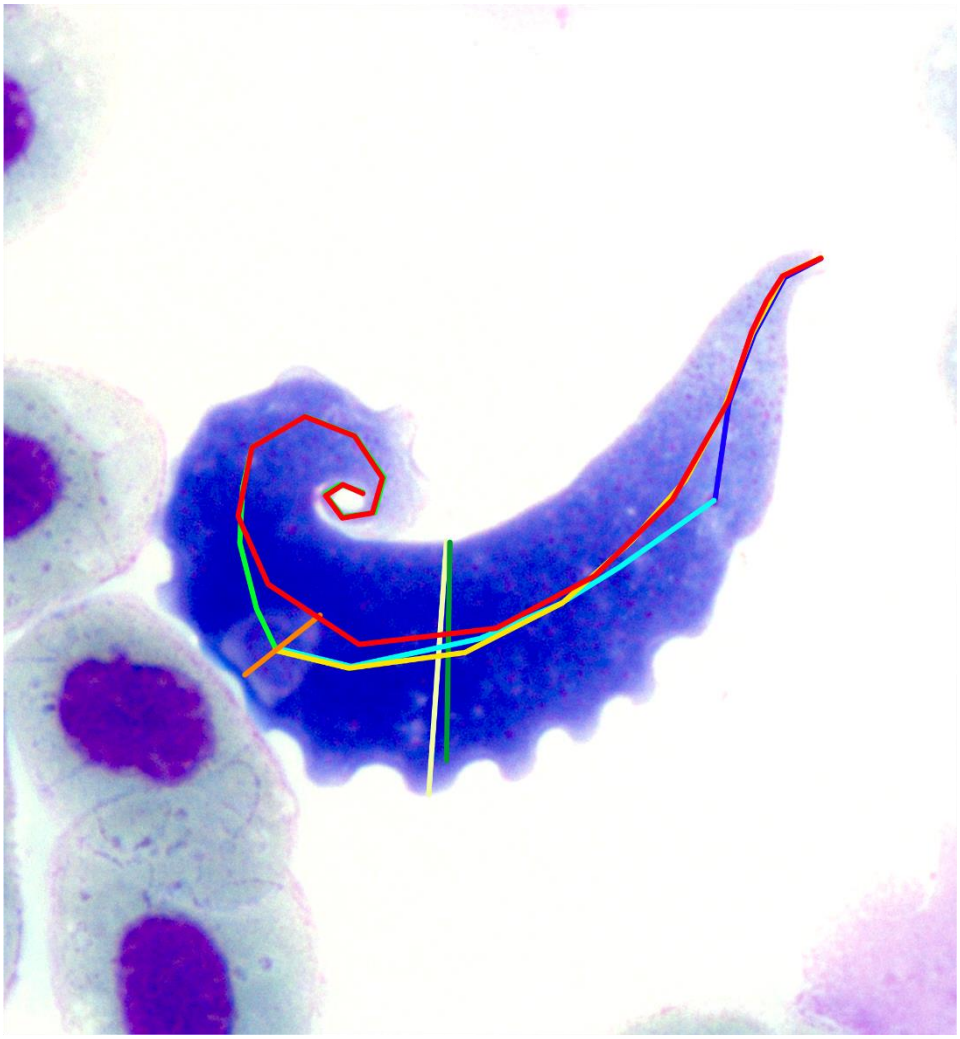

Figure 3.6 Example of measurements taken including total body length (red), total body width excluding undulating membrane (dark green), total body width including undulating membrane (white), nuclear length (orange), midnucleus to anterior (green), midnucleus to posterior (yellow), midnucleus to kinetoplast (light blue), kinetoplast to posterior (dark blue).

3.2.5 Molecular and phylogenetic analysis

Genomic DNA was extracted (Fig. 3.5 D) from all individuals identified as positive for trypanosomes during screening by microscopy using the KAPA Express Extract Kit (Kapa Biosystems, Cape Town, South Africa) following manufacturer's instructions for animal blood. The resulting supernatant was used as a template for PCR using 18S rRNA trypanosome-specific primers listed in Table 3.2. The conditions of the PCR with external primers are as follows: initial denaturation cycle of 95°C for 5 min, 50°C for 2 min, 72°C for 4 min, followed by 35 cycles of 94°C for 30 sec, 52°C for 30 sec, 72°C for 2 min 20 sec, a final extension of 72°C for 7 min. The conditions of the PCR with internal primers are as follows: initial denaturation cycle of 95°C for 5 min, 50°C for 2 min, 72°C for 4 min, followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 2 min 20 sec, a final extension of 72°C

for 7 min. All PCR reactions were performed with volumes of 25 µl, using 12.5 µl Thermo Scientific DreamTaq PCR master mix (2×) (2× DreamTaq buffer, 0.4 mM of each dNTP, and 4 mM $MgCl_2$), 1.25 µl of each primer (10 µM), and at least 25 ng of DNA. PCR grade nuclease free water (Thermo Scientific, Vilnius, Lithuania) was used to make up the final reaction volume. Reactions were undertaken in a SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Singapore). A 1 % agarose gel electrophoresis was produced, and the results visualised under ultraviolet light to determine whether DNA amplicons were obtained. PCR products were then sent to Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria, South Africa) for purification and sequencing in both directions.

Table 3.2 PCR primers used for amplification and sequencing of the 18S rRNA gene region.

Primer		Sequence	Reference
External	SLF	5'-GCTTGTTTCAAGGACTTAGC-3'	McInnes et al. (2009)
External	S762	5'-GACTTTTGCTTCCTCTAATG-3'	Maslov et al. (1996)
Internal	B	5'-CGAACAACTGCCCTATCAGC-3'	Hayes et al. (2014)
Internal	I	5'-GACTACAATGGTCTCTAATC-3'	Hayes et al. (2014)

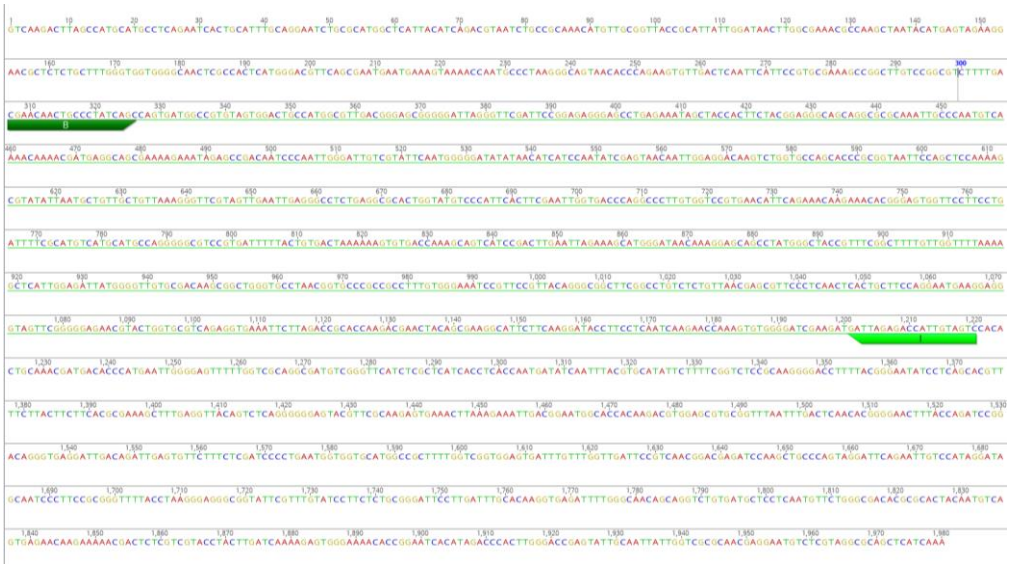

Figure 3.7 Figure showing where the internal primers anneal on the 18S gene region.

The quality of resultant sequences was assessed using Geneious ver. 11.1.4 (http://www.geneious.com, Kearse et al. 2012) before consensus sequences were generated from both forward and reverse sequence reads. Sequences obtained were deposited in the NCBI GenBank database under the following accession numbers (GenBank: MZ061638, MZ061640). Resultant sequences were identified using the Basic Local Alignment Search

Tool (BLAST) (Altschul et al. 1990) and an additional 23 comparative sequences of *Trypanosoma* were selected with *Trypanosoma avium* Danilewsky, 1885 (KT728402) as the outgroup following Hayes et al. (2014). Comparative sequences were aligned using the ClustalW alignment tool available on Geneious ver. 11.1.4. A model test was performed to determine the most suitable nucleotide substitution model, according to the Akaike information criterion using jModelTest 2.1.4 (Guindon and Gascuel 2003; Darriba et al. 2012). The best model for the alignment was the general time-reversible model incorporating invariant sites and gamma distributed among site-rate variations (GTR+I+G). A Bayesian Inference tree was constructed in Geneious ver. 11.1.4 using the MrBayes parameter with a four category Gamma distribution. A maximum likelihood tree was constructed using PhyML 3.0 (Guindon et al. 2010) and run on the ATGC bioinformatics platform (available from http://www.atgc-montpellier.fr/phyml/, Guindon et al. 2010) with support using 1000 rapid bootstrap inferences. Phylogenetic trees were visualised using FigTree v. 1.4.4 software (Rambaut 2012), the p-distance was calculated using MEGA 7 (Kumar et al. 2015) and the number of base pair differences was calculated using Geneious ver. 11.1.4.

The use of the cytochrome oxidase subunit 1 (COI) gene marker has been widely accepted as the target gene for animal barcoding studies, however, universal barcoding genes for eukaryotic groups have proven difficult due to (1) poorly understood genetic variability for each species, and (2) to date there is no agreement in the literature on which genes are the ideal markers for delimiting species groups (Hutchinson and Stevens 2017). The most widely accepted markers for trypanosome studies include 18S and 28S rRNA as well as fluorescent fragment length barcoding (FFLB). Although FFLB can detect novel trypanosome species, additional molecular studies are needed to identify those species. There are also very few points of reference available for this technique (Hutchinson and Stevens 2017). Therefore, the 18S rRNA is the most suitable marker of choice due to it being (1) a highly expressed multicopy gene, (2) present in all eukaryotes, and (3) has conserved and variable nucleotide arrangements (Hutchinson and Stevens 2017).

3.3 RESULTS

3.3.1 General observations of trypanosomes in the blood of sharks

Of the 98 individuals of four different species examined, 93 % were infected with trypanosomes. The metrical information, prevalence, and average numbers of trypanosomes per blood smear are recorded in Table 3.3. Of the 47 specimens of *H. pictus* and 13 specimens of *H. edwardsii* screened, 91 % and 100 %, respectively, were parasitised with a trypanosome species morphologically similar to *T. haploblephari*. All size classes were infected. A total of 14 *P. pantherinum* and 24 *P. africanum* were screened, with 100 % and

62.5 % prevalence, respectively, of a morphotype of the trypanosome species found in *H. pictus* in this study. Leeches were found on various surfaces not limited to a specific area on the host in four of the *H. pictus* individuals, two of the *H. edwardsii* individuals, seven of the *P. africanum* individuals and two of the *P. pantherinum* individuals. Table 3.4 provides the morphometrics of *T. haploblephari* observed in this study in *H. pictus* and *H. edwardsii* and that of the original description in Yeld and Smit (2006), as well as the morphotype parasitising *P. africanum* and *P. pantherinum* in the current study, along with measurements provided by the original descriptions of *T. humboldti* from *Schroederichthys chilensis* and *T. scylliumi* infecting *Scyliorhinus stellaris* (Laveran and Mesnil 1912; Pulsford 1984; Morillas et al. 1987). The following diagnosis and description of *T. haploblephari* will provide for a detailed description of trypanosome morphotypes in *H. pictus*, *H. edwardsii*, *P. africanum* and *P. pantherinum,* respectively.

Table 3.3 Information on elasmobranch hosts, including prevalence of peripheral blood trypanosomes.

	Sharks		Trypanosomes
Species	**N**	**ML ± SD (range) in mm**	**Prevalence**
Haploblepharus edwardsii	13	421.1 ± 34.6 (354 – 467) (N=9)	100 % (13/13)
Haploblepharus pictus	47	435.6 ± 101.6 (260 – 614)	91 % (43/47)
Poroderma africanum	24	767.1 ± 150.9 (501 – 1010)	62.5 % (15/24)
Poroderma pantherinum	14	511.4 ± 95.5 (363 – 725)	100 % (14/14)

N, number; ML, mean length; SD, standard deviation.

3.3.2 Description and diagnosis of stages found in the blood

Kinetoplastea (Honigberg, 1963) Vickerman, 1976
Trypanosomatida (Kent, 1880) Hollande, 1952
Trypanosomatidae (Doflein, 1901) Grobben, 1905
Trypanosoma haploblephari Yeld and Smit, 2006

Restricted synonymy: Yeld and Smit 2006: 829–833, figs. 1, 2; Hayes et al. 2006: 241; Hayes et al. 2014: 2; Smit and Hadfield 2015: 84; Schaeffner and Smit 2019: 2, 7, 16.

Type host: *Haploblepharus pictus* (Müller and Henle) (Chondrichthyes: Scyliorhinidae).
Other hosts: *Haploblepharus edwardsii* (Schinz), *Poroderma pantherinum* (Müller and Henle), *Poroderma africanum* (Gmelin) (Chondrichthyes: Scyliorhinidae)
Type locality: Granger Bay, Western Cape, South Africa (33° 52’ S 18° 24’ E).
Other localities: Hermanus, Western Cape, South Africa (34°25'15.76"S, 19°14'37.56" E).

3.3.2.1 Material studied in *H. pictus* and *H. edwardsii* (morphotype A):

Locality: Granger Bay, Cape Town (33°54'2.31"S, 18°24'56.38"E) and Hermanus (34°25'15.76"S, 19°14'37.56" E), Western Cape, South Africa.
Site in host: Peripheral blood.
Prevalence: 91 % (43/47).
Vector: Unknown. Possibly leech found on sharks preliminarily identified as *Pontobdella* cf. *macrothela* Schmarda, 1861 (Prof. E. Burreson, Virginia Institute of Marine Science, USA; pers. comm.).
Representative DNA sequence(s): Three partial sequences of the 18S rRNA gene; 1740 bp, 1155 bp and 920 bp in length respectively (GenBank accession numbers: MZ061638, MZ061640, MZ061642).

Diagnosis: Present specimens of *T. haploblephari* (Fig. 3.8 A–C) stained a deep blue with a wide and long body, and a distinct undulating membrane. The mean length of the trypanosome stages 64.7 ± 16.2 (26.6 – 112.2) (Table 3.4) correspond to the mean length of *T. haploblephari*; 70.4 ± 9.4 (53.7 – 99.4) reported by Yeld and Smit (2006). The karyosome is prominent within the nucleus, the latter situated in the anterior half of the parasite. Longitudinal striations were observed on larger specimens as reported for type specimens of *T. haploblephari* (Yeld and Smit 2006).

Description: Chromatic granules visible in cytoplasm, which stain a deep purple along with the kinetoplast and nucleolus. Karyosome visible in the nucleus (Fig. 3.8 A), a similar finding to that of Yeld and Smit, 2006. Kinetoplast distinct and located close to posterior region; 11.9 ± 6.1 (1.8 – 33.5 or 18.8 % of body length – this study) (Fig. 3.8 C) or 16.8 ± 4.5 (6.9 – 45.6 or 23.9 % of body length – Yeld and Smit, 2006). Similar to Yeld and Smit (2006), the flagellum can be observed but is not easily stained or measured. In smaller stages, the posterior end is more slender and pointed in comparison to the larger stages that often have a blunt and rounded end (Yeld and Smit 2006).

Remarks: The morphometrics of the trypanosome species observed from *H. pictus* and *H. edwardsii* in this study, closely resemble the data provided by Yeld and Smit (2006) for *T. haploblephari.* In addition to samples of the current study being collected from the type host and type locality, the species identification of *T. haploblephari* is further supported. This species of *Trypanosoma* was also found infecting *H. pictus* and *H. edwardsii* off the coast of Hermanus, a previously unknown distribution area of *T. haploblephari*, thus expanding the known biogeographical distribution of *T. haploblephari* to the southern Western Cape coast. Infection rates or parasitaemia vary among individuals of *H. pictus*, ranging from 0 to 200 trypanosomes per blood smear (45 trypanosomes on average), and 22 to 267 (89 trypanosomes on average) in *H. edwardsii* individuals, which is higher than Yeld and Smit's (2006) finding of 11 trypanosomes on average. As in Yeld and Smit (2006), all host size classes were infected, but in this study, prevalence was slightly lower at 91 % in *H. pictus* than the 100 % reported by Yeld and Smit (2006).

Table 3.4 Morphometrics of trypanosomes measured from the shark species examined and measurements provided by Morillas et al. (1987) and Pulsford (1984) for *Trypanosoma humboldti* Morillas, George-Nascimento, Valeria and Khan, 1987 and *Trypanosoma scylliumi* Laveran and Mesnil, 1902 respectively. Measurements have been rounded to the nearest whole number.

Species	***T. haploblephari* (morphotype B)**			***T. haploblephari* (morphotype A)**					***T. humboldti***		***T. scylliumi***	
Study	Present study			Present study			Yeld and Smit (2006)		Morillas et al. (1987)		Mesnil (1902)	
	N	Range	ML±SD	N	Range	ML±SD	Range	ML±SD	Range	ML±SD	ML (SF)	ML (LF)
MA	124	6–39	24 ± 8	147	12–60	28 ± 8	26–46	35 ± 4	22–30	26 ± 2	28	25
MP	124	8–69	28 ± 11	147	11–64	36 ± 10	–	–	47–64	55 ± 4	39	42
MK	124	8–52	24 ± 7	147	7–45	25 ± 7	–	19	31–46	37 ± 4	–	–
PK	124	0–24	7 ± 5	147	2–34	12 ± 6	7–46	17 ± 5	16–25	19 ± 2	9	10
NL	124	1–7	4 ± 1	147	2–8	5 ± 1	5–9	7 ± 1	5–6	5 ± 0	4	5
NI	124	0–3	1 ± 1	147	0–3	1 ± 0	–	–	2–3	2 ± 0	1	2
BW(N)	124	1–11	5 ± 2	147	4–20	11 ± 4	–	–	–	–	4	6
BW(UM)	79	2–16	7 ± 2	112	6–21	13 ± 3	13–24	17 ± 3	4–10	7 ± 2	6	10
TBL	124	20–93	52 ± 14	147	27–112	65 ± 16	54–99	70 ± 9	78–93	87 ± 4	54	59
FL	44	0–19	6 ± 4	126	–	–	–	–	5–11	7 ± 2	14	12

N, number; SD, standard deviation; ML, mean length; MA, mid-nucleus to anterior region; MP, mid-nucleus to posterior region; MK, mid-nucleus to kinetoplast; PK, posterior region to kinetoplast; NL, nuclear length (MP/MA); NI, nuclear index, BW(N), body width at nucleus; BW(UM), body width with undulating membrane; TBL, total body length; FL, flagellum length; SF, small form; LF, large form. Morphotype A from *H. edwardsii* and *H. pictus*, morphotype B from *P. africanum* and *P. pantherinum.*

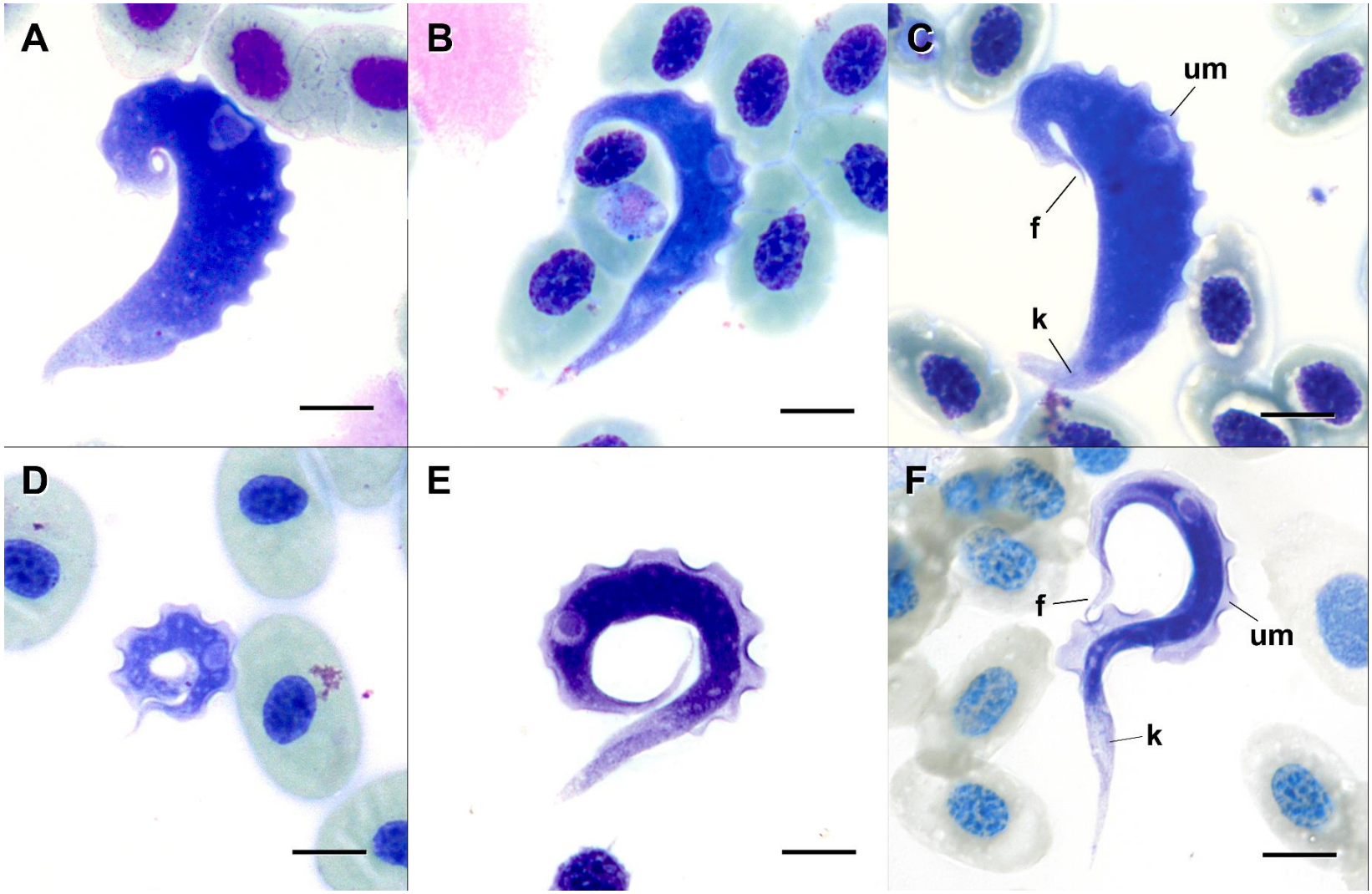

Figure 3.8 Micrographs of *Trypanosoma haploblephari* morphotype A (A–C) and *T. haploblephari* morphotype B (D–F) in Giemsa-stained blood films of *Haploblepharus pictus* and *Poroderma pantherinum*, respectively. Blood stage with kinetoplast (k) and undulating membrane (µm) visible (A–C); slender forms (B, E); presence of a flagellum (f) in deeply stained individuals (C, F). Scale bar: 10 µm

3.3.2.2 Material studied in *P. africanum* and *P. pantherinum* (morphotype B)

Type material: Parahapantotype: HE18-18, one blood film with 150 trypanosomes deposited in the National Museum, Bloemfontein, South Africa (accession number: NMB P 793).

Locality: Hermanus, Western Cape, South Africa (34°25'15.76"S, 19°14'37.56" E).

Site in host: Peripheral blood.

Prevalence: 100 % (14/14).

Representative DNA sequence(s): Partial sequence of the 18S rRNA gene; 1477 bp in length (GenBank accession number: MZ061641).

Diagnosis: A slender, blue-purple staining trypanosome (52.5 ± 14.1 in body length) with a short free flagellum (6.2). Body is slender (5.4 ± 2.0) and distinct kinetoplast is visible located on average 6.9 from the posterior region. Usually found curling in on itself in form of a doughnut (Fig. 3.9).

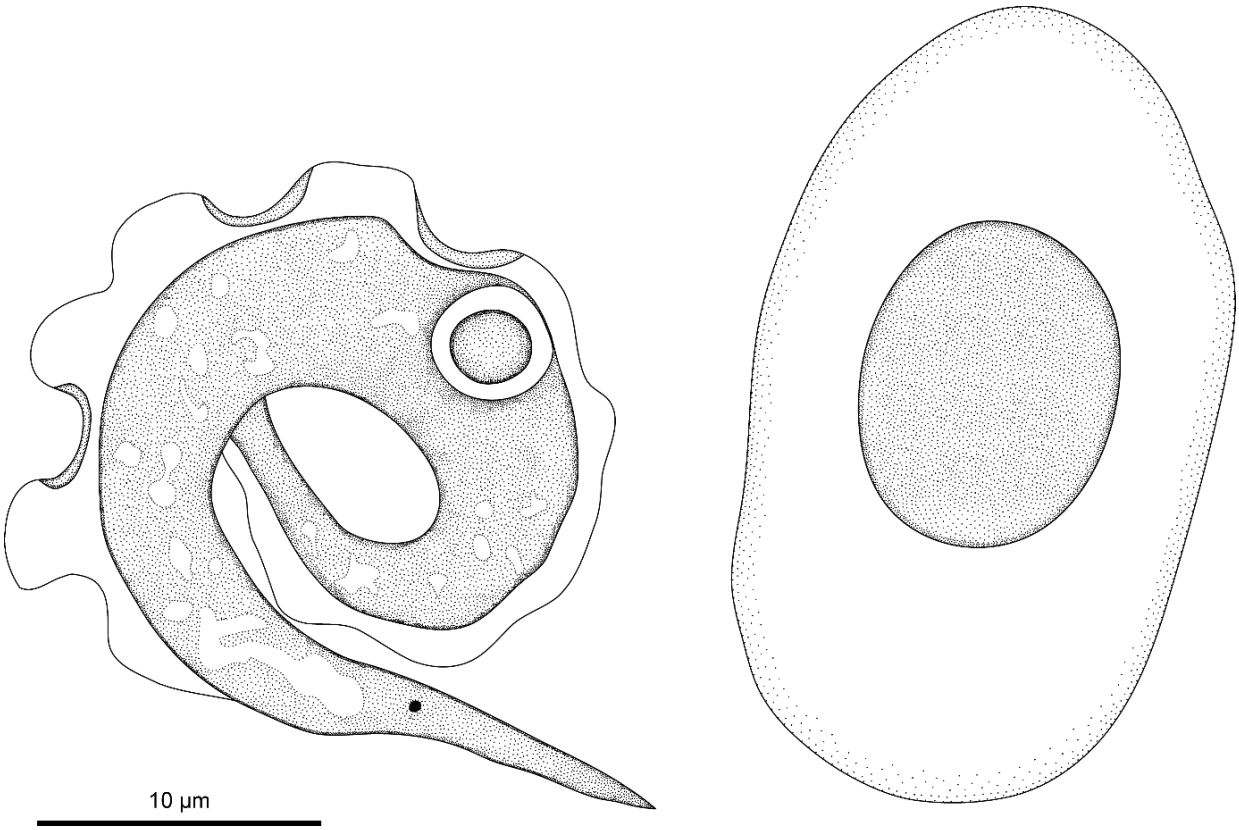

Figure 3.9 Line drawing of *Trypanosoma haploblephari* from the host *Poroderma pantherinum* (Slide HE18-18) next to a drawing of a red blood cell.

Description: Cytoplasm, kinetoplast and nucleolus are basophilic and stain deep blue-purple with numerous chromatic granules visible in the cytoplasm. The karyosome can be seen within the nucleus (Fig. 3.8 D, E) and a distinct kinetoplast, located close to the posterior part of the body, is visible; 6.9 ± 5.1 (0.1 – 23.6) from posterior region to kinetoplast or 13.3 % of the body length (Fig. 3.8 F). The undulating membrane is well developed with several undulations (Fig. 3.8 F) and a distinct, free flagellum is present; FL=6.2 ± 3.9 (0 – 19.3) (Fig. 3.8 F). In more deeply stained individuals, longitudinal striations are visible, often seen across the nucleus and anterior region. The location of the nucleus from the anterior end is 24.0 ± 7.7 (6.2 – 39.5, or 45.7 % of the body length, thus lies just anterior to the midpoint of the body for the majority of specimens examined, with a nuclear index of 1.2 ± 0.6 (0.3 – 3.0).

Remarks: Trypomastigote stages found in the blood of both *P. africanum* and *P. pantherinum* differ both in morphology and size to *T. haploblephari* morphotype A (Fig. 3.8) (Table 3.4). On average, morphotype B has a shorter body (52.5 ± 14.1) as compared to *T. haploblephari* morphotype A in both the present and Yeld and Smit's (2006) study (64.7 ± 16.2; 70.4 ± 9.4; respectively). A distinct, free flagellum (6.2 ± 3.9) is present as compared to morphotype A of *T. haploblephari* where the flagellum is longer and not easily seen in the present material (6.9). Furthermore, morphotype B is a slender trypanosome (5.4 ± 2.0), shown in both immature and mature stages, as compared to the characteristically wide form of *T. haploblephari* morphotype A [11.1 ± 3.7 (3.9 – 20.2)]. The undulating membrane is clearly visible in morphotype B with a body width including the undulating membrane measuring on average 6.8 ± 2.4 (2.3 – 15.9) (N=79), with the width of the undulating membrane approximately 1.4, in comparison to a body width of 13.5 (6.4 – 21.1) (N=111) for *T. haploblephari* morphotype A (present study). In the original description, measurements of the trypanosome including the undulating membrane were 17.4 ± 2.6 (12.6 – 24.3), with the width of the undulating membrane at between 1 and 4. The location of the nucleus of morphotype B is 24.02 from the anterior end, or 46 % of the body length, compared to 28.4 or 44 % of total body length of *T. haploblephari* morphotype A. The kinetoplast of morphotype B is located close to the posterior part of the body (PK=6.9 ± 5.1) or 13.1 % of the body length and 28.6 to the nucleus, or 54 % of the total body length, as compared to morphotype A at 11.9 (18.4 %) and 36.3 (56.1 %), respectively. Additionally, nuclear length of morphotype B is much shorter (3.9 ± 1.3) than that of morphotype A [6.5 in Yeld and Smit (2006), 5.3 in present study]. Morphotype B exhibits a unique characteristic by curling in on itself or appearing coiled, appearing almost circular in a doughnut-shape, with anterior and posterior ends situated closely together. This was not mentioned as a unique feature in either the immature or mature stages of *T. haploblephari* in the original description.

Yeld and Smit (2006) also observed dividing forms of *T. haploblephari* in the peripheral blood of *H. pictus* and *H. edwardsii*, however in this study, no dividing forms were observed in either *T. haploblephari* morphotype A or B. It was also proposed in the original description that *T. haploblephari* could be an endemic species, due to the restricted geographic distribution and high level of endemicity of the host species (Yeld and Smit 2006). Even though shark species collected from both localities showed trypanosome infections, trypanosomes infecting *H. pictus* and *H. edwardsii* always resembled morphotype A, with no stages resembling the morphotype isolated from *P. africanum* and *P. pantherinum.* Likewise, no trypanosome stages in *P. africanum* and *P. pantherinum* resembled morphotype A. Although pleomorphism is known to occur in species of *Trypanosoma*, this was not observed in *T. haploblephari*, even between the two species of hosts, *H. pictus* and *H. edwardsii*, infected with this trypanosome in the original description (Yeld and Smit 2006). As such, the now apparent pleomorphism may potentially be the result of the difference in host genus.

A trypanosome species *T. humboldti* was described in another catshark species, *S. chilensis*, off the Pacific coast of Chile (Morillas et al. 1987). Even though morphotype B in *P. africanum* and *P. pantherinum* does show 'C' and 'S' shaped forms when trypomastigotes are larger, as does *T. humboldti*, the latter is much larger (87 ± 3.8; including free flagellum) than that of *T. haploblephari* morphotype B (~57.9 including the free flagellum) (Table 4). Furthermore, *T. haploblephari* morphotype B does not conform with regards to nuclear position as compared to *T. humboldti* (NI= 1.2 vs. 2.1, respectively). Morphologically, especially with regards to the shape, *T. haploblephari* morphotype B conforms closely to *T. scylliumi*, found in the dogfish *Scyliorhinus canicula* (L.) and *Sc. stellaris* off Roscoff, France (Pulsford 1984). *Trypanosoma scylliumi* was later reported from *Sc. canicula* from British waters (Henry 1910; Coles 1914), and Pulsford (1984) provided additional measurements of this parasite from *Sc. canicula* from both Plymouth and the type locality Roscoff. Similarly, *T. scylliumi* and *T. haploblephari* morphotype B show trypanosome stages that are coiled when smaller or 'S' shaped when larger. *Trypanosoma haploblephari* morphotype B also conforms closely to *T. scylliumi* in length (52.5 ± 16.0; 54.1 – 58.6, respectively), nuclear length (3.9 ± 1.3; 3.7 – 5.0, respectively) and nuclear position (NI=1.2; 1.4 – 1.7 respectively). However, *T. haploblephari* morphotype B differs considerably in flagellum length (6.2 ± 3.9) as compared to that of *T. scylliumi* (12.0 – 13.5) (Table 3.4).

Furthermore, with regards to trypanosome infections, geographical proximity is often given priority (Khan 1977; Morillas et al. 1987). In this case, the distance between type localities of *T. haploblephari* and *T. scylliumi* are so distant that the potential of these species being

conspecific is considered extremely low. However, this should be investigated molecularly in future.

3.3.3 Molecular phylogeny

The sequences of *T. haploblephari* morphotype A isolated from *H. pictus* were approximately 800 nt long for both internal and external primers, respectively, and a consensus sequence was constructed of 1740 nt. Similarly, sequences of 800 nt were obtained for morphotype B from *P. pantherinum*, where three assembled sequences of the primer sets were used to construct a consensus sequence of 1477 nt. A consensus sequence of 1529 nt was constructed for morphotype B from *P. africanum* where sequences obtained from the internal and external primers consisted of 930 nt each. The alignment consisted of 26 trypanosome sequences (Table S1) with a final alignment length of 1582 nt. *Trypanosoma haploblephari* morphotype A and B showed a divergence of 0.5 % (p=0.005) and a 95 % similarity between isolates, with isolates from the two *Poroderma* species being more closely related with a divergence of 0.2% (p=0.002). *Trypanosoma haploblephari* morphotype A and B fall within the marine fish trypanosome clade (Fig. 3.10), showing a close relationship with *T. rajae* (p=0.01 – 0.04), with a divergence of 1 and 4 %, respectively (Table 3.5), described from various species of skates (*Raja*).

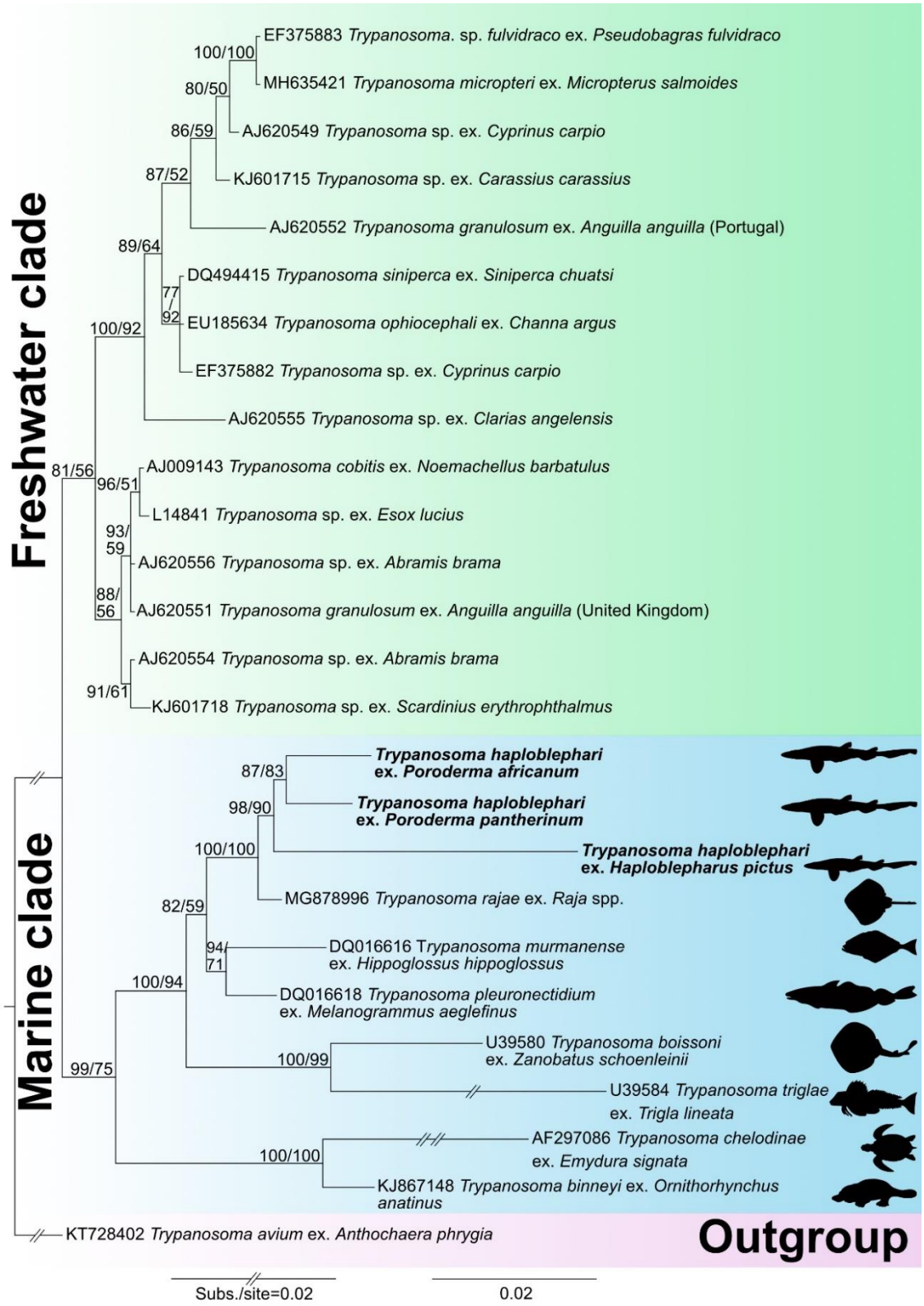

Figure 3.10 Bayesian Inference (BI) /Maximum Likelihood (ML) analysis showing the phylogenetic position of *Trypanosoma haploblephari* genotypes representing morphotypes A and B inferred from partial 18S rRNA gene sequences. Comparative sequences representing known *Trypanosoma* species, with *Trypanosoma avium* (KT728402) as outgroup, were obtained from GenBank. Tree topologies for both the BI and ML trees were identical; the nodal support values (BI/ML) are represented on the BI tree.

Table 3.5 Evolutionary differences of species of *Trypanosoma* Gruby, 1843 isolated from the 18S rRNA gene region of marine organisms included in the phylogenetic analysis presented in Fig. 3.9, expressed as percent similarity (%) (bottom left) and uncorrected pair-wise distance (p-distance) (top right).

	Accession number	*Trypanosoma* species	Host	1	2	3	4	5	6	7	8	9	10
1	MH635421	*Trypanosoma micropteri*	*Micropterus salmoides*		0.08	0.08	0.11	0.05	0.06	0.05	0.06	0.06	0.09
2	KT728402	*Trypanosoma avium*	*Anthochaera phrygia*	93		0.10	0.14	0.08	0.08	0.08	0.09	0.09	0.11
3	U39580	*Trypanosoma boissoni*	*Zanobatus schoenleinii*	94	92		0.07	0.05	0.05	0.05	0.06	0.06	0.08
4	U39584	*Trypanosoma triglae*	*Trigla lineata*	89	89	91		0.09	0.08	0.08	0.09	0.09	0.12
5	DQ016618	*Trypanosoma pleuronectidium*	*Melanogrammus aeglefinus*	96	93	95	90		0.02	0.02	0.03	0.03	0.05
6	DQ016616	*Trypanosoma murmanense*	*Hippoglossus hippoglossus*	95	93	95	90	98		0.02	0.03	0.03	0.06
7	MG878996	*Trypanosoma rajae*	*Raja* spp.	96	94	95	90	89	98		0.02	0.01	0.04
8	–	*Trypanosoma haploblephari*	*Poroderma africanum*	95	92	94	89	97	96	98		0.02	0.05
9	MZ061641	*Trypanosoma haploblephari*	*Poroderma pantherinum*	95	92	94	89	97	97	99	98		0.05
10	MZ061638	*Trypanosoma haploblephari*	*Haploblepharus pictus*	92	90	92	87	95	94	96	95	95	

3.4 DISCUSSION

To date, there is a lack of studies and knowledge on the trypanosomes of elasmobranchs, with only 12 reported species (Yeld and Smit 2006) compared to marine bony fishes of which at least 30 are known globally (Woo 1994). Most of the species infecting elasmobranchs have been recorded from skates and rays (Yeld and Smit 2006), while only five of the twelve species have been described from sharks. This paucity in knowledge of shark trypanosomes is particularly noticeable in elasmobranchs off the shores of southern Africa. Up until now, only a single species of *Trypanosoma* has been described infecting sharks from this region, *T. haploblephari*, which was described over a decade ago by Yeld and Smit (2006); this as compared to regions in the Mediterranean and Northern Atlantic from which four species have been described (Yeld and Smit 2006). Since the study by Yeld and Smit (2006), research on southern African elasmobranch trypanosomes has been neglected. Furthermore, this species was described solely on morphological characteristics. Given the minor morphological differences between trypanosome species and the tendency for pleomorphism in forms of a single species, morphological differentiation of species of trypanosomes can be challenging.

Yeld (2009) highlighted this with reference to *Trypanosoma gargantua* Laird, 1951, *T. giganteum* Neumann, 1909, *T. rajae* Laveran and Mesnil, 1902 and *T. murmanense* Nikitin, 1927, all well-known examples of species in which pleomorphism occurs. This, in the past, has produced a false representation of the true trypanosome biodiversity. Modern molecular techniques have proven useful in differentiating between closely related or even morphologically similar species (Borges et al. 2016; Davies et al. 2005). At the same time, these techniques have revealed the high levels of genetic diversity that can occur in a single species of trypanosome (Davies et al. 2005; Smit et al. 2020). Currently, most of the trypanosome diversity is described on morphometric data alone, particularly for elasmobranch trypanosomes (Laird 1951; Morillas et al. 1987; Yeld 2009). Furthermore, the set of morphometric characters used in trypanosome descriptions are not standardised leading to further challenges. Such challenges were experienced during the current study, but given the distinctive characteristics of *T. haploblephari* such as the species' body width, length and shape, as well as the collection of current samples from the type host and locality, current samples of *T. haploblephari* were easily identified and unequivocally assigned to this species.

During the current study, a trypanosome was found infecting *P. africanum* and *P. pantherinum* that was morphologically distinguishable from the original description of *T. haploblephari*, as well as to the specimens of *T. haploblephari* described from *H. pictus* and

H. edwardsii during the current study. Molecular data, however, indicated that the unknown trypanosome was closely related to *T. haploblephari* with a divergence of 0.5 %, well below the 3 % threshold considered necessary to differentiate between separate species (see Smit et al. 2020). Yeld and Smit (2006) described *T. haploblephari* as a trypanosome species in which pleomorphism does not appear to occur. However, the current study indicates that this is not the case, and that *T. haploblephari* demonstrates extreme pleomorphism, particularly between the two sympatric genera of catsharks. Additionally, the results showed a very low divergence between *T. pleuronectidium, T. murmanense* and *T. rajae*, all of which were described based on morphology alone (Laveran and Mesnil 1902; Robertson 1906; Nikitin 1927). With increasing efforts of trying to generate molecular data for species described on morphological characteristics, results may show that these species may turn out to be the same species with wide host and distribution ranges.

To date, the best-known example of extreme pleomorphism shown in a single species of marine trypanosome is *T. rajae* (Yeld and Smit 2006). Phylogenetic analysis places both genotypes of *T. haploblephari* in the same clade as *T. rajae*, with a divergence of less than 1 %, which strongly suggests that *T. haploblephari* and *T. rajae* are the same species. In the original description of *T. rajae*, authors collected this species off the coast of Roscoff, France, in two species of skates *Raja asterias* Delaroche (syn. *R. punctata* Risso) and *Raja undulata* Lacepède (syn. *R. mosaica* Lacepède) (Laveran and Mesnil, 1902). Both species of skates have an Eastern Atlantic distribution, from the Mediterranean to possibly the coasts off Mauritania and Senegal respectively (www.fishbase.org, Froese and Pauly 2020). Even though these two host species have not been reported off the coasts of southern Africa, they may overlap in distribution with species that do occur in this region. At least nine species of skate occurring off the coasts of southern Africa have a distribution range which spans to the Eastern Atlantic or may have a distribution which is bipolar in the former and latter regions (Compagno and Ebert 2009). As such, the potential for a multi-host species of trypanosome with an extensive distribution range cannot be excluded, and this may be the case with *T. rajae*.

As mentioned previously, geographical proximity is often given priority when attempting to differentiate between species, but this would not be applicable in a multi-host species with a wide distribution range. The *P. africanum* and *P. pantherinum* morphotype of *T. haploblephari* compared very closely to *T. scylliumi,* described from sharks of the same family (Scyliorhinidae) off the coasts of Roscoff. Given this and the possibility that *T. haploblephari* is a genotype of *T. rajae*, the extreme pleomorphism of the latter, and the same type locality of *T. rajae* and *T. scylliumi*, it calls into question whether *T. scylliumi* is yet

another morphotype of *T. rajae.* It also questions then the reliability of geographical proximity for differentiating between morphologically similar parasites. Unfortunately, the above remains hypothetical at present, as the study linked to both sequences of *T. rajae* (MG878996, MG878995) in GenBank has, as of yet, not been published, and as such, it is not possible to be certain that these sequences are in fact representative of *T. rajae* without the diagnosis that should accompany them.

Based on the morphological findings, it would be easy to describe the two morphotypes of *T. haploblephari* as separate species, as was done in the study by Sehgal et al. (2015) in which a new species of avian trypanosome was described based on distinct morphology, but from molecular findings is not a distinct species. This new species also showed an 18S sequence divergence of under 1 %, particularly when compared to other sympatric trypanosome species, which had been described from the same species of host. Attempts at the reconstruction of a phylogenetic tree were not possible by these authors as the sequence data lacked adequate variation. A similar issue was encountered during the study by Smit et al. (2020) on the freshwater fish trypanosome *Trypanosoma mukasai* Hoare, 1932. When molecularly characterising *T. mukasai* from various fish hosts of different genera and species as well as the probable leech vector, sequence variation was too low to allow for any definitive conclusions regarding the specific relationships between the taxa within these clades. According to these authors, this was accounted for by the close relationship of the sequences, all showing a divergence under the 3 % threshold, suggesting that they may not be separate species parasitising the different genera and species of host, but more likely a species of multi-host trypanosome which shows a high level of intraspecific genetic diversity. Similarly, an extensive molecular study on the trypanosome lineages of bats, did not differentiate between species or operational taxonomic units (OTU) when divergence was below 1 % (Clément et al. 2019).

As such, with our present knowledge on the trypanosomes of elasmobranchs, it would be best to be cautious and not describe the distinct morphotype of *T. haploblephari* as a new species. It is possible that both these morphotypes together with the probable *T. rajae* represent a single species with an extensive distribution range, such as the multi-host *T. mukasai,* which is considered to have a pan African distribution. As mentioned above *T. mukasai* demonstrates a high level of intraspecific genetic variation, with potentially emerging host-specific lineages (Davies et al. 2005; Smit et al. 2020). If the sequences included in the phylogenetic analysis of the current study do represent *T. rajae*, the current *T. haploblephari* genotypes could represent two of these host-specific lineages. Even though this cannot be determined at present without the diagnosis of these sequences as *T. rajae*,

this study does highlight the lack of molecular phylogenetic effort given to elasmobranch trypanosomes, and trypanosomes in general. Apart from *T. rajae*, only one other elasmobranch trypanosome has molecular data available, *T. boissoni* (U39580), isolated from *Zanobatus schoenleinii* off the coast of Senegal, this species showing an above threshold, but still low 3.8 % divergence from the *T. haploblephari* and *T. rajae* clade.

All *T. haploblephari* isolates from the current study, as well as the probable *T. rajae* fell within the marine fish *Trypanosoma* clade. *Trypanosoma binneyi* (KJ867148), described from a platypus, *Ornithorhynchus anatinus* Shaw as well as *T. chelodinae* (AF297086) from a turtle, *Emydura signata* Ahl, forms a subclade within the marine *Trypanosoma* clade. The same configuration was observed in other phylogenetic analyses including that of Hayes et al. (2014), Karlsbakk and Nylund (2006) as well as Gu et al. (2010). A possible reason for this occurrence could be due to insufficient taxon sampling and a lack of additional sequences of trypanosomes infecting other aquatic tetrapods. Only with additional survey efforts to characterise more trypanosome species infecting marine organisms as well as aquatic tetrapods on a molecular basis, can evolutionary histories be explained and more conclusive answers on the true phylogenies of aquatic trypanosomes be provided. *Trypanosoma haploblephari*, *T. rajae* and *T. boissoni* occupy a basal position, which could suggest that the trypanosomes from elasmobranchs are evolutionarily older than those parasitising other marine vertebrates.

It is difficult to determine if and to what degree trypanosome infections affect sharks, as there is no agreement in the literature on how to assess these impacts (Yeld 2009). In several species of amphibians, birds and reptiles, trypanosomes have been known to cause disease, however, in contrast it appears as if these parasites rarely cause any pathogenicity in fishes, especially marine cartilaginous or bony fishes (Pulsford 1984; Yeld 2009). Little information is known on the effect of trypanosomes on elasmobranchs, and it may be suggested that due to the long co-evolutionary time, the pathogenicity of trypanosomes seen in other vertebrate groups, might be absent in elasmobranchs. Parasitaemia in the blood of both *H. pictus* as well as *P. pantherinum* were notably high, a similar finding to that of Yeld and Smit (2006). It has been suggested that the high parasitaemia present in the blood could be attributed to the benthic-orientated and more sedentary behaviour of the shark hosts. This increases their exposure to marine leeches, the suggested vectors of these blood parasites. In contrast to other studies where trypanosomes were found infecting only hosts that are larger, and ultimately older (Aragort et al. 2005; Pulsford 1987), this study, along with that of Yeld and Smit (2006) found that sharks from all size classes were infected with trypanosomes. The infection rates of trypanosomes were high with an average of 43

trypanosomes per blood smear of *H. pictus,* 89 in *H. edwardsii*, 5 in *P. africanum* and 48 trypanosomes per blood smear of *P. pantherinum* in comparison to the low numbers reported by Aragort et al. (2005) (0 – 2), Pulsford (1984) (1 – 4) and Yeld and Smit (2006) (average of 11). It has also been suggested by Negm-Eldin (1998) that some trypanosomes might rather be vector-specific than vertebrate host-specific. This was concluded following the transmission experiments where the freshwater teleost infecting *T. mukasai* was successfully transmitted to eight different fish species using its vector *Batracobdelloides tricarinata* Blanchard. A similar finding was also observed for the marine teleost infecting *Trypanosoma cobitis* Mitrophanow 1883 and *T. murmanense*, both demonstrating a specificity to their vectors, *Hemiclepsis marginata* Müller and *Johanssonia arctica* Johansson, respectively (Negm-Eldin 1998). To date, life-cycle data of trypanosomes infecting marine fishes are scarce and studies on leeches infesting South African catsharks are entirely absent. As such, future work should include further research into identifying the leeches found on these sharks to species level and whether these invertebrates can act as vectors to these trypanosome species.

3.5 CONCLUSION

Many species of trypanosomes are known for their pleomorphism that, in the past, has created a false sense of their true biodiversity. This though appears to be a continuing dilemma. The current study draws attention to the need to be cautious in describing new trypanosome taxa based on new host and/or geographical distributions, as well as descriptions based on unique morphology or a combination of all these factors. This is particularly applicable to elasmobranch trypanosomes for which there is, at this time, too few molecular studies to begin to fully understand the phylogenetic relationships and taxonomy of this group of trypanosomes. More extensive sampling and molecular characterization of described species from elasmobranchs needs to occur before the degree of pleomorphism, as well as factors such as host-specificity, potential for mixed-infections, and distribution ranges can begin to be clearly understood. A further limit to unravelling the biodiversity and taxonomy of these parasites includes the use of one genetic marker, when likely it would be beneficial to apply multiple markers (Lemos et al. 2015; Clément et al. 2019; Smit et al. 2020). The above concerns may not only apply to elasmobranch or other aquatic species of trypanosome, such as those parasitising bony fishes, but to species of trypanosome in general.

Regardless, more effort needs to be placed in acquiring more data on trypanosomes from sharks, as until now there has been no sequence data on trypanosomes of sharks in general and from South Africa in particular. As South African waters present such a high diversity of

elasmobranchs, the potential of finding additional parasite species and revealing host-specific lineages of these is high, particularly with increased survey efforts. This study represents the first account on the molecular characterisation of trypanosomes parasitising sharks and the first screening of *P. pantherinum* for trypanosomes from South African waters.

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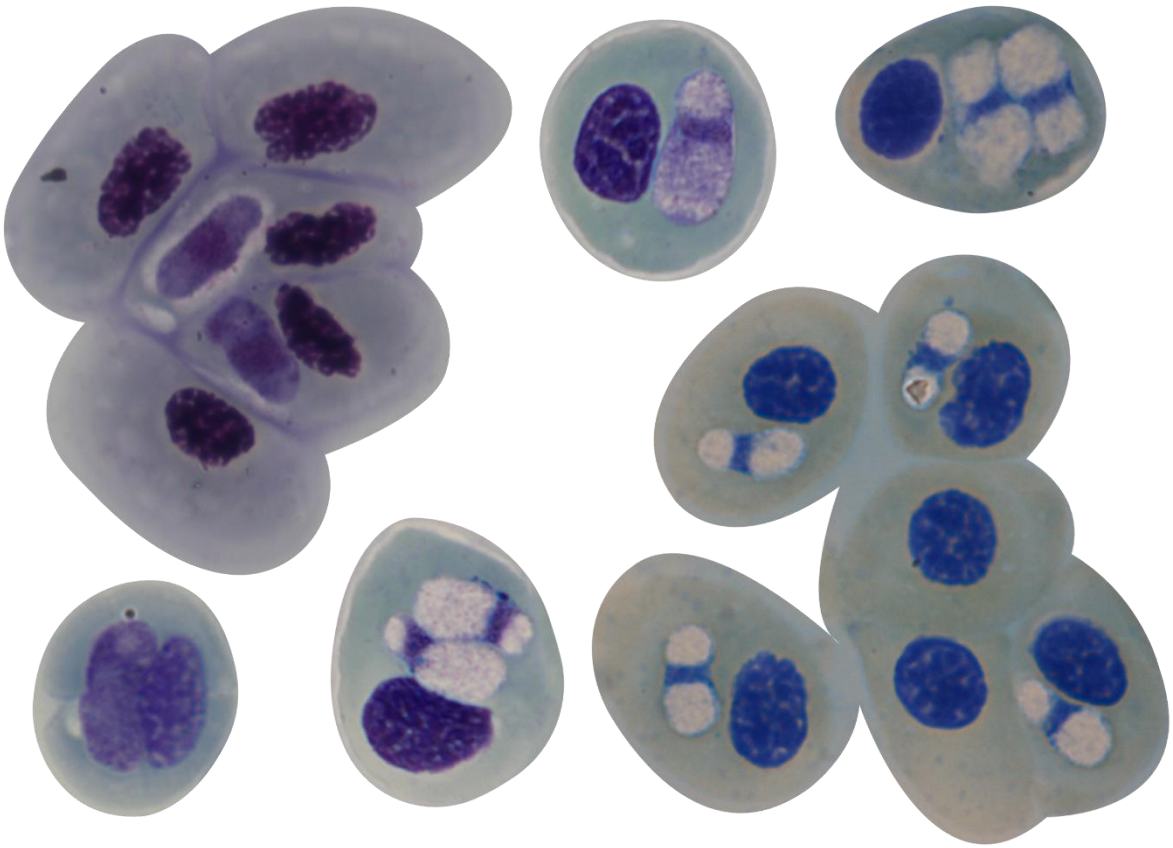

Chapter 4: Apicomplexans infecting elasmobranchs

4.1 INTRODUCTION

Blood parasitic apicomplexans (Apicomplexa: Adeleorina), are intracellular protozoans that can be found infecting almost every vertebrate and invertebrate class in both aquatic as well as terrestrial environments. As mentioned in Chapter 2, haemogregarines of the family Haemogregarinidae infecting fish are placed within *Haemogregarina* Danilewsky, 1885 and *Cyrilia* Lainson, 1981. In a review of the Haemogregarinidae by Siddall (1995) he transferred many of the members of *Haemogregarina* (*s.l.*) to a genus he named *Desseria* Siddall, 1995. Members of the genus *Desseria* are characterised as haemogregarines of fishes that do not undergo intraerythrocytic merogony (Siddall 1995). Genera of the family Dactylosomatidae also include fish parasitic species, these are *Dactylosoma* Labbé, 1894 and *Babesiosoma* Jakowska and Nigrelli, 1956. All these genera from both Haemogregarinidae and Dactylosomatidae can be found infecting marine vertebrate hosts, with the exception of *Cyrilia*, which to date has been identified from only freshwater hosts. The Dactylosomatidae typically infect both fish and anurans (Saunders 1960; Netherlands et al. 2020). This family was first erected by Jakowska and Nigrelli (1955) for haemogregarines from cold-blooded vertebrates where four to 16 merozoites are produced (Barta 1991). Being first described from amphibians (Barta 1991; Netherlands et al. 2020), members within this family have been poorly understood until life cycles were elucidated for some species (Negm-Eldin 1998; Barta and Desser 1989) and Kruse (1890) who showed that *Dactylosoma ranarum* (Kruse, 1890) possesses typical apicomplexan structures, including a complete conoid (Netherlands et al. 2020). The two genera *Dactylosoma* and *Babesiosoma* can be distinguished in several ways by morphological characteristics, including all stages showing a less granulated but more vacuolated cytoplasm, with a nucleus similar to those parasites in the genus *Babesia* Starcovivi, 1893, without a definite karyosome. Additionally, they reproduce by schizogony or binary fission and do not produce more than four merozoites (Netherlands et al. 2020). Currently, there are only two species of fish dactylosomes, *Dactylosoma lethrinorum* Saunders, 1960, and *Dactylosoma salvelini* Fantham, Porter and Richardson, 1942, and three species of fish babesiosomes, *Babesiosoma bettencourti* (França, 1908), *Babesiosoma mariae* (Hoare, 1930) and *Babesiosoma tetragonis* Becker and Katz, 1965 (Netherlands et al. 2020; Smit et al. 2003).

The status of whether fish haemogregarines should be placed within the genus *Haemogregarina* has been long disputed, especially following the finding of Davies and Smit

(2001) where it was demonstrated that *Haemogregarina bigemina* Laveran and Mesnil, 1901 is most likely transmitted to marine fish hosts via the parasitic gnathiid *Gnathia africana* Barnard, 1914, instead of the characteristic leech vectors transmitting species of *Haemogregarina* (Davies et al. 2004; Davies and Smit 2001; Smit and Davies 2006; Hayes and Smit 2019). Following that study, Hayes and Smit (2019) reported on the first molecular characterisation of a fish haemogregarine where their sequences of *H. bigemina* formed a marine clade along with undescribed species collected from the blood of *Stegastes* spp. and *Ophioblennius macclurei* (Silvester) from the Caribbean (Hayes and Smit 2019; Renoux et al. 2017; Sikkel et al. 2018). Only six species of haemogregarines have been described infecting elasmobranchs worldwide (Table 4.1), and these have been placed in two genera including *Haemogregarina* (*s.l.*) and *Cyrilia* from a freshwater elasmobranch host *Potamotrygon histrix* (Müller and Henle, 1839) (Magro et al. 2016; Oliviera et al. 2017). Knowledge of haemogregarines infecting elasmobranchs of South Africa is almost entirely absent, except for the work done by Yeld (2009) in an unpublished thesis. In her thesis, Yeld (2009) provided morphological descriptions of what she considered as two species of *Haemogregarina* infecting scyliorhinids off the South African coast, however no formal descriptions of these species were published as no molecular data were available at that time. The aim of this chapter is thus to molecularly characterise the two species of *Haemogregarina* which Yeld (2009) reported on, contributing to the diversity knowledge of elasmobranch haemogregarines, and determining their phylogenetic placement among other haemogregarines.

Table 4.1 Haemogregarine species described from elasmobranchs worldwide.

Haemogregarine species	Host (s)	Locality	Gamonts (Length x width)	Nucleus (Length x width)	Reference (s)
Cyrilia Lainson, 1981 sp.	*Potamotrygon motoro*, *P. hystrix*, *P. wallacei*	Brazil	Macro-gamonts: 15.8 ± 1.6 (11.9–21.0) x 5.8 ± 0.7 (4.2–7.8) / Micro-gamonts: 13.3 ± 1.6 (13.2–23.9) x 4.6 ± 1.0 (6.3–17.4)	Macro-gamonts: 4.3 ± 0.8 (2.9–7.0) x 5.3 ± 0.8 (3.2–6.9) / Micro-gamonts: 4.4 ± 0.8 (5.1–11.4) x 4.3 ± 0.9 (4.3–6.5)	Magro et al. (2016); Oliveira et al. (2017); Yeld (2009)
Haemogregarina dasyatis Saunders, 1958	*Hypanus americanus* (syn. *Dasyatis americana)*	Bahamas	Mature gametocyte: 13.0 x 3.8	Mature gametocyte: 3.6 x 3.6	Saunders (1958)
Haemogregarina heterodonti von Prowazek, 1910	*Heterodontus japonicus*	N/A	N/A	N/A	Von Prowazek (1910); Yeld (2009)
Haemogregarina torpedinis Neumann, 1909	*Torpedo ocellate, T. marmorata*	N/A	Macro-gametocytes: 18 x 4.5 Micro-gametocytes: 16 x 1.5	N/A	Neumann (1909)

Table 4.1 Continued.

Haemogregarine species	Host (s)	Locality	Gamonts (Length x width)	Nucleus (Length x width)	Reference (s)
Haemogregarina carchariasi Laveran, 1908	*Carcharias* sp.	Australia	20–27 x 7–10	N/A	Laveran (1908); Yeld (2009)
Haemogregarina hemiscyllii Mackerras & Mackerras, 1961	*Hemiscyllium ocellatum*	Australia	16–19 x 5–8	N/A	Mackerras and Mackerras (1961)
Haemogregarina delagei Laveran & Mesnil, 1912	*Raja punctata, Pavoraja mosaica, Leucoraja erinacea* (syn. *R. erinacea*), *Amblyraja radiata* (syn. *R. radiata*), *Malacoraja senta* (syn. *R. senta*), *Squalus acanthias*	North America	(6.3–13.7) x (1.6–3.6); (10.8–15.2) x (4.3–6.6)	(3.0 – 4.3) x (3.2 – 5.3)	Laveran and Mesnil (1902); Becker and Overstreet (1979)

Table 4.1 Continued.

Haemogregarine species	Host (s)	Locality	Gamonts (Length x width)	Nucleus (Length x width)	Reference (s)
Haemogregarina sp. A	*Haploblepharus pictus*, *Haploblepharus edwardsii*, *Poroderma africanum*	South Africa	13.94 ± 0.61 (12.72–15.18) x 7.47 ± 0.58 (6.57–8.63)	3.75 ± 0.48 (2.72–4.90) x 5.99 ± 0.50 (5.08–7.42)	Yeld (2009)**
Haemogregarina sp. B	*Haploblepharus pictus*, *Haploblepharus edwardsii*, *Poroderma africanum*	South Africa	14.15 ± 0.79 (12.24–15.62) x 8.00 ± 0.69 (4.39–6.84)	5.67 ± 0.69 (4.39–6.84) x 5.57 ± 0.62 (4.55–7.38)	Yeld (2009)**
Dactylosoma sp. A	*Haploblepharus pictus*, *Haploblepharus edwardsii*, *Poroderma africanum*, *Poroderma pantherinum*	South Africa	13.27 ± 1.23 (10.96–17.23) x 7.25 ± 1.07 (4.45–10.46)	2.39 ± 0.60 (1.27–4.34) x 5.51 ± 0.97 (3.18–7.81)	This chapter
Dactylosoma sp. B	*Haploblepharus pictus*, *Haploblepharus edwardsii*, *Poroderma africanum*, *Poroderma pantherinum*	South Africa	13.72 ± 0.95 (11.24–15.73) x 5.73 ± 0.84 (4.08–8.10)	4.43 ± 1.10 (2.22–6.70) x 4.12 ± 0.94 (2.45–5.83)	This chapter

**reported, but not formally described (in an unpublished thesis).

4.2 MATERIALS AND METHODS

4.2.1 Measurements and screening

Sharks were collected and their blood protozoans were screened and measured as described in Chapter 3. Additionally, slides were screened and the parasitaemia quantified for all morphotypes. Measurements are given in µm unless otherwise stated, and include length (L), width (W), anterior region to midnucleus (MA), posterior region to midnucleus (MP), nuclear length (NL) and nuclear width (NW) (Fig. 4.1). It is important to note that some shark blood samples contained only morphotype A, while all the samples with morphotype B also had morphotype A present. Upon selection of samples to be used for molecular analysis, shark blood samples that contained only morphotype A were used for the molecular typing of morphotype A and sharks with a ratio of 70:30 % for morphotype B, were selected for the molecular typing of morphotype B.

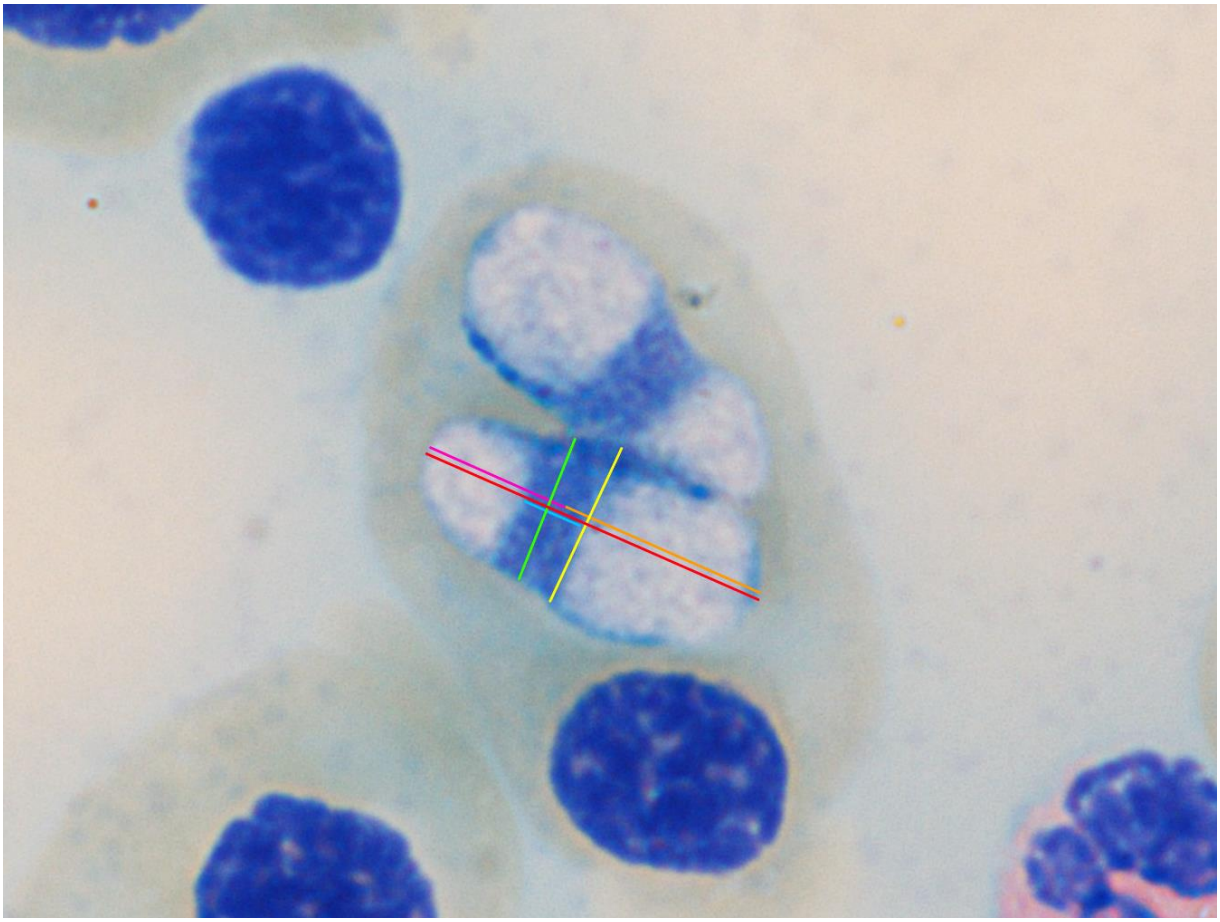

Figure 4.1 Example of measurements taken including length (L) (red line), width (W) (yellow line), anterior region to midnucleus (MA) (orange line), posterior region to midnucleus (MP) (pink line), nuclear length (NL) (blue line) and nuclear width (NW) (green line).

4.2.2 Molecular and phylogenetic analysis

The following methods are specific to this chapter, in addition to the methods discussed in Chapter 3. Extracted DNA was used as a template for PCR using 18S rRNA apicomplexan-specific primers 4558 (5′-GCTAATACATGAGCAAAATCTCAA-3′) (Mathew et al. 2000) and HepR900 (5′-CAAATCTAAGAATTTCACCTCTGAC-3′) (Ujvari et al. 2004). The PCR protocol is as follows: initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30s, 55.3°C for 30 sec, 72°C for 1 min and a final extension step of 72°C for 10 min.

Upon assessment of resultant sequences (Chapter 3), that is any positive PCR products were sequenced in both directions for the primer pairs as in Chapter 3, visualising and manually editing these in Geneious ver. 11.1.4, thereby producing consensus sequences. A total of four parasites were sequenced, including two consensus sequences generated from *Ha. pictus,* one from *P. africanum* and one from *P. pantherinum.* These sequences were identified using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/) and 54 comparative sequences were downloaded from the NCBI GenBank database for comparative analyses. Sequences were aligned using the MUSCLE alignment tool available on Geneious Ver. 11.1.4 with *Klossia helicina* (HQ224955), *Adelina grylli* (DQ096836) and *Adelina bambaroonidae* (AF494059) as outgroups following Cook et al. (2016). A model test was performed to determine the most suitable nucleotide substitution model, according to the Akaike information criterion using jModelTest 2.1.4 (Guindon and Gascuel 2003; Darriba et al. 2012). The best model for the alignment was the general time-reversible model incorporating invariant sites and gamma distributed among site-rate variations (GTR+I+G). A Bayesian Inference tree was constructed using MrBayes software (ver. 3.2.6) (Ronquist et al. 2012) run on the CIPRES portal (Miller et al. 2010). Markov chain Monte Carlo (MCMC) chains were run for 10,000,000 generations, log-likelihood scores plotted, and only the final 75% of trees were used to produce consensus trees by setting the ‘burn in’ parameter at 2500. A maximum likelihood tree was constructed in Geneious ver. 11.1.4 using the PhyML parameter with a four category Gamma distribution. Phylogenetic trees were visualised using FigTree v. 1.4.4 software (Rambaut 2012), the p-distance and number of base pair differences was calculated using Geneious ver. 11.1.4.

Even though the COI marker is ideal for studying recent phylogenetic events due to its rapid evolution in comparison to the slowly evolving 18S rRNA nuclear marker and would have aided in differentiating whether or not the two morphotypes represented distinct species, COI markers in protozoan research are still fairly new and understudied; 18S rRNA still being the most widely used gene region for studying protozoans (Hili et al. 2021). Based on a

GenBank search for COI and 18S rRNA sequences for apicomplexans, it was revealed that there were only 10 307 COI sequences in comparison to almost 21 000 18S rRNA sequences. This further emphasizes the point made in Chapter 3 that more gene markers are needed in order to fully understand the phylogeny of blood protozoans, including the apicomplexans. For this study, we used the 18S rRNA gene marker due to the large number of sequences available for comparison.

4.3 RESULTS

4.3.1 General observations of haemogregarines in the blood of sharks

Of the 98 individuals of four species examined, 85 (87%) were infected with the unnamed species of haemogregarines from Yeld (2009). Table 4.2 provides the metrical information, prevalence, and average number of haemogregarines per blood smear. Individuals were found to be parasitised by two morphologically distinguishable morphotypes of haemogregarines. Both morphotypes A and B were observed in *Haploblepharus edwardsii* (Schinz) and *Haploblepharus pictus* (Müller and Henle) with a prevalence of 100% and 91% for morphotype A and 38% and 53% for morphotype B, respectively. Only morphotype A was observed in *Poroderma africanum* (Gmelin) and *Poroderma pantherinum* (Müller and Henle) with a prevalence of 88% and 57%, respectively. The morphometrics of both morphotypes as well as the morphotypes reported in Yeld (2009) are included in Table 4.1 along with the morphometrics of other haemogregarines infecting elasmobranchs worldwide. The majority of shark blood samples contained only intraerythrocytic mature gamonts, with only a few individuals having infections where trophozoites and meronts were also present.

Table 4.2 Information on elasmobranch hosts, including prevalence of haemogregarines.

Sharks			Haemogregarines			
Species	**N**	**ML ± SD (range) in mm**	**Prevalence**		**Average***	
			Morphotype A	**Morphotype B**	**Morphotype A**	**Morphotype B**
Haploblepharus edwardsii	13	421.1 ± 34.6 (354–467) (N=9)	100% (13/13)	38% (5/13)	44	15
Haploblepharus pictus	47	435.6 ± 101.6 (260–614)	91 % (43/47)	53% (25/47)	21	10
Poroderma africanum	24	767.1 ± 150.9 (501–1010)	88% (21/24)	0% (0/0)	25	0
Poroderma pantherinum	14	511.4 ± 95.5 (363–725)	57% (8/14)	0% (0/0)	6	0

N, number; ML, mean length; SD, standard deviation. *Average prevalence per 500 erythrocytes.

4.3.2 Molecular characterisation and phylogenetic analysis

In order to elucidate the identity of the unnamed haemogregarines present, the molecular results are presented here first. Four consensus sequences of approximately 750 nt were constructed using the sequences obtained from each primer. When constructing the consensus sequences, the raw sequences were aligned for each primer (sequenced in both directions) and manually edited in Geneious ver. 11.1.4 for each isolate retrieved from the different host species and/or individual (section 4.2.2. above). The alignment consisted of 31 sequences with a final alignment length of 700 nt, and a list of the species used in the phylogenetic analysis are given in Table S2. Both haemogregarine morphotypes fall within the Dactylosomatidae clade (Fig. 4.2), forming a sister taxon to *Dactylosoma ranarum, Dactylosoma kermiti* Netherlands, Cook and Smit, 2020 and a *Dactylosoma* sp., all described from amphibian hosts, identifying the unknown haemogregarines from the present study as a *Dactylosoma* species*. Dactylosoma* morphotype A and *Dactylosoma* morphotype B show a divergence of only 1% (p=0.01) (Table 4.3), with only 6–8 nt differences in the phylogenetic alignment. As observed by Netherlands et al. (2020), several other species of haemogregarines have also shown a very low divergence. These include *D. ranarum* and *D. kermiti* showing a divergence of only 0.2–0.5 %, *Hepatozoon* cf. *clamatae*, *Hepatozoon chinensis* Han, Wu, Dong, Zhu, Li, Zhao, Wu, Pei, Wang, Huang, 2015 and *Hemolivia parvula* (Dias, 1953) all showing a divergence of only 0.3 %, while *Hemolivia mauritanica* (Sergent and Sergent, 1904) had a divergence of 0.7 %. With such a low divergence between described species of *Dactylosoma*, as well as between species of other haemogregarine genera, additionally also between some genera for instance *Hemolivia* and *Karyolysus* with a divergence of 3 % (Table 4.3) it can be easy to consider *Dactylosoma* morphotype A and B as separate species. In this case, it is best to be cautious in separating these morphotypes as different species when considering the widely accepted 3 % divergence rule between protist species. As such morphotype A and B will be considered a single species throughout the rest of this dissertation.

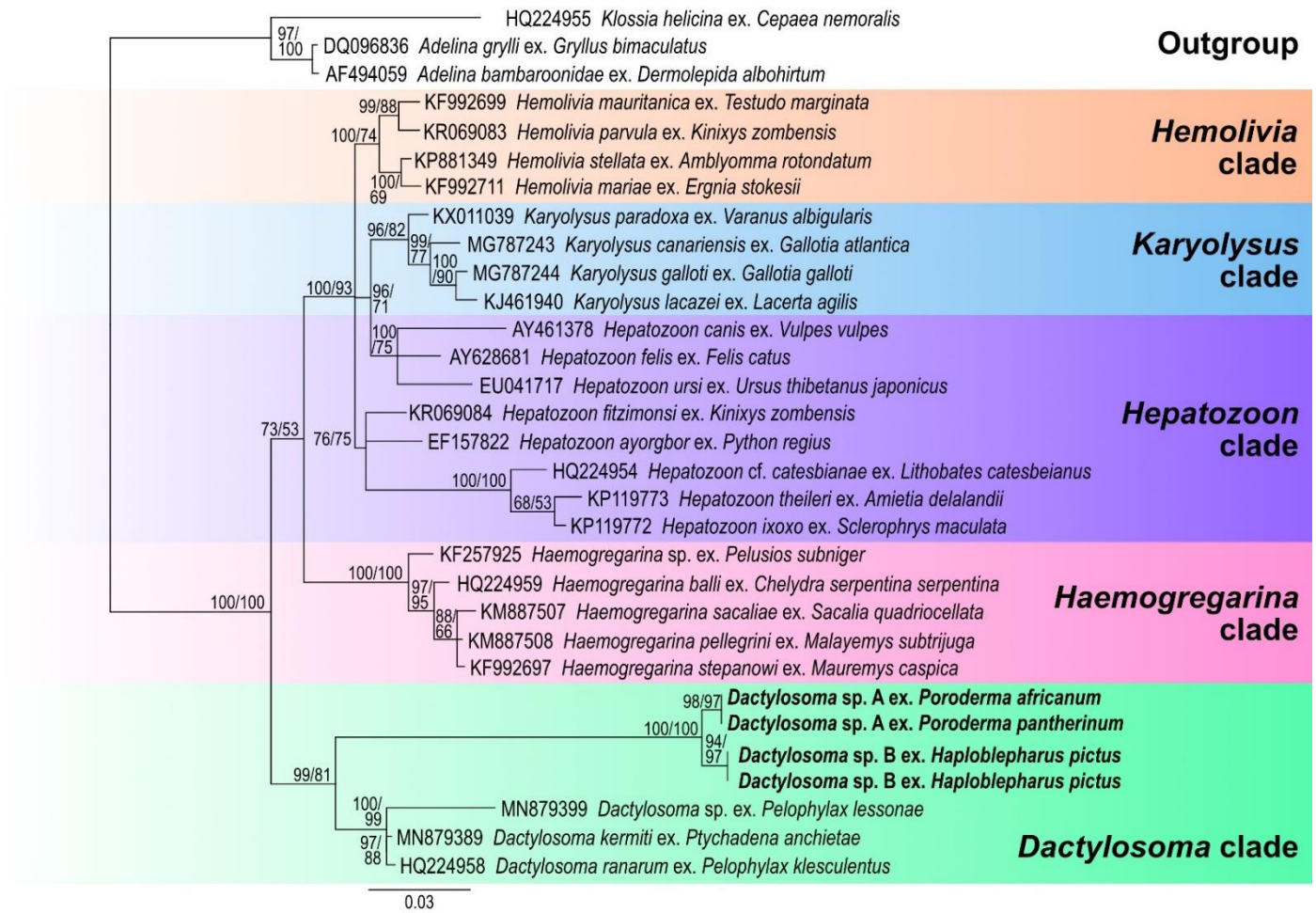

Figure 4.2 Bayesian Inference (BI)/Maximum Likelihood (ML) analysis showing the phylogenetic position of *Dactylosoma* species A and *Dactylosoma* species B inferred from partial 18S rRNA gene sequences. Tree topologies for both analyses were similar and are represented on the ML tree. Comparative sequences representing known haemogregarine species, with *Klossia helicina* (HQ224955), *Adelina grylli* (DQ096836) and *Adelina bambaroonidae* (AF494059) as outgroup, were obtained from GenBank. The scale bar represents 0.03 nucleotide substitutions per site.

Table 4.3 Evolutionary differences of various haemogregarine species isolated from the 18S rRNA gene region of organisms included in the phylogenetic analysis presented in Fig. 4.2, expressed as percent similarity (%) (bottom left) and uncorrected pair-wise distance (p-distance) (top right).

	Accession number	Haemogregarine species	Host	1	2	3	4	5	6	7	8	9	10	11	12
1	HQ224955	*Klossia helicina*	*Cepaea nemoralis*		0.10	0.10	0.10	0.11	0.10	0.11	0.10	0.16	0.16	0.16	0.16
2	KR069083	*Hemolivia parvula*	*Kinixys zombensis*	87		0.03	0.03	0.05	0.05	0.05	0.05	0.10	0.10	0.10	0.10
3	KX011039	*Karyolysus paradoxa*	*Varanus albigularis*	86	95		0.03	0.05	0.05	0.05	0.05	0.11	0.11	0.11	0.11
4	KR069084	*Hepatozoon fitzsimonsi*	*Kinixys zombensis*	87	96	94		0.05	0.05	0.06	0.06	0.10	0.10	0.10	0.10
5	KM8875017	*Haemogregarina sacaliae*	*Sacalia quadriocellata*	84	91	94	91		0.06	0.06	0.06	0.10	0.10	0.10	0.10
6	MN879399	*Dactylosoma* sp.	*Pelophylax lessonae*	81	87	90	87	86		0.00	0.01	0.09	0.09	0.09	0.09
7	HQ224958	*Dactylosoma ranarum*	*Rana esculenta*	86	93	92	93	90	91		0.01	0.09	0.09	0.10	0.09
8	MN879389	*Dactylosoma kermiti*	*Ptychadena anchietae*	86	93	92	93	91	91	99		0.09	0.09	0.09	0.09
9	XXX (isolate 354)	*Dactylosoma* morphotype A	*Poroderma pantherinum*	82	89	86	89	86	84	89	89		0.00	0.00	0.01
10	XXX (isolate 355)	*Dactylosoma* morphotype A	*Poroderma africanum*	82	89	86	89	86	84	89	89	100		0.00	0.01
11	XXX (isolate 409)	*Dactylosoma* morphotype B	*Haploblepharus pictus*	81	88	86	89	87	83	89	89	99	99		0.00
12	XXX (isolate 410)	*Dactylosoma* morphotype B	*Haploblepharus pictus*	81	88	87	89	87	83	89	89	99	99	100	

4.3.3 Description and diagnosis of blood stages

Apicomplexa Levine, 1970
Conoidasida Levine, 1988
Coccidiasina Leuckhart, 1879
Eucoccidiorida Léger, 1911
Adeleina Léger, 1911
Dactylosomatidae (Jakowska and Nigrelli, 1955) Becker, 1970
Dactylosoma Labbé, 1894

Restricted synonymy: Labbé, 1894: 100; Nöller, 1913: 169–240; Jakowska and Nigrelli, 1956: 8; Barta, 1991: 1–37; Netherlands et al. 2020: 246–260.

Type species: *Dactylosoma ranarum* (Kruse, 1890) syn*. Dactylosoma splendes* Labbé 1894

Diagnosis of the genus *Dactylosoma*:

Elongated, finger form-like hemocytozoa and short, ameboid forms with periodic pseudopodia. Protoplasm with hyaline, or translucent-like appearance which shows an areolar structure and vesicular nucleus after staining. No pigment, but presence of retractile granules. Minimal effect on host erythrocyte and nucleus. Merogony that presents 6–12 sporozoites, arranged in a fan-like or rosette-like structure.

4.3.3.1 *Dactylosoma* morphotype A

Restricted synonymy: *Haemogregarina* sp. A Yeld, 2009: 73–75, figs. 5.1, 5.2, 5.4.

Hosts from Yeld (2009): *Haploblepharus pictus* (Müller and Henle); *Haploblepharus edwardsii* (Schinz); *Poroderma africanum* (Gmelin) (Chondrichthyes: Scyliorhinidae).

Locality from Yeld (2009): Granger Bay, Western Cape, South Africa (33° 52’ S 18° 24’ E); Saldanha Bay (33º 02’ S 18º 02’ E); False Bay (34º 14’ S 18º 28’ E); De Hoop Nature Reserve (34º 28’ S 20º 30’ E).

Material studied:

Host(s): *Haploblepharus edwardsii* (Schinz); *Haploblepharus pictus* (Müller and Henle); *Poroderma africanum* (Gmelin); *Poroderma pantherinum* (Müller and Henle) (Chondrichthyes: Scyliorhinidae).

Locality: Granger Bay, Cape Town (33° 54' 2.31'' S, 18° 24' 56.38'' E) and Hermanus (34° 25' 15.76' 'S, 19° 14' 37.56'' E), Western Cape, South Africa.

Site in host: Peripheral blood.

Prevalence: 100% (13/13) in *Ha. edwardsii*; 91% (43/47) in *Ha. pictus*; 88% (21/24) in *P. africanum* and 57% (8/14) in *P. pantherinum*.

Vector: Unknown. Possibly leech found on sharks preliminarily identified as *Pontobdella* sp. (Prof. E. Burreson, Virginia Institute of Marine Science, USA; pers. comm.).

Representative DNA sequence (s): Two partial sequences of the 18S rRNA gene; both 750 nt in length, respectively (GenBank accession numbers: XXXX).

Diagnosis:

Gamonts large and round, nucleus towards posterior end and cytoplasm stains very lightly, with foamy or bubbly-like appearance.

Description:

Trophozoites (Fig. 4.3 A; Fig. 4.4 A–B) round and broad in shape, measuring 7.52 ± 1.44 (6.01–12.51) x 4.08 ± 0.57 (3.23–5.61) (N=17), a similar finding to Yeld (2009) (see Table 4.4). Usually found with a round anterior shape and slightly tapered at the posterior end. Nucleus stains a dark purple with Giemsa stain, elongated in shape, densely packed and located closest to the anterior end [MA: 3.13 ± 1.23 (1.33–7.35); MP: 4.38 ± 0.73 (3.30–5.82) (N=17) – this study; MA: 3.95 ± 0.75 (3.09–5.22) x 5.29 ± 1.02 (3.42–7.1) – Yeld (2009)]. Cytoplasm stains very light and a number of granules are visible, appearing almost foamy or bubbly-like.

Meronts (Fig. 4.3 B; Fig. 4.4 C–D) larger and rounded in shape measuring 9.32 ± 1.25 (7.24–12.52) x 5.31 ± 1.46 (3.13–11.11) (N=77). Both ends are rounded, and the nucleus has a slight bias towards the posterior end [MA: 5.73 ± 1.12 (3.78–9.79); MP: 3.59 ± 0.80 (1.64–5.23) (N=77)]. Nucleus is densely packed, staining a deep purple and is more elongated than round in shape [NL: 1.94 ± 0.69 (1.05–4.19); NW: 4.32 ± 1.09 (2.86–8.37) (N=77)]. Cytoplasm stains very lightly and bubble-like appearance seen in trophozoites also visible here.

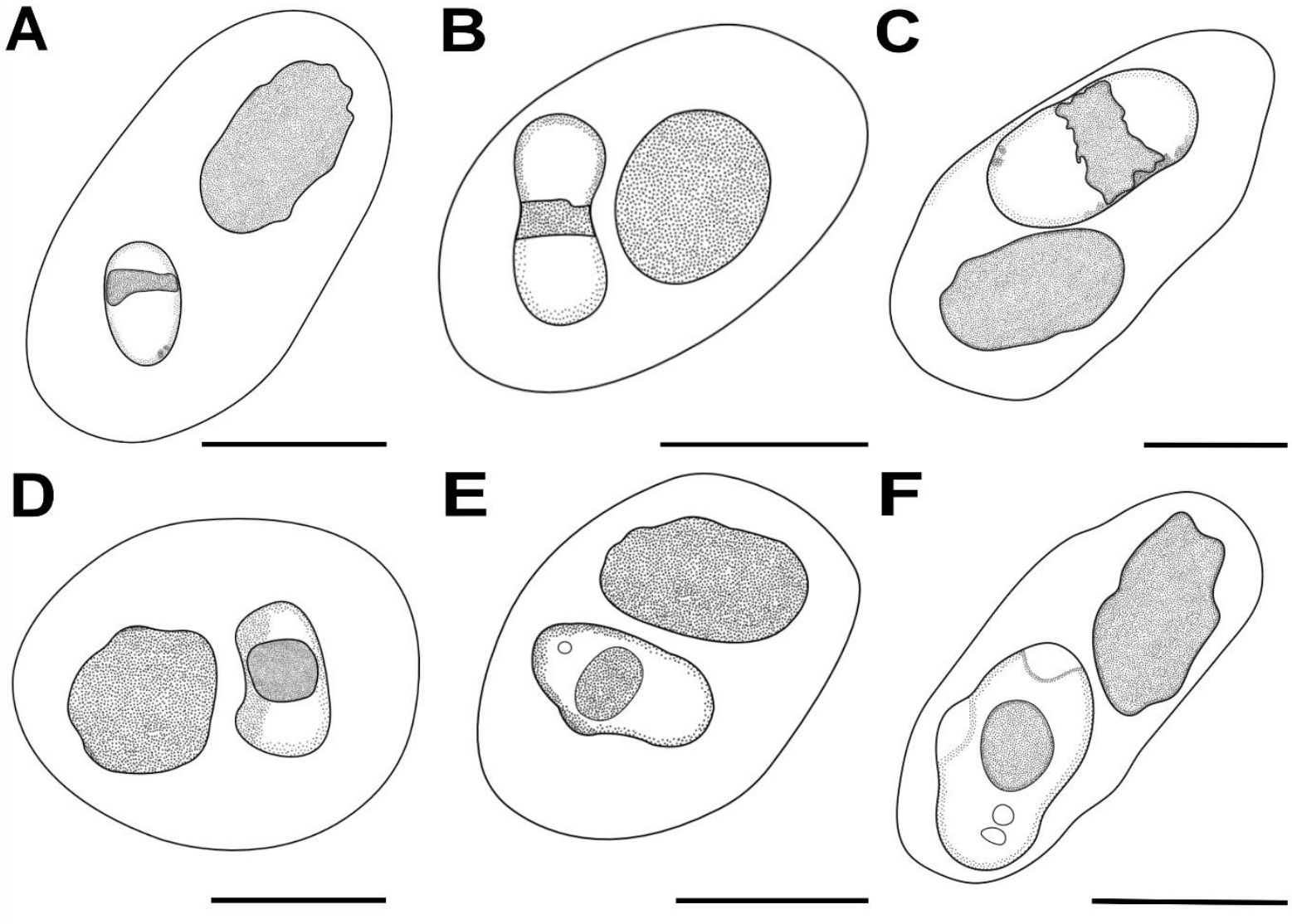

Figure 4.3 Line drawings of *Dactylosoma* morphotype A showing a trophozoite (A), meront (B) and mature gamont (C). Line drawings of *Dactylosoma* morphotype B showing a trophozoite (D), immature gamont (E), and mature gamont (F). Scale bar: 10µm

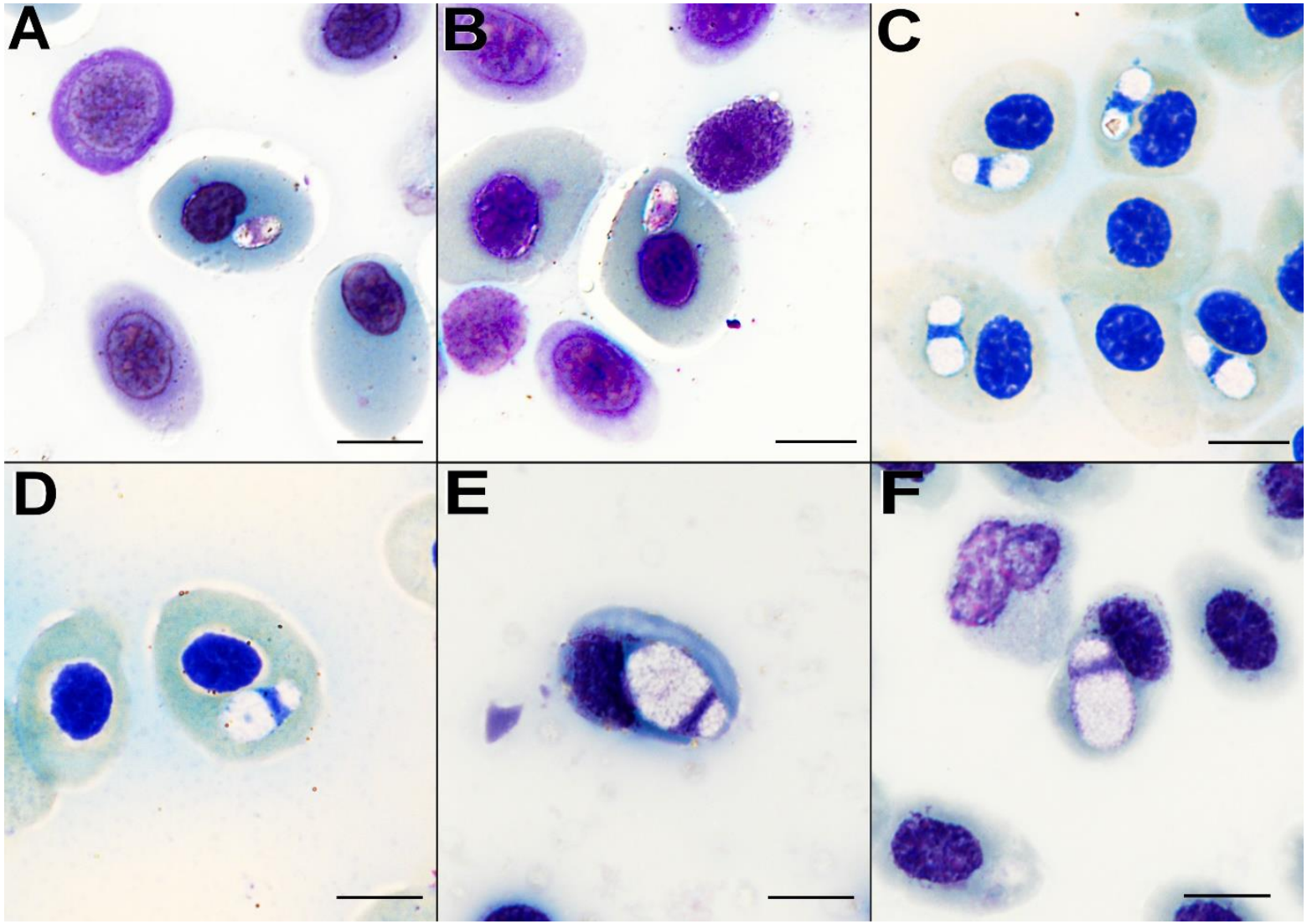

Figure 4.4 Micrographs of *Dactylosoma* morphotype A in Giemsa-stained blood films of *Poroderma pantherinum* (A–B), *Poroderma africanum* (C–D) and *Haploblepharus pictus* (E–F). Trophozoite (A–B), meronts (C–D) and gamonts (E–F). Scale bar: 10µm.

Table 4.4 Morphometrics of blood apicomplexans measured from the shark species examined along with measurements provided by Yeld (2009).

Stage	Measurement	*Haemogregarina* sp. A	*Haemogregarina* sp. B	*Dactylosoma* morphotype A	*Dactylosoma* morphotype B
Trophozoite	**L**	9.24 ± 1.11 (7.06–10.86)	9.72 ± 1.31 (7.13–12.71)	7.52 ± 1.44 (6.01–12.51)	10.98 ± 0.81 (9.83–11.70)
	W	5.17 ± 0.49 (4.34–6)	5.37 ± 0.64 (4.26–6.81)	4.08 ± 0.57 (3.23–5.61)	4.91 ± 0.90 (3.60–6.30)
	NL	4.118 ± 0.97 (3.05–5.78)	4.59 ± 0.83 (2.91–6.10)	1.53 ± 0.24 (1.00–1.87)	3.20 ± 0.74 (2.50–4.73)
	NW	3.91 ± 0.74 (2.8–5.43)	4.06 ± 0.59 (2.92–5.14)	3.28 ± 0.78 (2.04–5.15)	4.39 ± 0.89 (2.89–5.41)
	MA	3.95 ± 0.75 (3.09–5.22)	5.56 ± 0.93 (3.61–7.41)	3.13 ± 1.23 (1.33–7.35)	5.51 ± 0.76 (4.88–6.76)
	MP	5.29 ± 1.02 (3.42–7.1)	4.17 ± 1.05 (2.57–6.02)	4.38 ± 0.73 (3.30–5.82)	5.42 ± 0.98 (4.46–6.76)

Table 4.4 Continued.

Stage	Measurement	*Haemogregarina* sp. A	*Haemogregarina* sp. B	*Dactylosoma* morphotype A	*Dactylosoma* morphotype B
Meront	**L**	9.83 ± 1.87 (7.53–11.40)	9.90 ± 1.25 (7.12–12.40)	9.32 ± 1.25 (7.24–12.52)	N/A
	W	8.71 ± 0.74 (7.48–9.66)	8.90 ± 1.02 (7.20–11.66)	5.31 ± 1.46 (3.13–11.11)	N/A
	NL	5.15 ± 0.73 (4.14–6.34)	5.80 ± 0.88 (3.99–7.63)	1.94 ± 0.69 (1.05–4.19)	N/A
	NW	6.13 ± 1.10 (4.61–8.25)	6.26 ± 0.78 (4.28–7.60)	4.32 ± 1.09 (2.86–8.37)	N/A
	MA	N/A	N/A	5.73 ± 1.12 (3.78–9.79)	N/A
	MP	N/A	N/A	3.59 ± 0.80 (1.64–5.23)	N/A

Table 4.4 Continued.

Stage	Measurement	*Haemogregarina* sp. A	*Haemogregarina* sp. B	*Dactylosoma morphotype A*	*Dactylosoma morphotype B*
Gamont	**L**	13.94 ± 0.61 (12.72–15.18)	14.15 ± 0.79 (12.24–15.62)	13.27 ± 1.23 (10.96–17.23)	13.72 ± 0.95 (11.24–15.73)
	W	7.47 ± 0.58 (6.57–8.63)	8.00 ± 0.69 (4.39–6.84)	7.25 ± 1.07 (4.45–10.46)	5.73 ± 0.84 (4.08–8.10)
	NL	3.75 ± 0.48 (2.72–4.90)	5.67 ± 0.69 (4.39–6.84)	2.39 ± 0.60 (1.27–4.34)	4.43 ± 1.10 (2.22–6.70)
	NW	5.99 ± 0.50 (5.08–7.42)	5.57 ± 0.62 (4.55–7.38)	5.51 ± 0.97 (3.18–7.81)	4.12 ± 0.94 (2.45–5.83)
	MA	9.42 ± 0.88 (7.33–11.18)	8.10 ±1.36 (5.74–10.37)	9.16 ± 1.38 (5.46–14.07)	8.10 ± 2.09 (4.69–11.31)
	MP	4.52 ± 0.89 (3.25–6.52)	6.05 ± 1.43 (3.99–9.13)	4.09 ± 1.23 (1.38–8.12)	5.53 ± 1.79 (2.73–8.73)

L, length; W, width; NL, nucleus length; NW, nucleus width; MA, midnucleus to anterior region; MP, midnucleus to posterior region.

Gamonts (Fig. 4.3 C; Fig. 4.4 E–F) are large and rounded measuring 13.27 ± 1.23 (10.96–17.23) x 7.25 ± 1.07 (4.45–10.46) (N=237). Can be found intra-erythrocytic but was also observed outside of the host erythrocyte. Gamonts usually take up most space in the erythrocyte, often causing malformed nuclei. Nucleus located posteriorly [MA: 9.16 ± 1.38 (5.46–14.07); MP: 4.09 ± 1.23 (1.38–8.12) (N=237), and is elongated across the width of the parasite, measuring 2.39 ± 0.60 (1.27–4.34) x 5.51 ± 0.97 (3.18–7.81) (N=237). Nucleus stains a deep purple with Giemsa stain, due to it being densely packed. As seen in trophozoites and meronts, the cytoplasm stains very lightly with numerous granules giving it an almost bubbly-like appearance.

Remarks:

No dividing forms or early life-stages were observed during this study. Very few trophozoites were observed in comparison to meronts and gamonts, a possible reason for this could be that the majority of the sharks had advanced infections and, in the stage, where meronts would undergo gamontogony to transform into gamonts. *Dactylosoma* morphotype A is unique in the sense that the cytoplasm stains very lightly which gives the parasite a bubbly or speckled appearance, as well as an elongated nucleus situated closer to the posterior end. The morphometrics of the trophozoites, meronts and gamonts observed during this study, closely resemble the data provided by Yeld (2009) in an unpublished Ph.D thesis (Table 4.4). In addition to samples of the current study being collected from the same host species and locality, it is identified here as representing the same species as that referred to as *Haemogregarina* sp. A of Yeld (2009). Yeld (2009) placed this morphotype under the genus *Haemogregarina* Danilewsky, 1885 however, molecular results discussed above, show that this morphotype is closely related to the Dactylosomatidae, and is thus placed under *Dactylosoma*. From this point forward, *Haemogregarina* sp. A recorded by Yeld (2009) is therefore referred to as *Dactylosoma* morphotype A. This morphotype was also found infecting sharks collected at Hermanus, a previously unrecorded location, thus expanding the known biogeographical distribution of *Dactylosoma* morphotype A to the southern Western Cape coast. Infection rates varied among individuals ranging from 22 to 267 dactylosomes per 500 erythrocytes per bloodsmear in *Ha. edwardsii*, to 2 to 200 in *Ha. pictus,* 0 to 27 and 4 to 181 in *P. africanum* and *P. pantherinum*, respectively. In contrast to Yeld (2009) where all sharks from all size classes were infected, in this study, the prevalence was slightly lower (Table 4.2).

4.3.3.2 *Dactylosoma* morphotype B

Restricted synonymy: *Haemogregarina* sp. B Yeld, 2009: 75–78, figs. 5.1, 5.3, 5.4.

Hosts from Yeld (2009): *Haploblepharus pictus* (Müller and Henle); *Haploblepharus edwardsii* (Schinz); *Poroderma africanum* (Gmelin) (Chondrichthyes: Scyliorhinidae).

Locality from Yeld (2009): Granger Bay, Western Cape, South Africa (33° 52' S 18° 24' E); Saldanha Bay (33º 02' S 18º 02' E); False Bay (34º 14' S 18º 28' E); De Hoop Nature Reserve (34º 28' S 20º 30' E).

Material studied:

Host(s): *Haploblepharus edwardsii* (Schinz); *Haploblepharus pictus* (Müller and Henle); *Poroderma africanum* (Gmelin).

Locality: Granger Bay, Cape Town (33° 54' 2.31" S, 18° 24' 56.38" E) and Hermanus (34° 25' 15.76" S, 19° 14' 37.56" E), Western Cape, South Africa.

Site in host: Peripheral blood.

Prevalence: 38% (5/13); 53% (25/47) and 4% (1/24), respectively.

Vector: Unknown. Possibly leech found on sharks preliminarily identified as *Pontobdella* sp. (Prof. E. Burreson, Virginia Institute of Marine Science, USA; pers. comm.).

Representative DNA sequence(s): Two partial sequences of the 18S rRNA gene; 757 nt and 747 nt in length, respectively (GenBank accession numbers: XXXX).

Diagnosis:

Deep purple staining gamont, with circular nucleus often stained a deep purple or overstained and not easily seen. Vacuole can be seen in most gamonts, located between nucleus and posterior end.

Description:

Trophozoites (Fig. 4.3 D; Fig. 4.5 A) observed in the study were elongated and situated close to the host erythrocyte nucleus measuring 10.98 ± 0.81 (9.83–11.70) x 4.91 ± 0.90 (3.60–6.30) (N=7). The nuclei were densely packed and situated relatively centrally [MA: 5.51 ± 0.76 (4.88–6.76); MP: 5.42 ± 0.98 (4.46–6.76) (N=7)] and stained a dark purple with Giemsa stain. The nucleus was round in shape and measured 3.20 ± 0.74 (2.50–4.73) x 4.39 ± 0.89 (2.89–5.41) (N=7). The cytoplasm stained a light blue-purple.

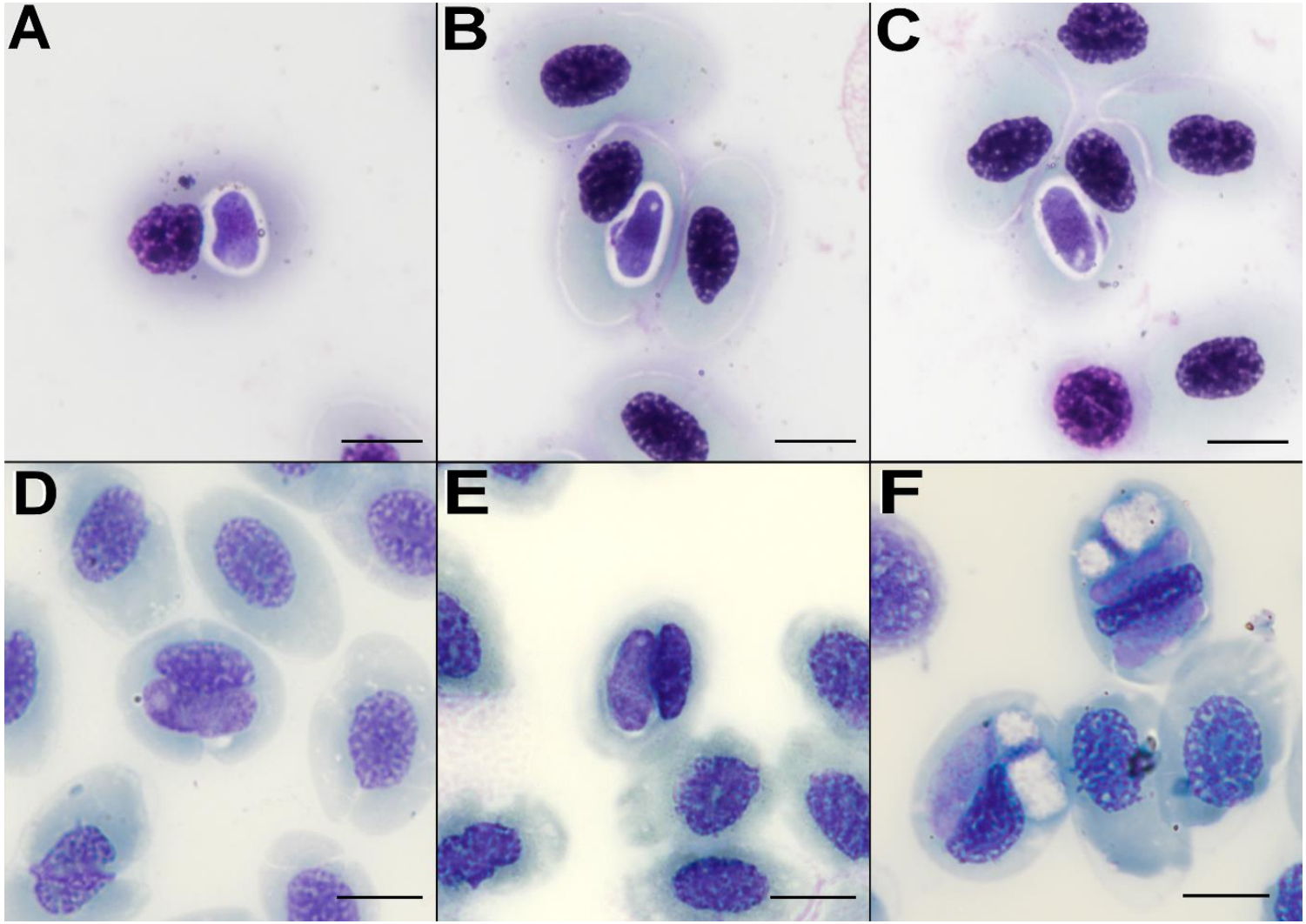

Figure 4.5 Micrographs of *Dactylosoma* morphotype B in Giemsa-stained blood films of *Haploblepharus pictus* (A–C), *Poroderma africanum* (D–E) and *Haploblepharus edwardsii* (F). Trophozoite (A), immature gamonts (B–C), gamonts (D–E) and a mixed infection of both *D.* morphotype A and *D.* morphotype B (F). Scale bar: 10µm.

No meronts were observed during this study. Gamonts (Fig. 4.3 E–F; Fig. 4.5 D–E) were elongated and oval in shape, with no apparent tapering towards the anterior or posterior end measuring 13.72 ± 0.95 (11.24–15.73) x 5.73 ± 0.84 (4.08–8.10) (N=85). The nucleus was situated towards the posterior end of the parasite [MA: 8.10 ± 2.09 (4.69–11.31); MP: 5.53 ± 1.79 (2.73–8.73) (N=16)] measuring 4.43 ± 1.10 (2.22–6.70) x 4.12 ± 0.94 (2.45–5.83) (N=16). Nucleus densely packed, staining a deep purple with Giemsa stain. Cytoplasm stains a blue-purple, and often stains too dark so nucleus is not visible. In most gamonts, a prominent vacuole is visible between the nucleus and posterior end of the parasite, as well as a dark purple-staining granule located on the lateral side.

Remarks:

No dividing forms or early life-stages were observed during this study. Few trophozoites were observed, and no meronts indicating that the sharks screened might be in a very early or very late stage of infection with only trophozoites and gamonts present. While the trophozoites stained a dark purple with the nucleus still visible to measure, the gamonts stained a very dark purple, often causing the nucleus not to be clearly visible for measurement. The morphometrics of the trophozoites and gamonts closely resemble the data provided by Yeld (2009) for the species she referred to as *Haemogregarina* sp. B*,* however molecular analysis placed this morphotype along with *Dactylosoma* morphotype B in the *Dactylosoma* clade, indistinguishable from *Dactylosoma* morphotype A. From this point forward, *Haemogregarina* sp. B will be referred to as *Dactylosoma* morphotype B. *Dactylosoma* morphotype B was also found infecting *Ha. edwardsii* and *Ha. pictus* off the coast of Hermanus, a previously unrecorded location, thus expanding the known biogeographical distribution of *Dactylosoma* morphotype B to the southern Western Cape coast. Infection rates also vary among individuals with 0 to 125 dactylosomes per 500 erythrocytes in *Ha. edwardsii,* 0 to 77 and 0 to 25 in *Ha. pictus* and *P. africanum*, respectively.

4.3.4 Remarks on *Dactylosoma* morphotype A and *Dactylosoma* morphotype B

Dactylosoma morphotype A and B can be distinguished by their difference in staining properties, the shape of the nucleus as well as the width of the gamonts. The cytoplasm of *Dactylosoma* morphotype A stains very lightly in comparison to *Dactylosoma* morphotype B which stains a deep purple and can easily be overstained to the point where the nucleus is no longer visible. The nucleus shape of *Dactylosoma* morphotype A is also more elongated than the circular nucleus of *Dactylosoma* morphotype B, appearing almost as a slender bar

across the gamont width. *Dactylosoma* morphotype B is also a more slender haemogregarine than *Dactylosoma* morphotype A [7.25 (4.45–10.46) and 5.73 (4.08–8.10) – respectively].

Even though the widths of both morphotypes of this specie's gamonts compared to those of *Haemogregarina carchariasi* Laveran, 1908, the gamonts of *Dactylosoma* morphotype A and B are considerably shorter in length [20–27 (Laveran 1908); 16–19 (Mackerras and Mackerras 1961); 13.94 (12.72–15.18) (Yeld 2009) and 13.27 (10.96–17.23); 13.72 (11.24–15.73) – this study, respectively], a similar finding to Yeld (2009). It also differs from the morphometrics provided for *Haemogregarina delagei* Laveran and Mesnil, 1912 [6.3–13.7 (Laveran and Mesnil 1902); 10.8–15.2 (Becker and Overstreet 1979)], yet *H. delagei* is also often found with more than one gamont per host erythrocyte (Becker and Overstreet, 1979; Yeld 2009). Yeld (2009) also stated that the nucleus of *Haemogregarina* sp. A (now *Dactylosoma* morphotype A) differs from the nuclei of other described elasmobranch haemogregarines, with the nucleus being more elongated than round (see Table 4.1). *Dactylosoma* morphotype B is also considerably more slender than other known elasmobranch haemogregarines [3.8 (Saunders 1958); 7–10 (Laveran 1908); 5–8 (Mackerras and Mackerras 1961); 1.6–3.6 (Laveran and Mesnil 1902); 5.73 (4.08–8.10) – this study].

Dactylosoma morphotype A conforms to the general diagnosis for the genus *Dactylosoma* by having a hyaline, or translucent-like cytoplasm that does not stain very clearly, however this does not apply to *Dactylosoma* morphotype B that does not display a hyaline cytoplasm and instead stains a deep purple. *Dactylosoma* morphotype B does exhibit elongated, slender forms, characteristic to those of the genus *Dactylosoma,* however, *Dactylosoma* morphotype A has a more oval form instead. Interestingly, both these morphotypes displayed a nucleus without a distinctive karyosome, an important characteristic of a *Dactylosoma* species. During this study, no developmental or dividing stages were found, resulting in no information available in relation to their development within the host and the invertebrate vector, thus no comparison could be made between the two morphotypes of this species observed during this study and those of other *Dactylosoma* species.

The trophozoites of both morphotypes observed during this study are considerably larger than those of *D. kermiti* (5.3–7.7 x 2.6–4.4 – Netherlands et al. 2020), *D. ranarum* (3.0–4.0 x 1.5–2.0 – Kruse 1890; Barta et al. 1987), and *D. sylvatica* (7.8–8.5 x 6.3–7.6 – Fantham et al. 1942). Trophozoites of *D. kermiti* display large vacuolated cytoplasms (Netherlands et al. 2002), which is somewhat similar to the vacuolated cytoplasm of *Dactylosoma* morphotype A, however this is not seen in *Dactylosoma* morphotype B. When comparing the meronts of

the dactylosomes found infecting fish, those from *Dactylosoma* morphotype A is considerably longer than that of *D. salvelini* (5.8 in comparison to 9.3 (7.2–12.5) – this study), while *D. salvelini* is wider than *Dactylosoma* morphotype A (8.5 in comparison to 5.3 (3.1–11.1 – this study). The meronts of *D. kermiti* have small, round, dense nuclei (Netherlands et al. 2020), which was not seen in the meront stages of *Dactylosoma* morphotype A, while no meront stages were observed in *Dactylosoma* morphotype B. The gamonts of both morphotypes of this species are larger in size as compared to those of *Dactylosoma sylvatica* Fantham, Porter and Richardson, 1942 [7.0–12.6 x 1.5–3.0 compared to 13.27 (10.9–17.2) x 7.25 (4.5–10.5) for morphotype A and 13.72 (11.2–15.7) x 5.73 (4.1–8.1) for morphotype B]. While the gamonts of *D. kermiti* are slender, with a slight curvature (Netherlands et al. 2020), this cannot be said for either of the morphotypes observed during this study with *Dactylosoma* morphotype A having broad, oval shaped gamonts and *Dactylosoma* morphotype B displaying elongated, bean-like shapes with no distinctive curvature or tapering towards the end.

4.4 DISCUSSION

Haemogregarines have been well-studied in most terrestrial vertebrate groups in contrast with vertebrates in the aquatic environment. Only seven species of haemogregarines have been described from elasmobranch hosts, four of which were recorded from skates and rays. The only other three species recorded from sharks include *Haemogregarina heterodonti* von Prowazek, 1910 from the Japanese bullhead shark, *Heterodontus japonicus* Miklouho-Maclay and Macleay; *Haemogregarina carchariasi* from an unknown species of *Carcharias* Rafinesque; and *Haemogregarina hemiscyllii* Mackerras and Mackerras, 1961 from *Hemiscyllium ocellatum* Bonnaterre. In South Africa, research on haemogregarines infecting elasmobranch hosts is almost completely absent, with the only haemogregarines infecting elasmobranchs being reported in an unpublished Ph.D thesis (Yeld 2009).

During this present study, two morphotypes of haemogregarines were found infecting all four of the shark species examined. These two morphotypes conformed to the morphology and morphometrics of the two haemogregarine species reported in Yeld (2009). Phylogenetic analysis placed the two morphotypes observed in this study within the adeleid group, most closely related to *D. ranarum* and *D. kermiti*. No sequences were available for aquatic haemogregarine species described from South Africa, or any of the species of haemogregarines described from elasmobranchs worldwide. This study therefore provides the first molecular characterisation of haemogregarines infecting elasmobranch hosts.

Kirmse (1979) reported that micro-gametocytes and macro-gametocytes were observed when studying the life cycle of *Haemogregarina simondi* Laveran and Mesnil, 1901. Dimorphism has also been reported in several other species, including *Haemogregarina torpedinis* (Neumann, 1909)*, Haemogregarina rovignensis* (Minchin and Woodcock, 1910), *Haemogregarina yakomovi-kohl* (Kohl-Yakimoff and Yakimoff, 1915), *Haemogregarina quadrigemina* (Brumpt and Lebailly, 1904), *Haemogregarina callioymi* (Brumpt and Lebailly, 1904) and *Cyrilia gomesi* (Neiva and Pinto 1926) Lainson 1981. Kirmse (1979) further stated that it could be assumed that haemogregarines infecting marine fish hosts show sexual dimorphism, however to date this has not been identified in species of dactylosomes from either fish or anuran hosts (Barta 1991; Netherlands et al. 2020). The present study supports the latter, as one morphotype of this *Dactylosoma* sp. was found in the absence of the other in both *Poroderma* spp. If sexual dimorphism was occurring, it would have been expected that both morphotypes of this *Dactylosoma* sp. occur together persistently as was found in the case of the *Haploblepharus* spp. Davies (1995) also noted that gamonts found occurring individually within host erythrocytes, could show either monomorphism or dimorphism. This was not observed during the present study, with frequent accounts of dual or even triple infections within shark erythrocytes. Laird (1952) described the “rovignensis group”, a group consisting of fish haemogregarines which have deep-staining caps. However, Davies (1995) mentioned that the staining properties of the caps might be influenced by either the staining procedure or that the caps might stain differently at different stages of development. In the present study the latter may be true, the morphotypes representing different stages of development. In fish haemogregarines, the most commonly found haemogregarines are monomorphic and often times shaped like a sausage (Davies 1995), whereas dimorphic types are rarely seen in fish hosts (Davies 1995). Dimorphism often occurs so that macro-gamonts (female) and micro-gamonts (male) can form, where they are usually either round in shape or they can take on S or U-shapes when their length exceeds the length of the host erythrocyte. Davies (1995) mentioned that in some haemogregarines dimorphism is visible within the fish host, while in others, the gamonts might be monomorphic until they reach the definite host (the invertebrate), however this might be difficult to prove in the absence of the definite host and the stages present therein (Davies 1995). It is therefore believed that the two morphotypes seen, do not represent dimorphism as sometimes seen in marine haemogregarines, but potentially different stages of development. However, based on observations by Yeld (2009), who considered these two morphotypes as two separate species, display differences in their developmental stages (trophozoites through to gamonts), which is also observable for trophozoite, meront and gamont stages in the present study. As in Yeld (2009), trophozoites of the now *Dactylosoma* morphotype B showed a

preference for erythroblasts (however, unlike that of Yeld (2009), in this study, trophozoites were observed in erythrocytes), with trophozoites and all other stages of now *Dactylosoma* morphotype A found only in erythrocytes. As alluded to by this author, this cannot be simply a display of dimorphism, but, unlike in Yeld (2009), in this study, it cannot be ruled out (looking at the molecular data), that this is not a representation of different stages of development.

Due to most shark individuals being infected with either only *Dactylosoma* morphotype A or both *Dactylosoma* morphotype A and B, with no shark individual displaying an infection of solely *Dactylosoma* morphotype B, the possibility arises that during PCR, only *Dactylosoma* morphotype A amplified and thus sequenced. The use of the 70:30 ratio for the selection of samples to sequence *Dactylosoma* morphotype B appeared to be effective, with sequences for *Dactylosoma* morphotype B being amplified easily with chromatograms suggestive of amplification of a single species. Furthermore, sequences for *Dactylosoma* morphotype A amplified from only species A infected individuals showed a 100% similarity further suggesting amplification of only morphotype B in those individuals with a co-infection of 70:30. In future, if the opportunity arises, it is suggested that hosts with only a single species infection, either *Dactylosoma* morphotype A or *Dactylosoma* morphotype B, be screened and additional molecular characterisation or single-cell sequencing should be performed along with further molecular analysis to confirm the present study's findings, making certain these two morphotypes are in fact variations in life cycle stages and that separate species were not missed. Additionally, molecular characterisation should be performed on a variety of genes, such as the ITS gene, a piece of non-functional RNA found between the small and large subunit of the rRNA, in order to more accurately determine whether species and particularly newly described species are in truth valid. Currently, most apicomplexan molecular studies rely on the 18S rRNA gene to provide insight into the relationships between apicomplexan blood parasites, however due to this region being known as conservative, the additional use of genetic markers, such as ITS, COI, COIII or cytochrome b, would help in differentiating between species on a molecular level (Gutierrez-Liberato et al. 2021). The use of ITS and mitochondrial markers has several advantages, most notably being a rapidly evolving region of the rRNA (Hili et al. 2021; Walker et al. 2022). At present, with the current use of the 18S gene and the low divergence below the accepted 3 % divergence rate, the two morphotypes cannot definitively be separated into two species. This molecular work should be revisited in the future, only when there is a larger database with faster evolving gene markers to use for comparisons, in order to accurately determine whether the two morphotypes are truly only one species. Future work should also include sequencing of a vast number more elasmobranch haemogregarines in order to get a more

comprehensive understanding on the placement of these parasites, and their relationships to other fish haemogregarines.

Infections of haemogregarines are known to modify the shape and size of host erythrocytes which often lead to a decrease in the oxygen carrying capacity, resulting in increased numbers of erythrocytes by the vertebrate host (Smit et al., 2006). Parasitaemia in the blood of *Ha. edwardsii* and *Ha. pictus* were notably high, most often with infections of *Dactylosoma* morphotype A. High parasitaemias could be indirectly attributed to host behaviour, as the benthic-orientated and sedentary behaviour of these sharks allow vectors to attach with ease and transmit these parasites to the shark host.

4.5 CONCLUSION

Haemogregarines of elasmobranchs remain a poorly studied group of organisms worldwide, and more effort needs to be placed in acquiring data to expand on the information available to date. It is important to expand on the knowledge that is currently known of haemogregarines and their effect on the various vertebrate hosts they infect, in order to get a more holistic understanding of how these parasites affect their hosts and whether the relationship between the parasites and their hosts are truly parasitic or more mutualistic than expected. With increased efforts of screening additional marine vertebrates for blood parasites, we could not only increase the number of known taxa in South African waters, but also broaden our understanding of South African biodiversity. By increasing our efforts of molecular characterisation of marine haemogregarines infecting both fish and elasmobranchs, we could form a better understanding of the haemogregarine phylogeny in general. This study represents the first account on the molecular characterisation of haemogregarines infecting elasmobranchs in South Africa. This is also the first study to identify and sequence haemogregarines, and more specifically *Dactylosoma,* infecting elasmobranchs worldwide, revealing that with increased molecular efforts, other marine haemogregarines presently assigned to the genus *Haemogregarina* may in fact represent a greater diversity of genera.

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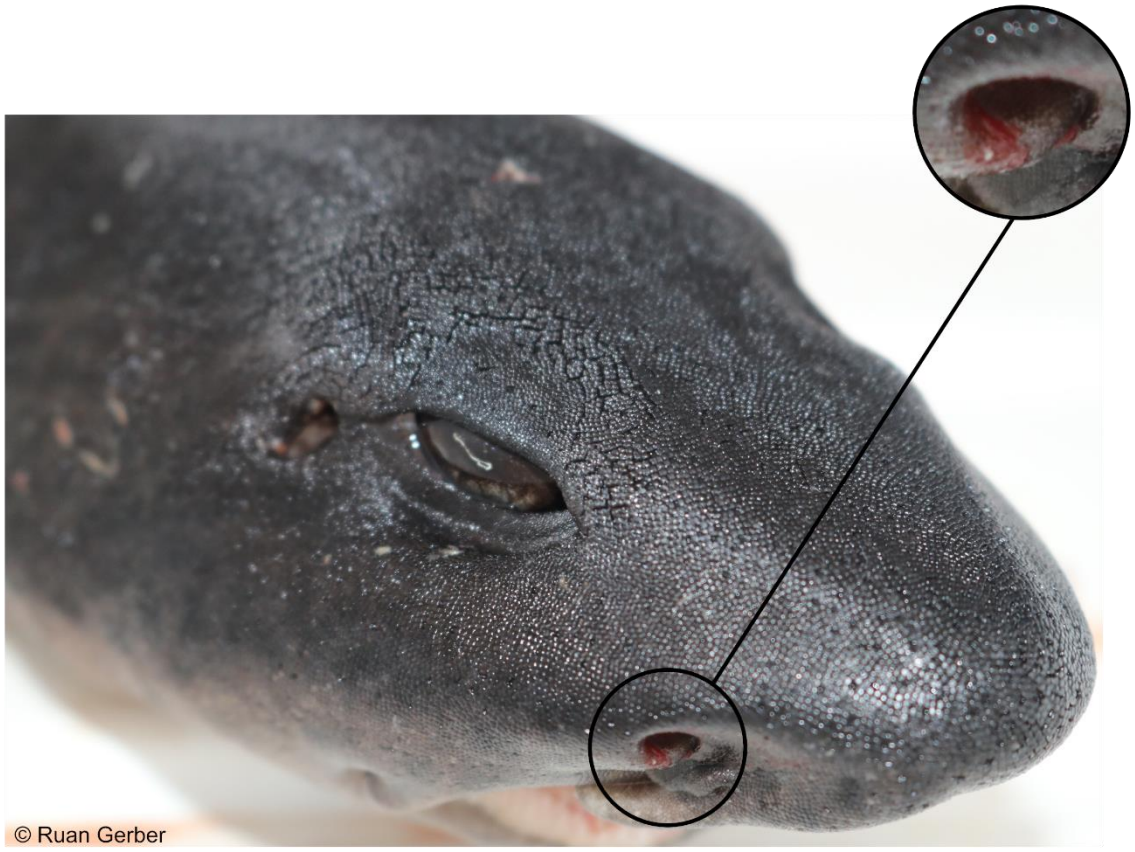
5

LEECH VECTORS



Chapter 5: Leech vectors

5.1 INTRODUCTION

Elasmobranchs are known to be parasitised by a wide range of parasites, one of which includes marine leeches from the family Piscicolidae (Daly et al. 2019; Keating-Daly et al. 2019). Over 23 species of marine leeches have been recorded from elasmobranchs worldwide, with the most abundant and cosmopolitan being *Pontobdella macrothela* Schmarda, 1861 (previously *Stibarobdella macrothela*) (see Burreson and Passarelli 2015; Daly et al. 2019; Keating-Daly et al. 2019). In South Africa, knowledge on marine leech biodiversity remains poorly explored, with only four species being recorded to date, including *Malmiana stellata* (Moore, 1958) from an unknown species of toby fish in Richards Bay, KwaZulu-Natal, *Austrobdella oosthuizeni* Utevsky, 2004 from the Cape rock lobster *Jasus lalandii* (Milne Edwards) from Bloubergstrand, *Lizabdella africana* Utevsky, 2007 from three species of mullets from the genus *Liza* Jordan and Swain, 1884 in the Eastern Cape (Utevsky 2004; 2007) and *Zeylanicobdella arugamensis* De Silva, 1963 from the intertidal fishes *Clinus cottoides* Valenciennes, *Clinus superciliosus* (L.), *Clinus taurus* Gilchrist and Thompson and *Parablennius cornutus* (L.) from the Western Cape (Hayes et al. 2006; 2014). Similar to other animals, species descriptions for leeches are based primarily on the internal anatomy and external morphology including number of body segments and number of annuli present. Siddall and Burreson (1998) were the first to include molecular analyses with their morphological descriptions of the Piscicolidae, Piscicolinae and Platybdellinae, which laid the foundation of providing molecular data along with new species descriptions (Tseng et al. 2017). The *co*1 gene is the most widely used genetic tool as a DNA barcode for species identification because of its high variability (Tseng et al. 2017; Hebert et al. 2003; Hebert et al. 2004; McGowin et al. 2011). This gene has been widely used in elucidating the phylogenetic relationships between leeches, which in turn has helped solve many taxonomic questions. However, research has been based primarily on medicinal and freshwater leeches (Tseng et al. 2017; Siddall and Burreson 1998; Utevsky and Trontelj 2004; Utevsky et al. 2007). As such, more research is needed into marine leeches to determine their phylogenetic placement and relationship to other leeches. This chapter therefore aims to provide more morphological and molecular information on leeches of elasmobranchs found on South African sharks.

5.2 MATERIALS AND METHODS

5.2.1 Host and leech collection, identification, and microscopy

Collection of sharks and screening were performed as reported in Chapter 3. Leeches present on sharks were removed following their release from the host using tweezers. Leeches were then placed in 70 % ethanol for further morphological and molecular analyses. For morphological analyses, leeches were screened on a Zeiss Stemi 508 stereo microscope (Carl Zeiss Microscopy, Jena, Germany) where photographs and measurements were taken with the attached camera and the Labscope Material Ver. 2.8.3 application on an Apple iPad 7 (Apple, California, USA). Measurements for leeches are given in mm unless stated otherwise and include total body length (TBL) (excluding anterior and posterior sucker), total body width (TBW), anterior sucker length (ASL), anterior sucker width (ASW), posterior sucker length (PSL), posterior sucker width (PSW), trachelosome length (TL) and urosome length (UL). Micrographs were captured of the following aspects of the external anatomy including oral sucker, posterior sucker, eyespots, and complete body form following Utevsky (2007) and Utevsky et al. (2019) for each leech individually and sent to Prof. Eugene Burreson (Virginia Institute of Marine Science, USA) for identification.

Scanning electron microscopy (SEM) was performed on two specimens in which the leech was cut in half so the anterior and posterior sucker could face upward. Fixed specimens were hydrated from 70% ethanol to 100% ethanol through a graded alcohol series (70%, 80%, 90%, 96%, 100%, 100%) for 10 min each followed by a graded series of Hexamethyl-disilazene (HMDS) (30%, 50%, 70%, 100%, 100%) for 5 min each, leaving the last 100% to completely evaporate. The anterior and posterior suckers were then mounted on an aluminium stub with double-sided carbon tape. The stubs were then sputtercoated for 2 min in a SPI module EIKO IB-2 ion coater (EIKO Engineering, Ltd., Yamazaki Hitachinaka, Japan) with gold palladium. Scanning electron microscopy was performed using a Phenom Pro Desktop scanning electron microscope (Thermo Fisher Scientific Inc., Waltham, USA).

5.2.2 Molecular and phylogenetic analysis

5.2.2.1 Molecular and phylogenetic analysis of leeches

For molecular analysis, the fixed leeches were stretched out, pinned on a pin pad, and placed under a dissection microscope [Zeiss Stemi 508 (Carl Zeiss Microscopy, Jena, Germany)] where the posterior sucker, crop and salivary glands were removed. No removal of the crop or salivary glands were done from fresh leech material. This was largely due to the sampling trips being constrained for time and equipment for dissections of the leeches

were not on hand during these trips. Also, given the large size of the leeches, fresh leech squashes on microscope slides to check for any developmental stages, could not be performed. Genomic DNA was extracted post-dissection of the fixed leeches from the removed organs using the KAPA Express Extract Kit (Kapa Biosystems, Cape Town, South Africa) following manufacturer's instructions for animal tissue. The resultant supernatant was used as a template using universal *co*1 mRNA primers for metazoan invertebrates LCO-1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO-2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994). The PCR protocol was as follows: initial denaturation step of 95°C for 3 min, followed by 35 cycles of 94°C for 1 min, 40°C for 1 min, 72°C for 1 min 30 s and a final extension step of 72°C for 7 min. All PCR reactions were performed with volumes of 25 µl, using 12.5 µl Thermo Scientific DreamTaq PCR master mix (2×) (final concentration: 2× DreamTaq buffer, 0.4 mM of each dNTP, and 4 mM $MgCl_2$), 1.25 µl of each primer (10 µM), and at least 25 ng of DNA. The final reaction volume was made up of PCR grade nuclease free water (Thermo Scientific, Vilnius, Lithuania). Reactions were undertaken in a SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Singapore). A 1 % agarose gel electrophoresis was performed, and the results visualised under ultraviolet light to determine whether DNA amplicons were obtained. PCR products were then sent to Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria, South Africa), a commercial sequencing company, for purification and sequencing.

BLAST results identified highly similar sequences from which 68 sequences were selected for phylogenetic analysis and downloaded from the NCBI GenBank database. Following Utevsky et al. (2019) the following sequences were selected as outgroup: *Hirudo orientalis* Utevsky and Trontelj, 2005 (EF405599), *Erpobdella monostriata* Lindenfeld and Pietruszynski, 1890 (KP300764), *Ozobranchus margoi* (Apáthy, 1890) (AF003268), *Ozobranchus branchiatus* (Menzies, 1791) (GU985466), *Ozobranchus jantseanus* Oka, 1912 (KY861060), *Theromyzon tessulatum* (Müller, 1774) (AY047318), *Hemiclepsis marginata* (Müller, 1774) (MH643798), *Glossiphonia complanata* L. (AF003277) and *Helobdella europea* Kutschera, 1987 (AY856048) (Utevsky et al. 2019). Sequences were aligned using the ClustalW tool available in Geneious Ver. 11.1.4 with reference to the amino acid translation, using the invertebrate mitochondrial code (translation table 5, frame 1) for a final alignment length of 657 nt. A model test was performed to determine the most suitable nucleotide substitution model, according to the Bayesian information criterion (BIC) using jModelTest 2.1.4 (Guindon and Gascuel 2003; Darriba et al. 2012). The model with the best BIC score was the general time-reversible model incorporating invariant sites and gamma distributed among site-variations (GTR+I+G). A Bayesian Inference (BI) analysis was implemented in Geneious Ver. 11.1.4 using the MrBayes 3.2.2 parameter (Huelsenbeck

and Ronquist 2001) with a four category Gamma distribution to infer phylogenetic relationships. Phylogenetic trees were visualised using FigTree ver. 1.4.4 software (Rambaut 2012) and the p-distance, percentage identity and number of nucleotide (nt) differences were calculated from the alignment using the Geneious Ver. 11.1.4.

5.2.2.2 Molecular and phylogenetic analysis of haemoprotozoans from leeches

Genomic DNA was extracted as detailed in Chapter 3. For the amplification of potential trypanosome DNA, PCRs were conducted using the same 18S rRNA trypanosome-specific primers and protocol as detailed in Chapter 3. Similarly, for the amplification of potential haemogregarine DNA PCR was conducted using the same primer set (4558 and HepR900) and protocol as detailed in Chapter 4.

5.3 RESULTS

5.3.1 General observations of leeches present on sharks

Of the 98 individuals of four species of sharks collected, 15% (15/98) were infested with leeches. Table 5.1 provides the metrical data and prevalence of leeches on the shark species examined. *Poroderma africanum* (Gmelin) was the species with the highest number of leeches present (7/24), followed by *Haploblepharus pictus* (Müller and Henle) (4/47) and both *Poroderma pantherinum* (Müller and Henle) and *Haploblepharus edwardsii* (Schinz) with only two shark individuals respectively infested with leeches. Based on the photographs, the leeches were preliminary identified as *Pontobdella* sp. (Prof. E. Burreson, pers. comm.; N=26). The measurements taken for all leech specimens are recorded in Table 5.2.

Table 5.1 Information on elasmobranch hosts, including prevalence of leeches.

Shark Species	N	ML ± SD (range) in mm	Prevalence (leech)
Haploblepharus edwardsii	9	421.1 ± 34.6 (354–467)	15 % (2/13)
Haploblepharus pictus	47	435.6 ± 101.6 (260–614)	8.5 % (4/47)
Poroderma africanum	24	767.1 ± 150.9 (501–1,010)	2.9 % (7/24)
Poroderma pantherinum	14	511.4 ± 95.5 (363–725)	1.4 % (2/14)

Table 5.2 Morphometrics of leeches measured from species of sharks.

Leech measurements	N	*Pontobdella* sp. – this study		*Pontobdella macrothela* (Soto, 2000)[1]
	N	ML ± SD (range) in mm	N	Length in mm
Total body length (TBL)	26	10.6 ± 3.3 (5.9–18.4)	1	67
Total body width (TBW)	26	2.5 ± 1.5 (0.9–6.2)	1	14
Anterior sucker length (ASL)	26	0.8 ± 0.3 (0.3–1.4)	1	Diameter: 5
Anterior sucker width (ASW)	26	0.9 ± 0.3 (0.4–1.6)		
Posterior sucker length (PSL)	26	1.1 ± 04 (0.4–1.9)	1	Diameter: 12
Posterior sucker width (PSW)	26	1.4 ± 0.4 (0.5–2.1)		
Trachelosome length (TL)	26	2.1 ± 1.2 (0.9–5.5)		Not provided
Urosome length (UL)	26	8.5 ± 2.3 (4.7–13.7)		Not provided

[1] The only morphometrics available for this species is from Soto (2000). Other articles identify the leech as *Pontobdella macrothela* and only provide measurements for length (Keating-Daly et al. 2019; Daly et al. 2019; Burreson 2020). A full species description can be found in Llewellyn (1966), however the only measurement given, is an approximate body length.

5.3.1.1 Description of leeches collected from sharks

Annelida Lamarck, 1802
Clitellata Michaelsen, 1919
Hirudinea Savigny, 1822
Rhynchobdellida Blanchard, 1894
Piscicolidae Johnston, 1921
Pontobdella Leach, 1815
Pontobdella sp. A

Material studied:

Host(s): *Haploblepharus edwardsii* (Schinz), *Haploblepharus pictus* (Müller and Henle), *Poroderma africanum* (Gmelin), *Poroderma pantherinum* (Müller and Henle) (Chondrichthyes: Scyliorhinidae).

Locality: Granger Bay, Cape Town (33°54'2.31"S, 18°24'56.38"E) and Hermanus (34°25'15.76"S, 19°14'37.56" E), Western Cape, South Africa.

Site on host: Nostrils, head, gills, claspers, pectoral fins, anal fin.

Prevalence: 15 % (2/13) on *H. edwardsii*, 8.5 % (4/47) on *H. pictus*, 2.9 % (7/24) on *P. africanum* and 1.4 % (2/14) on *P. pantherinum*.

Diagnosis:

Present specimens large with prominent wart-like tubercules on both dorsal and ventral side of all annuli of the trachelosome and urosome. Oral sucker smaller than posterior sucker with distinctive eyespots visible near base of oral sucker. Eyespots are conspicuous and easily visible on all specimens collected.

Description:

Leeches tapered towards anterior sucker (Fig. 5.1 A–C), division between trachelosome and urosome can be seen clearly. Mean body length 10.6 ± 3.3 (5.9 – 18.4) (Table 5.2) with large warts visible on dorsal and ventral side, alternating large and small tubercules on annuli. Tubercules round and fluctuate in colour with smaller tubercules usually being brownish, while larger tubercules appear white. Annulations clearly visible with annuli in the trachelosome usually biannulate, while annuli on the urosome are triannulate. Oral sucker relatively small [0.8 ± 0.3 (0.3 – 1.4) x 0.9 ± 0.3 (0.4 – 1.6)] (Fig. 5.1 E–F, Fig. 5.2 A) in comparison to size of posterior sucker [1.1 ± 04 (0.4 – 1.9) x 1.4 ± 0.4 (0.5 – 2.1)] (Fig. 5.2

B). Oral sucker also has a fringe with two papillae per side with two eyespots at the base of the oral sucker which are clearly visible (Fig. 5.1 D). Position of the mouth is at the centre of the oral sucker (Fig. 5.2 C). Edges of the posterior sucker revealed sensory organs, possibly used to help attach to the host (Fig. 5.2 D). Posterior sucker often shows light brown colour stripes on the outside (Fig. 5.1 G–I). Scanning electron microscopy (SEM) micrographs also showed the position of a reproductive pore, possibly the male gonopore with the position being at the bottom of the clitellar region, close to the start of the urosome (Fig. 5.2 E, F). No female gonopore was observed with SEM.

Remarks:

Leeches present on the collected shark individuals were morphologically the most similar to *Pontobdella macrothela* (Schmarda, 1861) that is a known parasite of at least 20 known elasmobranch hosts with a distribution ranging from the warm waters of the Indian, Pacific and Atlantic oceans. However, the South African specimens differ considerably in size to other known specimens of *P. macrothela*, with reported sizes for this species (67 mm in Soto, 2000; 7.5 – 12.0 × 1.8 x 2.8 mm in Yamauchi et al. 2012; 25 x 5 mm in Keating-Daly et al. 2019; 24 mm and 48 mm in Daly et al. 2019; and up to 16 cm in Llewellyn 1966) being much larger than the individuals measured within this study [10.6 ± 3.3 (5.9 – 18.4)]. Morphologically, the leeches are very similar to the description of *P. macrothela* by Llewellyn (1966) specifically in having a prominent wart-like tubercule shape, the posterior sucker being 2 – 3 times larger than the oral sucker, which also has an oval shape and two prominent eyespots (as seen by Llewellyn 1966).

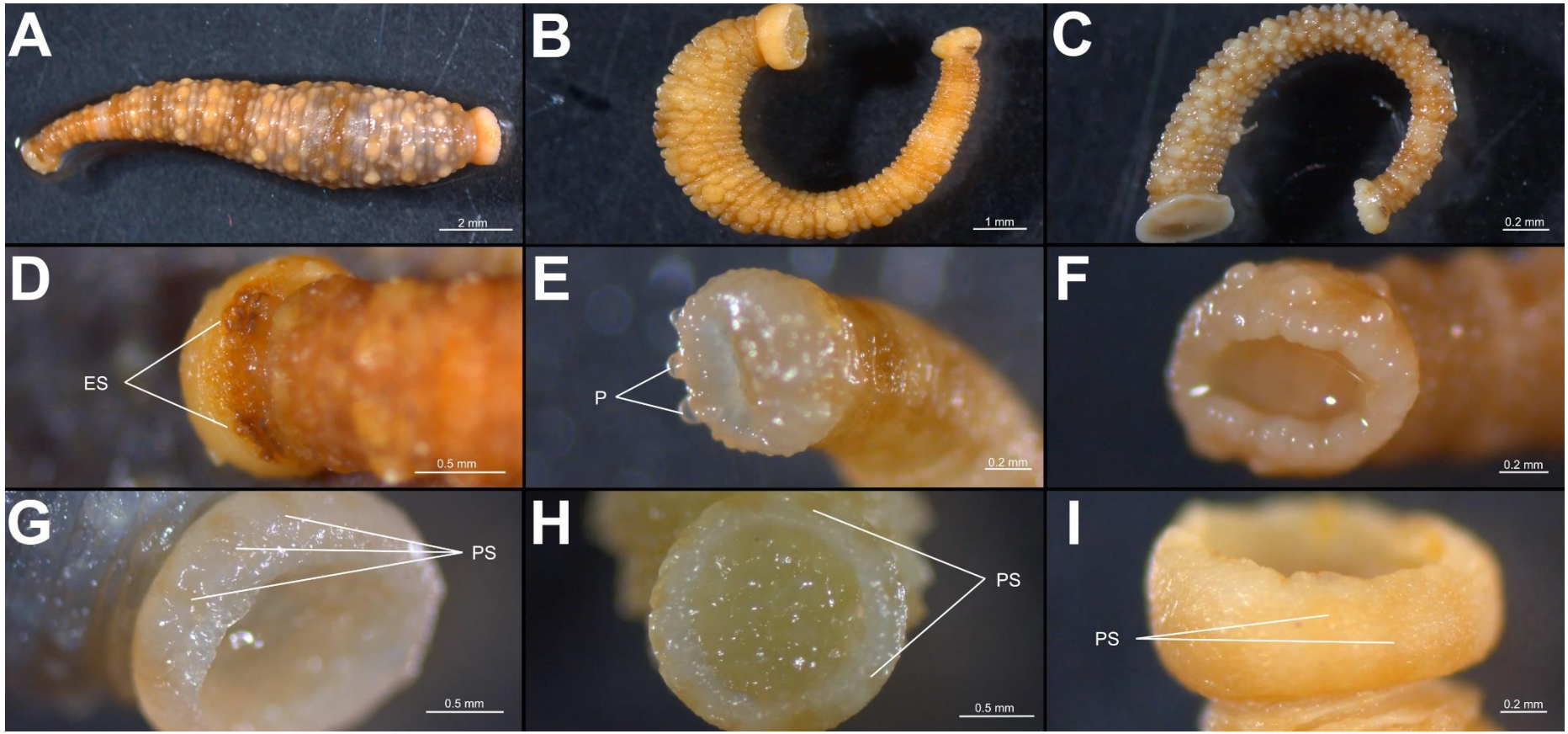


Figure 5.1 Photographs of leeches collected during this study illustrating the leech body shape (A–C), different views on the anterior/oral sucker (D–F), showing the eyespots (ES) (D), and papillae (P) (E), and the posterior sucker (G–I) showing pigment stripes (PS).

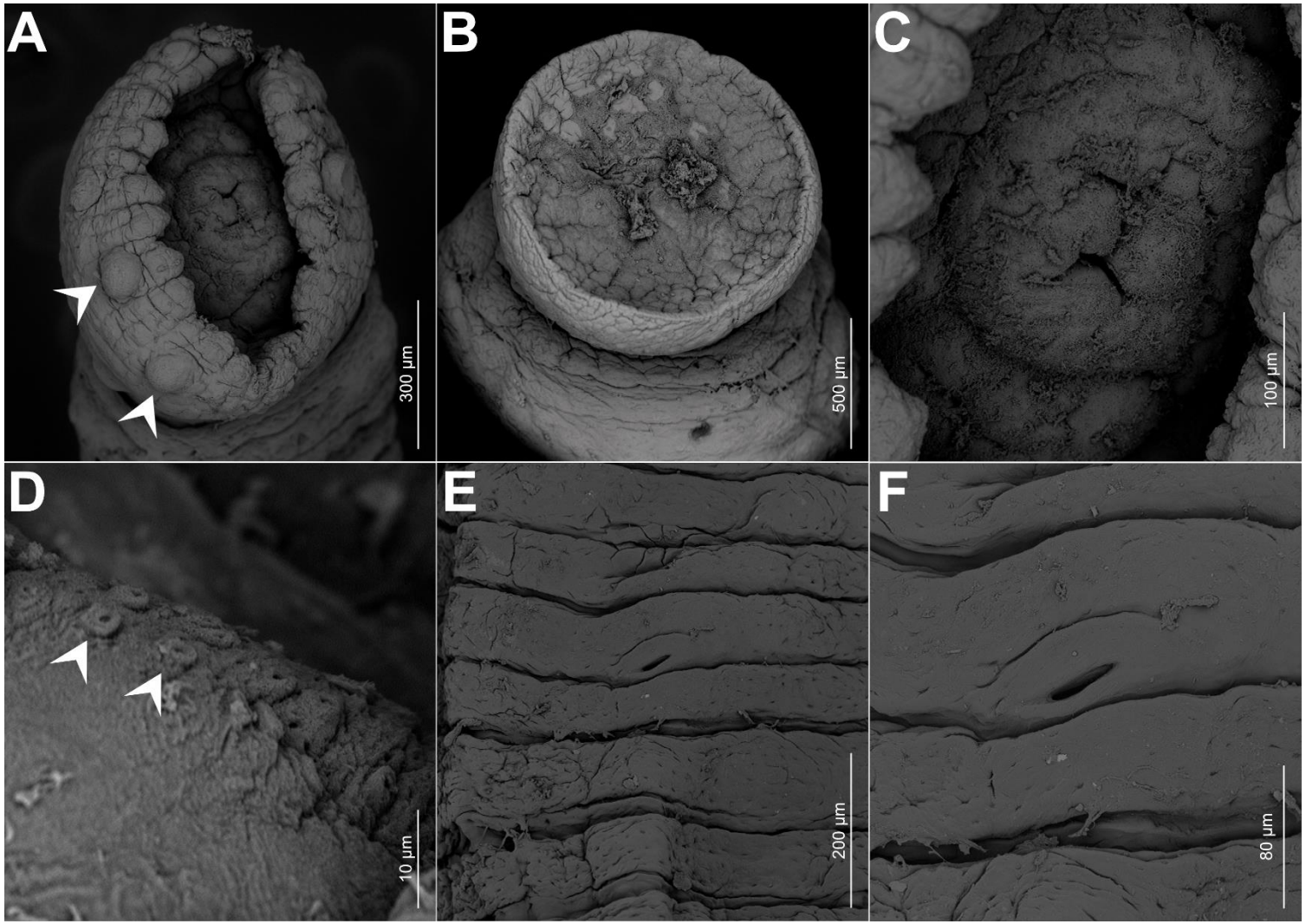


Figure 5.2 Scanning electron micrographs illustrating the anterior sucker (A), posterior sucker (B), position of the mouth within the anterior sucker (C), sensory organs on the anterior sucker (D), and the position of the genital pore, possibly the male gonopore (E, F).

5.3.2 Molecular characterisation and phylogenetic analysis

5.3.2.1 Molecular results of leeches

A consensus sequence of approximately 700 nt was constructed using the aligned and manually edited sequence chromatograms obtained for each primer. The alignment consisted of 73 sequences (Table S3) with a final alignment length of 657 nt. *Pontobdella* sp. A fall within the larger clade of the Piscicolidae family, forming a separate clade from the sequences of *Pontobdella macrothela* [syn. *Stibarobdella macrothela* (Schmarda, 1861)] provided for by other studies (Fig. 5.3). Table 5.3 provides the evolutionary differences between the sequences of *Pontobdella* used in the phylogeny, including four additional sequences from this study which were not included in the phylogeny due to the presence of stopcodons in the middle of the sequences. *Pontobdella* sp. A isolate 1 and 2 are molecularly identical (p=0.00, 100 %), while *Pontobdella* sp. A isolates 3, 5, 6, 7, 9, 10 and 11 were molecularly similar (p=0.00 – 0.02, 99 % – 100 %) (Table 5.3), differentiating the isolate 1 and 2 group from the latter group. Even though *Pontobdella* sp. A isolate group 1 and 2 and isolate group 3, 5, 6, 7, 9, 10 and 11 individuals were morphologically indistinguishable from the description provided by Llewellyn (1966) of *P. macrothela*, they were molecularly distant from other sequences included of this species (p=0.21 – 0 .26, a divergence of 21 – 26 %).

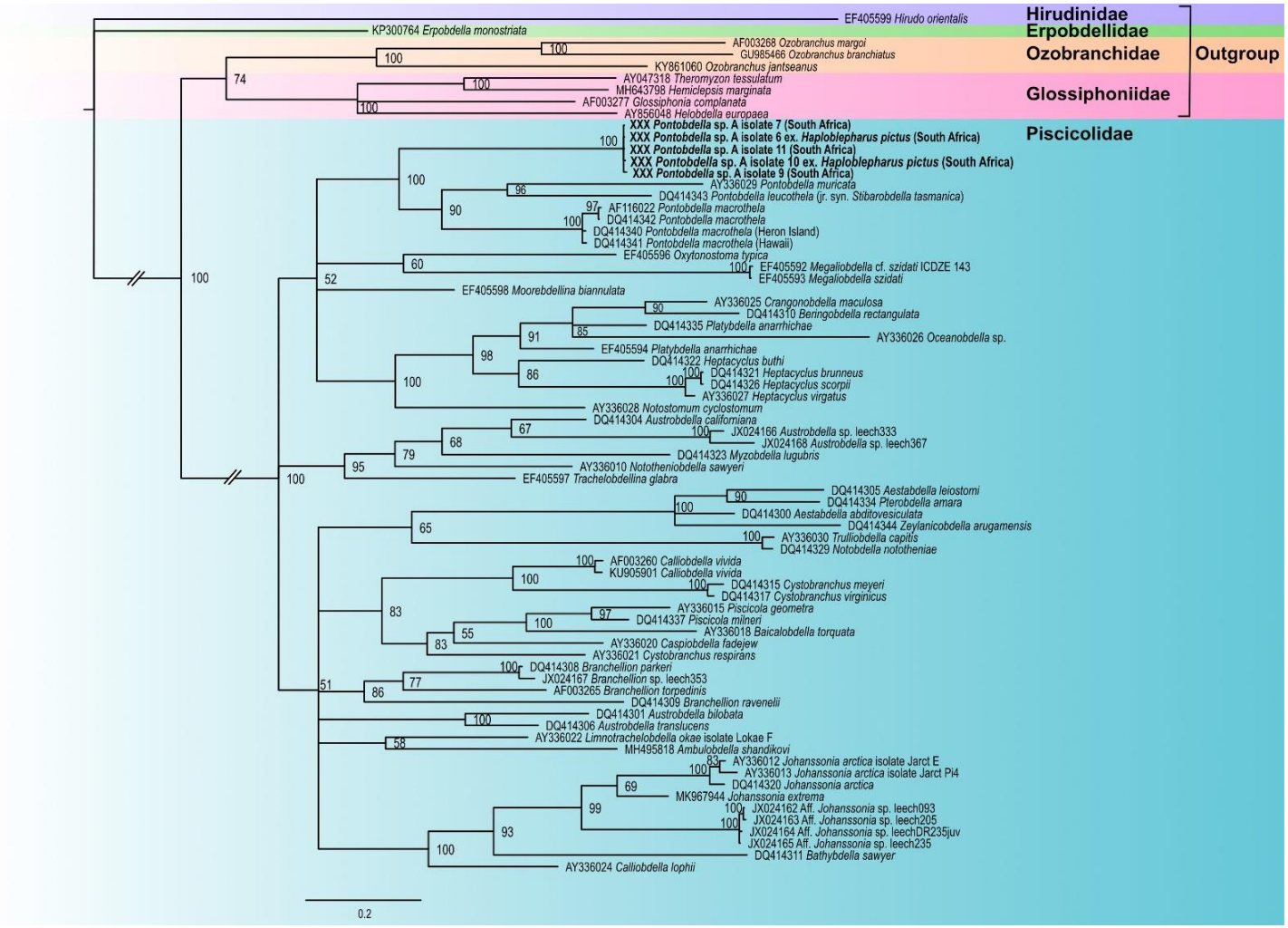


Figure 5.3 Bayesian Inference (BI) analysis of representatives of leech species inferred from *co*1 mRNA gene sequences showing the phylogenetic position of the *Pontobdella* sp. A. Comparative sequences representing known leech species were obtained from GenBank. *Hirudo orientalis* (EF405599), *Erpobdella monostriata* (KP300764), *Ozobranchus margoi* (AF003268), *Ozobranchus branchiatus* (GU985466), *Ozobranchus jantseanus* (KY861060), *Theromyzon tessulatum* (AY047318), *Hemiclepsis marginata* (MH643798), *Glossiphonia complanata* (AF003277) *and Helobdella europea* (AY856048) serve as outgroup taxa following Utevsky (2019).

Table 5.3 Evolutionary differences of species of *Pontobdella* Leach, 1815 isolated from the *co*1 mRNA gene region of leeches included in the phylogenetic analysis presented in Fig. 5.3, including four additional sequences (nos. 7–10) not included in the phylogeny, expressed as percent similarity (%) (bottom left) and uncorrected pair-wise distance (p-distance) (top right).

	Accession number	Leech species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	EF405599	*Hirudo orientalis*		0.51	0.50	0.50	0.51	0.58	0.55	0.55	0.59	0.59	0.58	0.58	0.58	0.57	0.58
2	AF116022	*Pontobdella macrothela*[2]	77		0.03	0.03	0.00	0.25	0.22	0.22	0.26	0.26	0.25	0.24	0.25	0.24	0.24
3	DQ414340	*Pontobdella macrothela*[4]	78	97		0.00	0.02	0.24	0.21	0.21	0.25	0.25	0.24	0.24	0.24	0.23	0.24
4	DQ414341	*Pontobdella macrothela*[4]	77	97	99		0.02	0.24	0.21	0.21	0.25	0.25	0.24	0.24	0.24	0.24	0.24
5	DQ414342	*Pontobdella macrothela*[4]	77	100	97	97		0.25	0.22	0.22	0.25	0.25	0.24	0.24	0.25	0.24	0.24
6	DQ414343	*Pontobdella leucothela*[3]	76	87	86	97	97		0.21	0.21	0.24	0.24	0.23	0.23	0.24	0.23	0.23
7	XXX (isolate 1)	*Pontobdella* sp.	78	88	88	87	88	86		0.00	0.06	0.06	0.05	0.05	0.06	0.05	0.05
8	XXX (isolate 2)	*Pontobdella* sp.	78	88	88	87	88	86	100		0.06	0.06	0.05	0.05	0.06	0.05	0.05
9	XXX (isolate 3)	*Pontobdella* sp.	77	87	86	86	87	86	96	95		0.01	0.02	0.01	0.02	0.02	0.01
10	XXX (isolate 5)	*Pontobdella* sp.	77	87	86	86	87	86	96	96	99		0.02	0.01	0.02	0.02	0.01
11	XXX (isolate 6)	*Pontobdella* sp.	77	87	86	86	87	86	96	96	100	100		0.00	0.01	0.01	0.00
12	XXX (isolate 7)	*Pontobdella* sp.	77	87	86	86	87	86	96	96	99	99	100		0.01	0.01	0.00
13	XXX (isolate 9)	*Pontobdella* sp.	77	87	86	86	87	86	96	96	99	99	100	100		0.01	0.01
14	XXX (isolate 10)	*Pontobdella* sp.	77	87	86	86	87	86	96	96	99	99	100	100	99		0.01
15	XXX (isolate 11)	*Pontobdella* sp.	77	87	86	86	87	86	96	96	99	99	100	100	100	100	

[2] Appears as *Stibarobdella macrothela* in GenBank.
[3] Appears as *Stibarobdella leucothela* in GenBank.

5.3.2.2 Molecular results of haemoprotozoans from leeches

It is important to note that from all the PCRs conducted, although the sharks the leeches fed on were infected by both haemogregarines and trypanosomes (see chapters 3 and 4), only sequences of trypanosomes were obtained, with no sequences or results obtained for any haemogregarines. Each primer yielded a sequence length of approximately 870 nt and were subsequently assembled into a consensus sequence of approximately 890 nt. The alignment consisted of 14 sequences (Table S4) with a final alignment length of 956 nt. The trypanosomes isolated from the dissected leech crop forms a clade with *Trypanosoma haploblephari* Yeld and Smit, 2006 morphotype A and *T. haploblephari* morphotype B (Fig. 5.4). The three *Trypanosoma* isolates group with morphotype B*,* even though it was isolated from *H. pictus* and *H. edwardsii* in which infections correspond to *T. haploblephari* morphotype A (see Chapter 3). The trypanosomes observed during this study (*T. haploblephari* morphotype A, *T. haploblephari* morphotype B; *Trypanosoma* sp. isolate 17, *Trypanosoma* sp. isolate 21, *Trypanosoma* sp. isolate 23) were almost identical with a divergence of only 1 % (p=0.01) (Table 5.4), indicating that the trypanosomes isolated from the leeches are the same species.

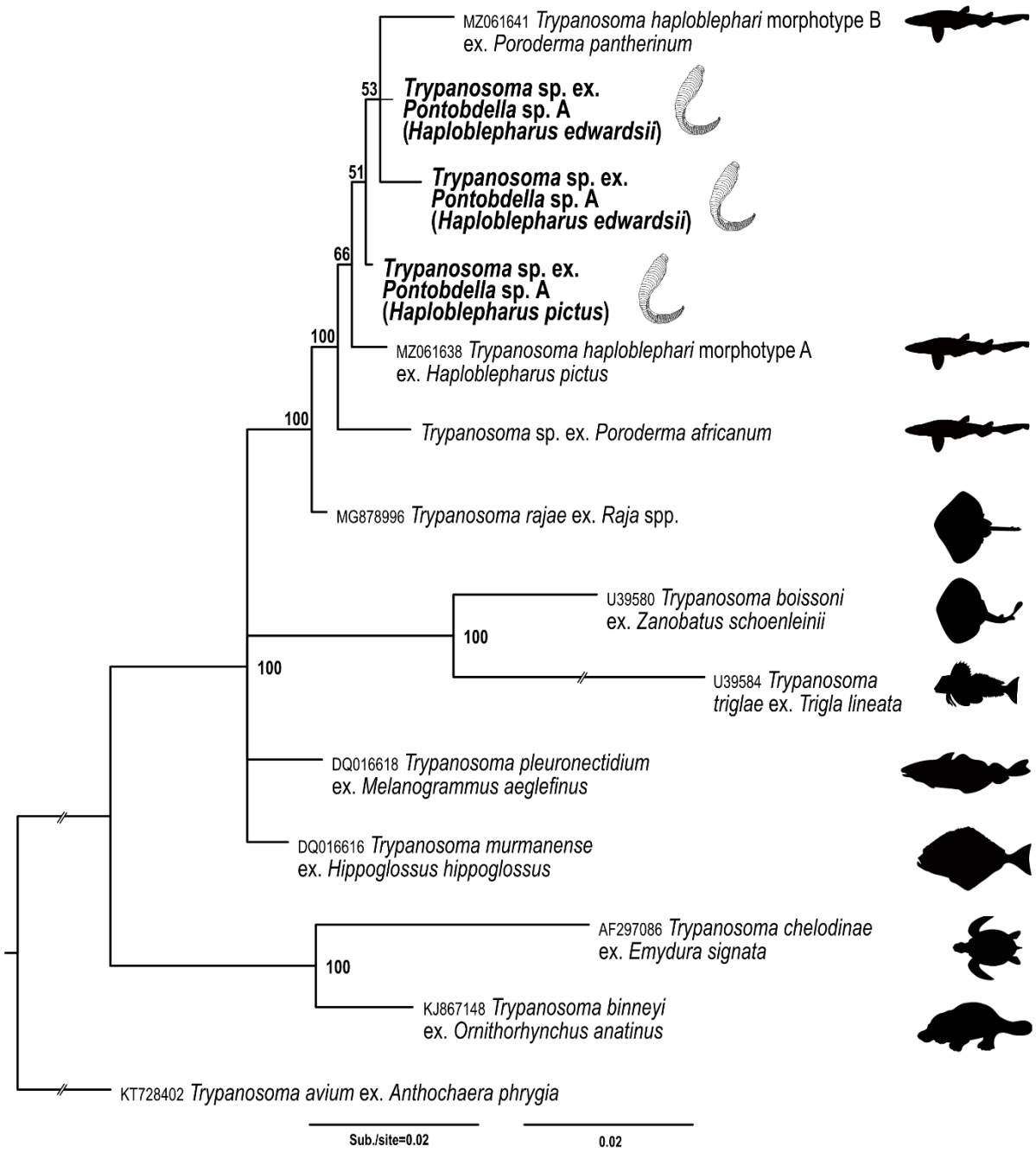


Figure 5.4 Bayesian Inference (BI) analysis showing the phylogenetic position of the *Trypanosoma* sp. inferred from partial 18S rRNA gene sequences. Comparative sequences representing marine trypanosome species, with *Trypanosoma avium* (KT728402) as outgroup, were obtained from GenBank.

Table 5.4 Evolutionary differences of species of *Trypanosoma* Gruby, 1843 isolated from the crop of dissected leeches used in the phylogenetic analysis presented in Fig. 5.4, expressed as percent similarity (%) (bottom left) and uncorrected pair-wise distance (p-distance) (top right).

	Accession number	*Trypanosoma* species	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	KT728402	*Trypanosoma avium*		0.11	0.13	0.09	0.09	0.17	0.13	0.10	0.11	0.10	0.11	0.10	0.10	0.11
2	KJ867148	*Trypanosoma binneyi*	90		0.04	0.06	0.06	0.14	0.09	0.06	0.07	0.07	0.08	0.07	0.07	0.07
3	AF297086	*Trypanosoma chelodinae*	89	95		0.08	0.08	0.16	0.11	0.08	0.09	0.09	0.10	0.09	0.09	0.09
4	DQ016616	*Trypanosoma murmanense*	92	95	93		0.01	0.09	0.05	0.01	0.02	0.02	0.03	0.02	0.02	0.03
5	DQ016618	*Trypanosoma pleuronectidium*	91	95	93	99		0.10	0.05	0.02	0.03	0.03	0.03	0.02	0.03	0.03
6	U39584	*Trypanosoma triglae*	85	86	84	88	88		0.08	0.10	0.11	0.10	0.11	0.10	0.10	0.11
7	U39580	*Trypanosoma boissoni*	90	92	90	94	94	89		0.05	0.06	0.06	0.07	0.06	0.06	0.06
8	MG878996	*Trypanosoma rajae*	92	95	93	99	98	88	94		0.01	0.01	0.02	0.01	0.01	0.01
9		*Trypanosoma* sp.	90	94	92	97	97	86	92	98		0.01	0.02	0.01	0.02	0.02
10	MZ061638	*Trypanosoma haploblephari* morphotype A	92	94	92	98	98	87	93	99	98		0.02	0.01	0.01	0.01
11	MZ061641	*Trypanosoma haploblephari* morphotype B	90	94	92	97	97	86	92	99	98	99		0.01	0.01	0.01
12	XXX (isolate 17)	*Trypanosoma* sp.	91	94	93	98	98	87	93	99	98	99	99		0.00	0.01
13	XXX (isolate 21)	*Trypanosoma* sp.	91	94	93	98	98	87	93	99	98	99	99	100		0.01
14	XXX (isolate 23)	*Trypanosoma* sp.	91	94	92	98	98	87	93	99	98	99	99	99	99	

5.4 DISCUSSION

With increasing efforts of studies being conducted on leeches to elucidate their phylogeny, knowledge on marine leeches is becoming more readily available (Keating-Daly et al. 2019). To date, 23 species are known to infect elasmobranch hosts, with *Pontobdella macrothela* being the most widespread and commonly identified leech species parasitising 20 known species of elasmobranchs (Daly et al. 2019). With this widespread distribution and infection of predominantly littoral hosts (Yamauchi et al. 2008; Wunderlich et al. 2011), the question arises whether *P. macrothela* is a cosmopolitan species or if it represents a species complex (Keating-Daly et al. 2019). Studies on genetic variation, which this chapter contributes to, are needed in order to resolve this question. During this study, leech specimens collected were morphological most similar to *P. macrothela*, however phylogenetic analysis showed that the specimens collected were only 87 % identical to previously sequenced *P. macrothela* leeches from Virginia, USA and Heron Island, Australia, with a divergence of 21 – 26 %. This divergence is far above the >2 % sequence divergence considered to differentiate between species using the mitochondrial *co*1 gene (Kaygorodova et al. 2014; Hebert et al. 2003; Hebert et al. 2004), which would strongly suggest that the leeches in this study are not *P. macrothela*. Interestingly, leeches from this study were more closely related to *Stibarobdella tasmanica* (Hickman, 1947) from Tasmania, Australia, a junior synonym of *Pontobdella leucothela* Schmarda, 1861, than to the known sequences of *P. macrothela*. From the nine leech individuals which were sequenced, it appears that these may represent two different species. Furthermore, sequences of the leech specimens of this study were not all identical, with isolates 1 and 2 (being identical) showing a divergence from isolates 3, 5, 6, 7, 9, 10, 11 of 5 – 6 %. The latter isolates showed no more than a 2 % divergence between each another, suggesting an intraspecific divergence, but not enough to differentiate between species. The divergence of >2 % between isolates 1, 2 and the remaining isolate group would suggest that these are representative of two different species.

In comparison to the round ribbontail ray, *Taeniura meyeni* (Müller and Henle) and the nurse shark, *Ginglymostoma cirratum* (Bonnaterre) where infections of 33 and 10 leeches were found respectively, this study in comparison found a maximum of three leeches per host individual, a similar finding to that of Keating-Daly et al. (2019), Williams (1982) and Yamauchi et al. (2008). It has been suggested that behavioural and environmental factors could influence infection rates, with sharks such as the silky sharks *Carcharhinus falciformis* (Müller and Henle), blacktip shark *Carcharhinus limbatus* (Müller and Henle) and tiger shark *Galeocerdo cuvier* Péron and Lesueur being more active swimmers, resulting in low infections of leeches, in comparison to slow-moving, benthic-orientated elasmobranchs such

as the round ribbontail ray *Ta. meyeni* (Müller and Henle), nurse shark (*Gi. cirratum*) and sicklefin lemon shark *Negaprion acutidens* (Rüppell), in which higher infections are observed (Keating-Daly et al. 2019).

The phylogeny of the leeches showed that the leech specimens of this study fall outside of the clade containing *P. leucothela*, *P. macrothela* and *P. muricata*, forming a separate sister clade, further supporting the possibility that these individuals may represent a new species [when a cut-off divergence of 2 – 3 % is considered (Evans and Paulay 2012)].

The phylogenetic results showed that the trypanosomes isolated from the various leeches in this study, groups closely together with *T. haploblephari* morphotype B, and not *T. haploblephari* morphotype A as expected. A possible explanation for this finding could be attributed to leeches being opportunistic feeders and may be found on all four of the shark species examined. Leeches might have fed on a *P. pantherinum* individual infected with *T. haploblephari* morphotype B before moving on to a different individual of *H. pictus* or *H. edwardsii*, where it was collected from, possibly before it could feed on this individual. However, these results further support the hypothesis presented in Chapter 3 that *T. haploblephari* from the different sharks is a single species that shows pleomorphism. A recent study by Smit et al. (2020) where they morphologically and molecularly characterised a trypanosome species from both the fish host and the leech vector, found that the trypanosome sequences generated from both hosts showed a divergence of 0.7 %, still below the 3 % divergence threshold that is used to distinguish between protists (Smit et al. 2020). Similarly, Davies et al. (2005) found two different genotypes of trypanosomes infecting freshwater fish in the Okavango Delta, further strengthening the point that fish trypanosomes from Africa demonstrate a high genetic diversity within a single species of parasite which are able to infect multiple host species. To date, the only study of leeches parasitising marine hosts in South Africa, is that of Hayes et al. (2014) where it was shown that the leech *Zeylanicobdella arugamensis* is the vector for *Trypanosoma nudigobii* Fantham, 1919 in the Koppie Alleen and Tsitsikamma areas of the Western Cape. From the present study, the trypanosome sequences generated from both the shark hosts and the leeches show a divergence of 1 % (p=0.01), indicating that these leeches might be the vector for *T. haploblephari*.

During the phylogenetic analysis of the haemoprotozoans from the dissected leeches, only trypanosomes were sequenced while haemogregarines were not detected. This finding is surprising, not only because there was a haemogregarine presence in the blood of the infected sharks, but also since haemogregarines, identified as species of *Dactylosoma*

(Chapter 4), were readily sequenced from shark blood. A possible explanation for this finding could be that the primers or PCR protocol needs to be refined for sequencing the haemogregarines from the crop or salivary glands of leeches. During this study, there were various challenges when trying to generate DNA sequences for both the trypanosomes and haemogregarines. One of these challenges, were because the trypanosome-specific primers alone did not amplify the parasite DNA, but actually amplified the host shark's DNA. Only when combining those primers with the external primers listed in Chapter 3, could DNA sequences be generated. Thus, the primers used for trying to isolate the blood parasite sequences from the leech host could potentially not be as effective as developing specific primers for the amplification of the parasites from the leech host. Additionally, the PCR protocol could not be at the optimal temperature for the sequences to be amplified as expected. No haemoprotozoans could be sequenced from the salivary glands, which could suggest, to a lesser extent, that there were no developmental stages present, but more likely it suggests that the fixation method used during this study was not sufficient for haemoprotozoan DNA extraction from the leech. When specimens were present on a shark host, the leech was removed, immediately placed in 70 % ethanol, and only much later dissected. If the ethanol did not infiltrate the tissues of the leeches rapidly enough to fix the internal organs, DNA of the intracellular haemoprotozoans may have been compromised. Alternatively, it is suggested that when the leeches are removed, they should be placed in a container filled with seawater and kept for a few days [similarly following the method of 7 – 10 days from Negm-Eldin's (1998) study on the life cycle of *Trypanosoma mukasai* (Hoare, 1932) and its leech vector *Batracobdelloides tricarinata* (Blanchard, 1897)] in order to allow for the blood digestion to take place and developmental stages to potentially form within the crop, intestine and the salivary glands. Additionally, following Hayes et al. (2014), leech squashes of the intestine and proboscis should be made at 1, 30, 31 and 32 d.p.f. (days past feeding) in order to observe developmental stages such as amastigotes in the crop, epimastigotes in the intestine and metacyclic trypomastigotes in the proboscis. On the other hand, for haemogregarines such as *Dactylosoma*, leeches should be dissected from 3 – 14 days post feeding, based on studies such as Barta (1991) in order to observe developmental stages. Once developmental stages, particularly those stages mentioned above for trypanosomes and sporozoites and merozoites for *Dactylosoma*, have been observed, the remaining tissues of the crop, intestine, proboscis and salivary glands can be fixed for further molecular analysis.

5.5 CONCLUSION

Further, a more detailed study into the morphology of the leeches collected here will need to be done, taking into account not only external morphology, but internal morphology as well (as in Moser et al. 2013; Hopkins et al. 2014), before being able to confirm whether these specimens represent a new species. With the high biodiversity of elasmobranchs present in South Africa, if more effort is placed on screening a larger group of these hosts, the potential of discovering more leech species may be high. Increased efforts on molecular characterisation of leeches are needed to elucidate whether the *P. macrothela* species is cosmopolitan and may represent a species complex or if it is a number of species of similar morphology. This study represents the first account on the molecular characterisation of a *Pontobdella* species infecting elasmobranchs in South Africa. This is also the first study worldwide to sequence trypanosomes from the crop of leeches that had fed on elasmobranchs.

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6

SIMULATIVE CONCLUSION AND RECOMMENDATIONS

Chapter 6: Simulative conclusion and recommendations

6.1 INTRODUCTION

During the present study, the diversity of haemoprotozoans of elasmobranchs were researched, along with their possible vectors of transmission. This was achieved by using morphological techniques such as light microscopy and scanning electron microscopy (SEM) to identify and describe morphological characteristics. Additionally, molecular techniques such as DNA extraction, PCR amplification, sequencing and phylogenetic analyses were applied to compare haemoprotozoans and their vectors with other known species in order to get a better understanding of the relationships between these species. The overarching aim of the research from this dissertation was to contribute to the known diversity of haemoprotozoans globally and to serve as a basis on which elasmobranch haemoprotozoan research in South Africa could expand. To achieve this, the following aims for this project were set: (1) determine the biodiversity of haemoprotozoans infecting scyliorhinid sharks in the coastal waters of the Western Cape, (2) determine the phylogenetic placement and relationships of these parasites within their respective groups, subsequently determining their taxonomy, and (3) determining the potential vector(s) for these parasites, contributing to determining transmission routes and assisting with the elucidation of their complex life cycles.

6.2 MAIN FINDINGS FROM THE RESEARCH AND RECOMMENDATIONS FOR FUTURE WORK

6.2.1 Aim 1

Research on marine haemoprotozoans globally is very scarce, in particular research on elasmobranch haemoprotozoans. Aim 1 of the present study was addressed in two different chapters, Chapter 3 focussing on trypanosomes and Chapter 4 on the haemogregarines.

During the present study four different scyliorhind (Elasmobranchii: Scyliorhinidae) species were examined for the presence of *Trypanosoma haploblephari* Yeld and Smit, 2006, the only known South African shark trypanosome, and for other potentially different trypanosome species. Samples from all four shark species examined, particularly *Haploblepharus pictus* (Müller and Henle) and *Haploblepharus edwardsii* (Schinz), the two main hosts of *T. haploblephari* in the original description, yielded high parasitaemias of trypanosomes (Chapter 3). Blood smears of individuals of *Poroderma africanum* (Gmelin) and *Poroderma*

pantherinum (Müller and Henle) yielded trypanosomes which did not compare morphologically to *T. haploblephari*. This latter morphotype differed considerably in size and shape and was unique in the sense that it was always found curling up on itself in a doughnut or rosette-form. A full morphological and morphometrical description of the morphotype was made and compared to the original description of *T. haploblephari* and based on morphology may have easily been considered a separate species. However, with the incorporation of molecular tools, it was considered to be a morphotype of *T. haploblephari* (see Aim 2 below), a species now considered to demonstrate extreme poly- or pleomorphism [as per research recorded in Chapter 3 and published in Pretorius et al. (2021)].

South Africa represents a high diversity of elasmobranch species, and the potential of finding additional parasites unknown to science is high. Additional sampling of shark species in a wider geographic area should also be conducted to better understand the relationship between elasmobranchs and trypanosomes. While this study focused on the elasmobranch hosts along the Western Cape coast of South Africa, it would also be beneficial for future studies to explore elasmobranch hosts along the eastern coast of South Africa, where the water is warmer. Due to the difference in the ecosystem of the eastern coast, one might find a different variety of haemoprotozoans and vectors which may have adapted to a warmer climate than those in the cold water of the western coast. A wider sampling of more species of sharks might also yield either more information on the distribution and hosts of *T*. *haploblephari* or might potentially discover other species of trypanosomes.

In addition to trypanosome diversity, haemogregarines were also identified infecting sharks in this study (Chapter 4). Two morphotypes of haemogregarines were observed in the blood of three shark species examined, with individuals of *H. pictus* and *H. edwardsii* having the highest parasitaemias. Interestingly, individuals of *P. pantherinum* showed no haemogregarine infection. The two morphotypes were morphologically described in this chapter, differing by size, staining properties as well as only one morphotype displaying a vacuole in the gamont stage. Both these morphotypes were morphologically compared to the two *Haemogregarina* species described in the Ph.D thesis of Yeld (2009), comparing closely in morphological aspects. With the use of molecular tools in the present study it was determined that these two morphotypes should be considered one species until additional gene markers can prove otherwise and belong to the genus *Dactylosoma* rather than the genus *Haemogregarina* (see Aim 2 below).

6.2.2 Aim 2

Even though the two trypanosome morphotypes were considerably different in size and shape, molecular characterisation of these parasites showed that there is only a 0.5 % difference between the sequences, which is lower than the 3 % divergence used to discriminate between species. A new host and locality record was added to the distribution of *T. haploblephari* and its close genetic relationship with another elasmobranch trypanosome *Trypanosoma rajae* Laveran and Mesnil, 1902 from skates in Europe (see Chapter 3 and Pretorius et al. 2021). The divergence of only 0.6 % between *T. haploblephari* and *T. rajae* strengthened the point that researchers should be cautious in describing new species based only on morphological methods and geographical proximity, particularly for those of elasmobranch hosts as very little information is available on the distribution and host record of the trypanosomes already described in previous years. It is therefore extremely important that new species descriptions should have accompanying molecular data in order to prove that the new species is in fact novel. It was also found in the abovementioned study that *T. haploblephari* could in fact represent a different genotype of *T. rajae*, suggesting it may be a multi-host species with a wide distribution range, a similar finding to that of the freshwater fish trypanosome *Trypanosoma mukasai* Hoare, 1932 which also infects multiple host species and shows low sequence variation at the 18S rRNA gene level (Smit et al. 2020; Pretorius et al. 2021).

To date, the most popular gene to use in haemoprotozoan research is the conservative 18S rRNA nuclear gene, with very few sequences of other gene regions available to differentiate between trypanosome species. With the addition of other gene regions, differentiations between species could ultimately be made and the phylogeny of fish trypanosomes could be better understood. It is therefore recommended that more gene regions such as 5S, 16S and 24S nuclear genes (Grisard et al. 2003; Gurgel-Gonçalves et al. 2012; Kelly et al. 2014) as well as the NADH mitochondrial gene (Kelly et al. 2017) should be sequenced in order to get a better understanding of *T. haploblephari* and whether the morphotype infecting *P. africanum* and *P. pantherinum* is potentially a separate species.

Despite earlier studies (Yeld 2009) placing the haemogregarine morphotypes (Chapter 4) in the *Haemogregarina* (*s.l.*) Danilewsky, 1885, molecular characterisation of these parasites placed them in the Dactylosomatidae, or more specifically the genus *Dactylosoma* Labbé, 1894. Molecular analysis showed a very low divergence, something also experienced by Netherlands et al. (2020) when differentiating between species. However, in the present study, it has been decided to err on the side of caution and abide by the 3% divergence cut off for protist species. The use of the 70:30 ratio for sample selection of *Dactylosoma*

morphotype B were effective, with DNA for this parasite being amplified easily. Additionally, future studies should focus on DNA amplification for sharks that harbour only a single *Dactylosoma* infection or what appears to be a single infection, and if this is found not to be possible, explore the use of single-cell sequencing in order to ensure that the DNA sequence generated is truly the sequence of the desired parasite species. These species are the first report of dactylosomes described from elasmobranchs worldwide as most other haemogregarines are placed in the Haemogregarinidae genera, *Haemogregarina* (*s.l.*) or *Desseria* Siddall, 1995. While previous descriptions of these haemogregarines were based on taxonomy and no molecular data was generated, it is recommended that the other elasmobranch species previously described be molecularly characterised to determine their true molecular and taxonomic placement. Additionally, the genus diagnosis of the *Dactylosoma* should also be revised to include the morphological description of those infecting sharks, as even though molecular analysis placed these morphotypes in the *Dactylosoma*, they did not correspond to the current diagnosis of members of the *Dactylosoma*. With additional survey efforts, and sampling of other species of elasmobranch haemogregarines, it might show elasmobranch haemogregarines to belong to a completely new genus within the Dactylosomatidae. This study is also the first to sequence species of dactylosomes infecting elasmobranch hosts.

6.2.3 Aim 3

In Chapter 5, the aim was to collect and provide more morphological and molecular information on the leeches infecting South African elasmobranchs and their possible role as vectors for haemoprotozoans. Photographs of all required characteristics for identification were sent to leech taxonomist and expert, Prof. Eugene Burreson (Virginia Institute of Marine Science, USA). Professor Burreson primarily identified these leeches as species of *Pontobdella* as their characteristics conformed most closely to the basic morphological description of the cosmopolitan elasmobranch leech, *Pontobdella macrothela* Schmarda, 1861, with only a difference in size being observed. Leeches collected during this study were found to be significantly smaller than *P. macrothela*. Molecular characterisation concluded that the leeches from the present study are no more than 87 % identical to known sequences of *P. macrothela* and formed a separate clade to these. Additionally, molecular analysis showed that there was variation between the leeches collected during this study, with a divergence of 5 – 6 % between isolates. This large divergence would suggest that there are two different leech species represented in the current study and as such need further investigation.

In this study, the salivary glands and crop of the leeches were dissected to detect the potential presence of haemoprotozoans through PCR methods (Chapter 5). Sequences generated from the dissected leech organs showed only a divergence of 1 % to the sequences generated from the trypanosomes present in the shark hosts, further supporting the results that the trypanosomes are the same species. Additionally, sequence data from the leeches provides very strong evidence that while feeding on the sharks, trypanosomes were taken up with the blood meal. Unfortunately, only sequences of trypanosomes could be generated from the leech crop, with no sequences of haemogregarines detected. This was an unanticipated finding given that sequences for the haemogregarines could easily be generated from the blood of the shark individuals. Also, no sequences could be generated from the salivary glands for either parasite groups. A possible explanation for this could be that since the leeches were fixed in ethanol upon collection, therefore possibly not providing enough time for the development of haemoprotozoans to take place. It is therefore recommended that fresh leeches be collected and immediately dissected for their salivary glands and proboscis (for older infections), and other organs such as the crop and intestine, so as to identify developmental stages. It is further recommended that leeches be kept alive longer in order to process their blood meal. Only after a period of 3 – 14 days, can leeches be dissected to look for developmental stages through blood smears and squashes of the above organs (given that the leeches are too large for whole or sectional squashes) following closely the methods of Barta (1991) for *Dactylosoma* development, and Hayes et al. (2014) for *Trypanosoma* Gruby, 1843 development. Furthermore, presence of stages and the identity of these infections should be tested through the use of PCR methods, particularly if stages are observed during microscopy. Focus should be placed in elucidating transmission and life cycle information of both haemoprotozoan groups, in particular for those of the genus *Dactylosoma,* as to date only the life cycle information of one species of *Dactylosoma* has been elucidated. By doing this, essential information will be provided towards a better understanding of haemoprotozoans and their vectors infecting elasmobranch hosts.

6.3 CONCLUSION

With South Africa known as the third most biodiverse country in the world, the potential of discovering new species is extremely high. This is especially true for parasite species, as it has been mentioned that every single species harbours at least one parasite. This would mean that there is a large hidden diversity within South Africa as most animal species have not been thoroughly inspected for parasites. As experienced during this study, haemoprotozoans are very difficult to study, especially due to their morphology being very

similar and their genetic differences rather low with the currently available and most utilised molecular tools. This study lays the foundation for future elasmobranch haemoprotozoan parasitology research in South Africa and draws attention to the potential diversity of these parasites, particularly with the finding of the first molecularly identified species of *Dactylosoma* in elasmobranch hosts. It also further highlights the need for more research into, not only the haemoprotozoans studied here, but also the need to screen other elasmobranch species along a wider distribution within the Temperate Southern African marine ecoregion. Only with more information on the biology of elasmobranchs, and the parasites that live within them, can better conservation strategies be developed for both elasmobranchs and heamoprotozoans, as both are integral components of their respective ecosystems (Dunne et al. 2013). Now, more than ever, better conservation strategies are needed in order to conserve the unique biodiversity that is present in South African waters for future generations the world over.

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APPENDICES

Appendix 1
Chapter 3

Table

Table S1 Species of *Trypanosoma* implemented in the phylogenetic analysis (Figure 3.9).

***Trypanosoma* species**	**Host**	**Locality**	**Reference**	**Accession number**
Trypanosoma avium	*Anthochaera phrygia*	Australia	Šlapeta et al. (2016)	KT728402
Trypanosoma binneyi	*Ornithorhynchus anatinus*	Australia	Paparini et al. (2014)	KJ867148
Trypanosoma boissoni	*Zanobatus schoenleinii*	Senegal	Maslov et al. (1996)	U39580
Trypanosoma chelodinae	*Emydura signata*	Australia	Jakes et al. (2001)	AF297086
Trypanosoma cobitis	*Noemacheilus barbatulus*	England	Stevens et al. (1999)	AJ009143
Trypanosoma fulvidraco	*Pseudobagras fulvidraco*	China	Gu et al. (2007b)	EF375883
Trypanosoma granulosum	*Anguila anguila*	Portugal	Unpublished	AJ620552
Trypanosoma granulosum	*Anguila anguila*	United Kingdom	Unpublished	AJ620551
Trypanosoma micropteri	*Micropterus salmoides*	China	Jiang et al. (2019)	MH635421
Trypanosoma murmanense	*Hippoglossus hippoglossus*	Norway	Karlsbakk and Nylund (2006)	DQ016616
Trypanosoma ophiocephali	*Channa argus*	China	Gu et al. (2010)	EU185634
Trypanosoma pleuronectidium	*Melanogrammus aeglefinus*	Norway	Karlsbakk and Nylund (2006)	DQ016618
Trypanosoma rajae	*Raja* spp.	–	Unpublished	MG878996
Trypanosoma siniperca	*Siniperca chuatsi*	China	Gu et al. (2007a)	DQ494415
Trypanosoma sp.	*Carassius carassius*	Ukraine	Grybchuk-Ieremenko et al. (2014)	KJ601715
Trypanosoma sp.	*Scardinius erythrophthalmus*	Ukraine	Grybchuk-Ieremenko et al. (2014)	KJ601718
Trypanosoma sp. carpio	*Cyprinus carpio*	China	Gu et al. (2007b)	EF375882
Trypanosoma sp. CLAR	*Clarias angelensis*	Africa	Hamilton et al. (2004)	AJ620555
Trypanosoma sp. EL-CP	*Esox lucius*	Czech Republic	Maslov et al. (1996)	L14841

***Trypanosoma* species**	**Host**	**Locality**	**Reference**	**Accession number**
Trypanosoma sp. Marv	*Cyprinus carpio*	–	Unpublished	AJ620549
Trypanosoma sp. R6	*Abramis brama*	Poland	Unpublished	AJ620554
Trypanosoma sp. Ts-Ab-TB	*Abramis brama*	Czech Republic	Unpublished	AJ620556
Trypanosoma triglae	*Trigla lineata*	Senegal	Maslov et al. (1996)	U39584
Trypanosoma haploblephari	*Poroderma africanum*	South Africa	This chapter	–
Trypanosoma haploblephari	*Poroderma pantherinum*	South Africa	This chapter	MZ061641
Trypanosoma haploblephari	*Haploblepharus pictus*	South Africa	This chapter	MZ061638 MZ061640 MZ061642

Appendix 2
Chapter 4

Table

Table S2 Species of haemogregarines implemented in the phylogenetic analysis (Figure 4.2).

Haemogregarine species	Host	Locality	Reference	Accession number
Klossia helicina	*Cepaea nemoralis*	France	Barta et al. (2012)	HQ224955
Adelina grylli	*Gryllus bimaculatus*	Bulgaria	Kopecna et al. (2006)	DQ096836
Adelina bambarooinidae	*Dermolepida albohirtum*	–	Unpublished	AF494059
Hemolivia mauitanica	*Testudo marginata*	Greece	Kvicerova et al. (2014)	KF992699
Hemolivia parvula	*Kinixys zombensis*	South Africa	Cook et al. (2015)	KR069083
Hemolivia stellata	*Amblyomma rotondatum*	Brazil	Karadjian et al. (2015)	KP881349
Hemolivia mariae	*Ergnia stokesii*	Australia	Kvicerova et al. (2014)	KF992711
Karyolysus paradoxa	*Varanus albigularis*	South Africa	Cook et al. (2016)	KX011039
Karyolysus canariensis	*Gallotia atlantica*	Spain	Tome et al. (2018)	MG787243
Karyolysus galloti	*Gallotia galloti*	Spain	Tome et al. (2018)	MG787244
Karyolysus lacazei	*Lacerta agilis*	Poland	Haklová-Kočíková et al. (2014)	KF461940
Hepatozoon canis	*Vulpes vulpes*	Spain	Criado-Fornelio et al. (2006)	AY461378
Hepatozoon felis	Felis catus	Spain	Criado-Fornelio et al. (2006)	AY628681
Hepatozoon ursi	*Ursus thibetanus japonicus*	Japan	Kubo et al. (2008)	EU041717
Hepatozoon fitzimonsi	*Kinixys zombensis*	South Africa	Cook et al. (2015)	KR069084
Hepatozoon ayorgbor	*Python regius*	Ghana	Sloboda et al. (2007)	EF157822
Hepatozoon cf. *catesbianae*	*Lithobates catesbeianus*	France	Barta et al. (2012)	HQ224954
Hepatozoon theileri	*Ametia delalandii*	South Africa	Netherlands et al. (2014)	KP119773
Hepatozoon ixoxo	*Sclerophrys maculata*	South Africa	Netherlands et al. (2014)	KP119772
Haemogregarina sp.	*Pelusios subniger*	Mozambique	Dvořáková et al. (2014)	KF257925

Haemogregarine species	Host	Locality	Reference	Accession number
Haemogregarina balli	*Chelydra serpentina serpentina*	France	Barta et al. (2012)	HQ224959
Haemogregarina sacaliae	*Sacalia quadriocellata*	Vietnam	Dvořáková et al. (2015)	KM887507
Haemogregarina pellegrini	*Malayemys subtrijuga*	Vietnam	Dvořáková et al. (2015)	KM887508
Haemogregarina stepanowi	*Mauremys capsica*	Turkey	Kvicerova et al. (2014)	KF992697
Dactylosoma sp.	*Pelophylax lessonae*	Belgium	Netherlands et al. (2020)	MN879399
Dactylosoma kermiti	*Ptychadena anchietae*	South Africa	Netherlands et al. (2020)	MN879389
Dactylosoma ranarum	*Pelophylax klesculentus*	France	Barta et al. (2012)	HQ224958
Dactylosoma sp. A	*Poroderma pantherinum*	South Africa	This chapter	XXX
Dactylosoma sp. A	*Poroderma africanum*	South Africa	This chapter	XXX
Dactylosoma sp. A	*Haploblepharus pictus*	South Africa	This chapter	XXX
Dactylosoma sp. B	*Haploblepharus pictus*	South Africa	This chapter	XXX
Dactylosoma sp. B	*Haploblepharus pictus*	South Africa	This chapter	XXX

Appendix 3
Chapter 5

Tables

Table S3 List of leech species implemented in the phylogenetic analysis (Fig. 5.3).

Leech species	Locality	Reference	Accession number
Aestabdella abditovesiculata (Moore, 1952)	Hawaii, USA	Williams and Burreson (2006)	EF405599
Aestabdella leiostomi Burreson, 1991	Virginia, USA	Williams and Burreson (2006)	KP300764
Aff*. Johanssonia* sp. leech 093	California, USA	Goffredi et al. (2012)	AF003268
Aff. *Johanssonia* sp. leech 205	California, USA	Goffredi et al. (2012)	GU985466
Aff. *Johanssonia* sp. leech 235	California, USA	Goffredi et al. (2012)	KY861060
Aff. *Johanssonia* sp. leech DR235 juv	California, USA	Goffredi et al. (2012)	AY047318
Ambulobdella shandikovi A. Utevsky and S. Utevsky, 2018	Ross Sea, Antarctica	Utevsky and Utevsky (2018)	MH643798
Austrobdella bilobata Ingram, 1957	Tasmania, Australia	Williams and Burreson (2006)	AF003277
Austrobdella californiana Burreson, 1977	California, USA	Williams and Burreson (2006)	AY856048
Austrobdella sp. leech 333	California, USA	Goffredi et al. (2012)	AY336029
Austrobdella sp. leech 367	California, USA	Goffredi et al. (2012)	DQ414343
Austrobdella translucens Badham, 1916	South Australia, Australia	Williams and Burreson (2006)	AF116022
Baicalobdella torquata (Grube, 1871)	Russia	Utevsky and Trontelj (2004)	DQ414342
Bathybdella sawyer Burreson, 1981	East Pacific Rise	Williams and Burreson (2006)	DQ414340
Beringobdella rectangulata (Levinsen, 1881)	Bering Sea	Williams and Burreson (2006)	DQ414341
Branchellion parkeri Richardson, 1949	Tasmania, Australia	Williams and Burreson (2006)	EF405596
Branchellion ravenelii (Giard, 1851)	Gulf of Mexico	Williams and Burreson (2006)	DQ414309
Branchellion sp. leech 353	California, USA	Goffredi et al. (2012)	JX024167
Branchellion torpedinis Savigny, 1822	Virginia, USA	Siddall and Burreson, 1998	AF003265

Leech species	Locality	Reference	Accession number
Calliobdella lophii van Beneden and Hesse, 1863	Hordaland, Norway	Utevsky and Trontelj (2004)	AY336024
Calliobdella vivida (Verrill, 1872)	Virginia, USA	Siddall and Burreson (1998)	AF003260
Calliobdella vivida (Verrill, 1872)	Maryland, USA	Unpublished	KU905901
Caspiobdella fadejew (Epstein, 1961)	Ukraine	Utevsky and Trontelj (2004)	AY336020
Crangonobdella maculosa S. Utevsky, 2005	Tatar Strait, Russia	Utevsky and Trontelj (2004)	AY336025
Cystobranchus meyeri Hayunga and Grey, 1976	Tennessee, USA	Williams and Burreson (2006)	DQ414315
Cystobranchus respirans (Troschel, 1850)	Slovenia	Utevsky and Trontelj (2004)	AY336021
Cystobranchus virginicus Hoffman, 1964	North Carolina, USA	Williams and Burreson (2006)	DQ414317
Erpobdella monostriata Lindenfeld and Pietruszynski, 1890	Voronezh Oblast, Russia	Utevsky et al. (2015)	KP300764
Glossiphonia complanata L. 1758	Ontario, Canada	Siddall and Burreson (1998)	AF003277
Helobdella europaea Kutschera, 1987	N/A	Siddall and Budinoff (2005)	AY856048
Hemiclepsis marginata (O. F. Müller, 1774)	N/A	Utevsky and Utevsky (2018)	MH643798
Heptacyclus brunneus (Johannson, 1896)	New Brunswick, Canada	Williams and Burreson (2006)	DQ414321
Heptacyclus buthi (Burreson and Kalman, 2006)	California, USA	Williams and Burreson (2006)	DQ414322
Heptacyclus scorpii (Burreson and Kalman, 2006)	New Brunswick, Canada	Williams and Burreson (2006)	DQ414326
Heptacyclus virgatus Vasiliev, 1939	Sakhalin, Russia	Utevsky and Trontelj (2004)	AY336027
Hirudo orientalis S. Utevsky and Trontelj, 2005	Uzbekistan	Utevsky et al. (2007)	EF405599

N/A; not available.

Leech species	Locality	Reference	Accession number
Johanssonia arctica (Johansson, 1898)	Newfoundland, Canada	Williams and Burreson (2006)	DQ414320
Johanssonia arctica isolate Jarct E (Johansson, 1898)	Varangerfjord, Norway	Utevsky and Trontelj (2004)	AY336012
Johanssonia arctica isolate Jarct Pi4 (Johansson, 1898)	Sea of Okhotsk, Russia	Utevsky and Trontelj (2004)	AY336013
Johanssonia extrema	Kuril-Kamchatka Trench	Utevsky et al. (2019)	MK967944
Limnotrachelobdella okae isolate Lokae F (Moore, 1924)	Russia	Utevsky and Trontelj (2004)	AY336022
Megaliobdella cf. *szidati* ICDZE 143 Meyer and Burreson, 1990	Ross Sea, Antartica	Utevsky et al. (2007)	EF405592
Megaliobdella szidati Meyer and Burreson, 1990	Ross Sea, Antarctica	Utevsky et al. (2007)	EF405593
Moorebdellina biannulata (Moore, 1957)	Ross Sea, Antarctica	Utevsky et al. (2007)	EF405598
Myzobdella lugubris	Virginia, USA	Williams and Burreson (2006)	DQ414323
Notobdella nototheniae Benham, 1909	South Georgia Island, Antarctica	Williams and Burreson (2006)	DQ414329
Notostomum cyclostomum Johansson, 1898	Tatar Strait, Russia	Utevsky and Trontelj (2004)	AY336028
Nototheniobdella sawyer A. Utevsky, 1993	Ross Sea, Antarctica	Utevsky and Trontelj (2004)	AY336010
Oceanobdella sp. S. Utevsky, 2009	Sakhalin, Russia	Utevsky and Trontelj (2004)	AY336026
Oxytonostoma typica Malm, 1863	Finmarken Bank, Barents Sea	Utevsky et al. (2007)	EF405596
Ozobranchus branchiatus (Menzies, 1791)	Florida, USA	McGowin et al. (2011)	GU985466
Ozobranchus jantseanus Oka, 1912	Guangdong province, China	Liu et al. (2017)	KY861060
Ozobranchus margoi (Apathy, 1890)	Virginia, USA	Williams and Burreson (2006)	AF003268
Piscicola geometra (L., 1758)	Ukraine	Utevsky and Trontelj (2004)	AY336015

Leech species	Locality	Reference	Accession number
Piscicola milneri (Verrill, 1874)	Quebec, Canada	Williams and Burreson (2006)	DQ414337
Platybdella anarrhichae (Diesing, 1859)	Bering Sea	Williams and Burreson (2006)	DQ414335
Platybdella anarrhichae (Diesing, 1859)	Gusinaya Bank, Barents Sea	Utevsky et al. (2007)	EF405594
Pontobdella leucothela Schmarda, 1861[4]	Tasmania, Australia	Williams and Burreson (2006)	DQ414343
Pontobdella macrothela Schmarda, 1861[5]	Virginia, USA	Apakupakul et al. (1999)	AF116022
Pontobdella macrothela Schmarda, 1861[3]	Hawaii, USA	Williams and Burreson (2006)	DQ414341
Pontobdella macrothela Schmarda, 1861[3]	Virginia, USA	Williams and Burreson (2006)	DQ414342
Pontobdella macrothela Schmarda, 1861[3]	Heron Island, Australia	Williams and Burreson (2006)	DQ414340
Pontobdella muricata L. 1758	Gulf of Piran, Slovenia	Utevsky and Trontelj (2004)	AY336029
Pterobdella amara Kaburaki, 1921	Queensland, Australia	Williams and Burreson (2006)	DQ414334
Theromyzon tessulatum (O. F. Müller, 1774)	N/A	Light and Siddall (1999)	AY047318
Trachelobdellina glabra Moore, 1957	Antarctica	Utevsky et al. (2007)	EF405597
Trulliobdella capitis Brinkmann, 1948	Ross Sea, Antarctica	Utevsky and Trontelj (2004)	AY336030
Zeylanicobdella arugamensis da Silva, 1963	Borneo	Williams and Burreson (2006)	DQ414344

[4] Appears as *Stibarobdella tasmanica* (junior synonym) on GenBank.
[5] Appears as *Stibarobdella macrothela* on GenBank.

Table S4 Species of *Trypanosoma* Gruby, 1843 implemented in the phylogenetic analysis (Fig. 5.4).

***Trypanosoma* species**	**Host**	**Locality**	**Reference**	**Accession number**
Trypanosoma avium	*Anthochaera phrygia*	Australia	Šlapeta et al. 2016	KT728402
Trypanosoma binneyi	*Ornithorhynchus anatinus*	Australia	Paparini et al. 2014	KJ867148
Trypanosoma boissoni	*Zanobatus schoenleinii*	Senegal	Maslov et al. 1996	U39580
Trypanosoma chelodinae	*Emydura signata*	Australia	Jakes et al. 2001	AF297086
Trypanosoma murmanense	*Hippoglossus hippoglossus*	Norway	Karlsbakk and Nylund 2006	DQ016616
Trypanosoma pleuronectidium	*Melanogrammus aeglefinus*	Norway	Karlsbakk and Nylund 2006	DQ016618
Trypanosoma rajae	*Raja* spp.	–	Unpublished	MG878996
Trypanosoma triglae	*Trigla lineata*	Senegal	Maslov et al. 1996	U39584
Trypanosoma haploblephari	*Haploblepharus pictus*	South Africa	Pretorius et al. 2021	MZ061638 MZ061640 MZ061642
Trypanosoma morphotype A	*Poroderma pantherinum*	South Africa	Chapter 3	MZ061641
Trypanosoma sp.	*Poroderma africanum*	South Africa	This chapter	

Appendix 4

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Address: 18 Versfeld str, Yzerfontein, 7351
Telephone: 082 855 4784
Email: hdek@iafrica.com

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Signed at __Yzerfontein_____ on this _18th day of ___May_____ 2021_.

Photographer's signature: ____ _____

Photo (s) to be used:

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Photographer's name: Madelein Wolfaardt
Address: 33 Nautiluss Village, Gordon's Bay, WCape
Telephone: 082 890 8956
Email: madi@justscuba.co.za

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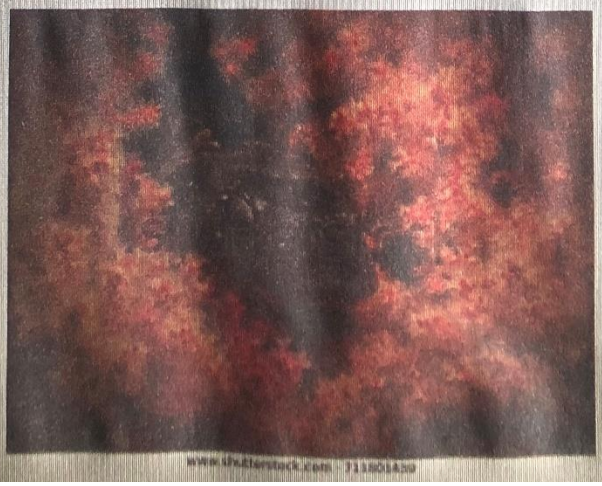
Photographer's signature: MWolf

Photo to be used:



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Photographer's name: Graeme Kruger
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Telephone:
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Photographer's name: Arno Jansen
Address: Erprather Str. 130, D-41466 Neuss, Germany
Telephone: +49 2131 475322
Email: mail@arno-jansen.de

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Photos of catsharks, taken in South Africa, near Simon´s Town

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