Morphology and functioning of attachment organs of the Polystomatidae (Monogenea)

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"The heavens are yours, and yours also the earth;
you founded the world and all that is in it."

- Psalm 89:11

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ABSTRACT

Monogeneans are mainly ectoparasitic on fish, but the family Polystomatidae radiated onto tetrapods and can be found on the skin and gills of the Australian lungfish, in the urinary bladder of frogs, gills and skin of salamanders, cloaca and phalodeum of caecileans, on the eye, nostrils, mouth, cloaca or urinary bladder of freshwater turtles, and on the eye of the hippopotamus. These host organisms are ecologically related through their association with freshwater habitats that favour parasite transmission. Firm attachment is critical to maintain a close relationship with their hosts. Attachment organs usually comprise of several units that are semi related to each other due to the need to form a functional unit. Interactions between subunits are expected to be under stabilising selection, and therefore hinder evolutionary change. Monogeneans are renowned for their effective posterior attachment structures in the form of hooks or hamuli and suckers that secure them, permanently or semi-permanently, to their hosts. The aim of this study was to investigate the morphology and functioning of attachment organs of selected polystomes representing different genera. A number of genera were selected in the study of attachment structures, genera included: *Protopolystoma*, *Polystoma*, *Eupolystoma*, *Neopolystoma*, *Polystomoides* and *Oculotrema*. Light microscopy and scanning electron microscopy was used to study the external morphology. Histology followed by light microscopy, confocal microscopy and enzyme digestion techniques followed by scanning electron microscopy was used to study the internal morphology. It was found that variation in haptoral components do exist, even among congeners, living for example in the bladder and oral cavity of the same host. Environmental factors relating to host ecology need to be taken into account when studying the morphology of monogenean haptors. Such factors play an important role in the adaptation of monogeneans and have possibly led to the change in microhabitats, which in turn explain the variation of haptoral components between parasites. Not all haptoral structures necessarily function in attachment throughout the entire life of the parasite and different haptoral structures are important for attachment to the host at different developmental stages of the parasite.

Key words: Polystomatidae, Monogenea, Morphology, Attachment organs, Functioning of attachment organs, and Adaptation.

OPSOMMING

Parasiete in die klas Monogenea is hoofsaaklik ektoparasieties op vis gashere, maar spesies in die familie Polystomatidae kan op die vel en kieue van die Australiese longvis, in die uriene blaas van paddas, kiewe en vel van salamanders, kloaak en geslagsbuis van wurmamfibieërs, op die oog, neus, mond, kloaak of blaas van varswater skilpaaie en op die oog van die seekoei gevind word. Die ekologiese verwantskap tussen al die bogenoemde gasheer organismes is hul assosiasie met varswaterhabitatte, wat ‘n vereiste is tydens die oordrag van parasiete. Die stewige vashegting van ‘n parasiet op die spesifieke gasheer is krities om 'n noue verbinding met hul gasheer te verseker. Vashegtingsorgane bestaan gewoonlik uit verskeie eenhede wat semi verwant is aan mekaar om uiteindelik 'n funksionele eenheid te vorm. Daar is ‘n moontlikheid dat interaksies tussen subeenhede onder stabeliserende seleksie is en daarom die kanse vir evolusionêre verandering verminder. Monogeniëers is bekend vir hul effektiewe posterior veshegtingsorgane, in die vorm van hake of hamuli en suiers, wat hulle permanente of semi-permante vashegting op spesifieke gashere verseker. Die doel van hierdie studie was om die morfologie en funksionering van die vashegtingsorgane van verskeie geselekteerde polystoom genera van die familie Polystomatidae te ondersoek. Die lys van geselekteerde genera is as volg: *Protopolystoma*, *Polystoma*, *Eupolystoma*, *Neopolystoma*, *Polystomoides* en *Oculotrema*. Lig- en skandeerelektronmikroskopie tegnieke is gebruik om die uitwendige morfologie te bestudeer. Histologie gevolg deur ligmikroskopie, konfokalemikroskopie en ensiemverteringstegnieke gevolg deur die skandeer-elektronmikroskopie is gebruik om die interne morfologie te bestudeer. Daar is gevind dat variasie in vashegtingskomponente bestaan, selfs onder spesies wat in dieselfde gasheer woon, byvoorbeeld in die blaas en mondholte van dieselfde gasheer. Dit is belangrik om die omgewingsfaktore wat verband hou met die ekologie van die gasheer in ag te neem wanneer die morfologie van monoginiëer vashegtingsorgane bestudeer word. Sulke faktore speel 'n belangrike rol in die aanpassing van die monogeniëerparasiete en het moontlik gelei tot die verandering in mikrohabitatte, wat op sy beurt die variasie van vashegtingskomponente tussen parasiete verduidelik. Nie alle vashegtingsstrukture funksioneer deurlopend in die hele lewe van die parasiet nie en verskillende vashegtingsstrukture is belangrik vir vashegtinging op die gasheer by verskillende ontwikkelingstadiums van die parasiet.

Sleutelwoorde: Polystomatidae, Monogenea, Morfologie, Vashegtingsorgane, Funksionering van vashegtingsorgane, en Aanpassing.

“A picture is worth more than a thousand words”

Chapter 1

General introduction and literature overview

CONTENTS

1.1 General introduction to parasitism:

Life is far from simple and it contains a multitude of different species within a fluctuating environment. Adaptation is the key driving force for the survival of species, and species that do not adapt are likely to perish, and are greatly influenced by natural selection. Adaptation leads to speciation, an on-going process where an ancestral species gives rise to two daughter species that cease to interbreed (Combes, 2005). Interactions between species are inevitable and associations are not restricted to members of the same species and can occur between two species that have followed two different lineages, this process is known as symbiosis. Parasitism occurs in a great majority of the cases. Parasites are organisms that find their ecological niche in or on another organism - the host, by feeding on it and displaying a certain degree of structural adaptation towards it (Araújo *et al*., 2003; Poulin, 2011). Parasitism is common among multicellular organisms, and more organisms are parasitic than non-parasitic. Natural selection favour hosts that are not only capable of successfully transmitting their genes to subsequent generations, but are also likely to defend themselves against parasites, and *vice versa* – parasites that transmit their genes best to the next generation are those that best exploit their host (Combes, 2005).

In the light of host-parasite evolution, hosts seem to 'accommodate' parasites while parasites 'do not seem to harm the host too much' (Van der Linde *et al*., 1984). Hosts seem to offer a more predictable environment than the stochastic environment they find themselves in. Even though the environments seem more stable, parasites that generally infect these host organisms have complex nervous systems and sensory structures that are similar or more advanced than their hosts, demonstrating that parasite environments may also contain different levels of heterogeneity (Thomas *et al*., 2002). Parasites' morphology may appear simplified, but over time have become progressively adapted to their parasitic way of life (Combes, 2005). The relationship between host and parasite, along with parasite and the external environment has profoundly changed over time, especially in terms of nutrition and reproduction.

Every living organism has a life cycle, a beginning and an end. Several changes take place during the life cycle, from birth until maturity, especially with regards to morphological and physiological changes. An increase in intensity of such changes take place when a species exploits more than one environment during the course of its life, for example a tadpole initially inhabits an aquatic environment before undergoing metamorphoses into an adult that subsequently inhabits a terrestrial or semi-terrestrial environment. Parasites exploit at least two different habitats; one being in or on the host (immediate environment) and the other during switching between two individual hosts (the ecosystem) (Thomas *et al*., 2002). Transitional habitats can either be the external environment or in or on another living organism. Many parasites have intermediate hosts. Humans, for example, are intermediate hosts for many parasites such as *Schistosoma* and *Plasmodium*. Some species, such as the trematode, *Halipegus ovocaudatus* have up to five stages (Combes, 2005). More than one life stage is a result of natural selection and increases, rather than decreases, the probability of successful completion of a parasite's life cycle.

Interaction between the parasite and host starts after the parasite successfully encounters the host by means of signals broadcasted by the host. Signals vary and can either be visual, olfactory, or acoustic. Parasites are specifically adapted to recognise certain characteristics and signals from a certain host, especially strict host-specific parasites. Once a parasite survives the encounter process, it migrates to the precise microhabitat within the host and reproduces (Kennedy, 1975; Combes, 2005). Compatibility is described by Combes (2005) as a lasting, intimate interaction between two partners. Natural selection works in the parasite genome to open two filters, namely encounter and compatibility; while on the contrary, natural selection works to close the filters in the host (Combes, 2005). The host's two lines of defence are behavioural and immunological. Behavioural defence aids in prohibiting parasites to encounter hosts, while immunological defence helps protect the host after an invasion of parasites have taken place. However, the encounter and compatibility filters are furthermore strongly influenced by environmental factors.

Parasites respond to a variety of cues, and to fully understand the sensory perception of parasites is almost impossible, however, their behavioural responses to environmental signals provide but a slight insight into their worlds. One such example of behavioural adaptation is among the bladder-inhabiting monogeneans, namely *Pseudodiplorchis americanus* (Rodgers and Kuntz, 1940) Yamaguti, 1963, whose transmission is limited to a window of opportunity as short as a single day in a year when their amphibian hosts emerge from underground to breed in water (Tinsley, 1982; Tocque and Tinsley, 1994). This supports the idea that parasites have the ability to detect and respond to subtle changes in the immediate environment of the host (Thomas *et al*., 2002).

Host-specificity or the restriction of parasites to a particular host species, or group of host species, is common worldwide. Specificity does not only involve close interaction with the host, but also structural and physiological adaptation, and is furthermore dependent on the length of association and stability of the environment (Hayunga, 1991). Specificity is said to improve the time it takes a parasite to recognise and respond to a particular habitat (Combes, 2005). It is also suggested that specificity improves a parasite's fitness (the reproductive success of species), since parasites only focus on specific organs, and consist over a method to cope with the immune system of a single host species or limited range of hosts (Whittington *et al*., 2000). A parasite's life cycle is not complete through simply finding a definite host, parasites also have to find the specific site of infection within the host, a site where the parasite can survive and reproduce. In some cases this site has very specific boundaries, even within a large organ, such as the intestine. Fitness is also reliant on how close parasites get to and attach to the optimal attachment site – achieved by effective attachment organs (Poulin, 2011). Attachment organs usually comprise of several units that are semi related to each other due to the need to form a functional unit. Interactions between subunits are expected to be under stabilising selection, and therefore hinder evolutionary change. Most host and micro-habitat restriction is universal among parasites, although its degree varies between the species and groups (Rohde, 1979).

The occurrence of parasites within different groups of living organisms vary, for example, among metazoans most Platyhelminthes (flatworms) are parasitic, within nematodes (roundworms) approximately half are parasitic and the other half free-living species while none parasitic species are found among Echinoderms (sea urchins and starfish) (Combes, 2005). Within parasitic flatworms (Platyhelminthes), four main lineages are identified, namely: Turbellaria (free living flatworms), Monogenea (monogenetic flukes), Trematoda (digeneric flukes) and Cestoda (tapeworms). Turbellarians include free-living and parasitic species, while the other three classes are entirely parasitic.

1.2 Monogenea:

Monogeneans belong to one of the largest classes within the phylum Platyhelminthes, estimated to comprise roughly of 20 000 species, and are among the most host-specific of parasites in general (Ramasamy and Brennan, 2000; Whittington *et al*., 2000). Odhner (1912) classified monogenean subclasses into two groups, namely: Monopisthocotylea and Polyopisthocotylea; Monopisthocotylea feed on epithelia and Polyopisthocotylea feed predominantly on blood (Halton and Jennings, 1965). This group of parasites maintain a direct life cycle with no intermediate hosts. Eggs are deposited either inside the host or in the external aquatic environment from which free swimming ciliated larvae (better known as oncomiracidia) emerge. The lifespan of an oncomiracidium has been documented as no longer than 24 h (Llewellyn, 1963) and consists of two phases. Firstly a free swimming phase in search of a host and secondly a gliding or looping phase on or in the host in search for specific attachment site. Oncomiracidia are released in order to infect other host individuals or possibly the same host. Within internal life cycles of monogeneans such as certain *Eupolystoma* species, eggs are deposited in the bladder of the host between urinations in order for unciliated oncomiracidia to emerge and attach themselves directly to the

bladder wall of the same host (autoinfection) (Tinsley, 1977, Tinsley, 1990). According to Kearn (1994), the development of a permanent association between early monogeneans and their hosts had conservation of energy as result since the parasites no longer need to actively search the external environment for food.

Once established on a host, oncomiracidia discard ciliated epidermis cells (hair-like structures on the surface of oncomiracidia used for locomotion through water in search of a suitable host), when it is no longer of use during development of the parasite (Du Preez and Kok, 1987). Parasites subsequently move across the surface of hosts in a leech-like fashion, known as looping; the extension and contraction of the body and temporary attachment. Monogeneans might provoke the host's anti-parasite defences and the host has the ability to recognise foreign molecules or groups of molecules that are not part of its own and resist these attacks through immune responses. The immune system mechanisms apply immense selective pressure against pathogenic agents. Parasites that are fit enough survive and reproduce (Combes, 2005). Monogeneans successfully inhabit a variety of surfaces on or within their hosts, but are mainly ecto-parasitic on the branchial cavities of fish and are highly adapted for attachment to the external surfaces.

Firm attachment is critical for many parasites to maintain a close relationship with their hosts and suitable attachment organs will greatly determine the success of species. Many groups of parasites have perfected this system. Monogeneans are renowned for their effective posterior attachment structures that secure them, permanently or semi-permanently, to their hosts. Fish-infecting monogeneans live on the epidermis, scales, fins, lip folds, nares, branchiostegal membranes and gills (Whittington *et al*., 2000).

1.3 Adaptations of monogeneans to different environmental pressures:

Adaptive processes have led to the presence of high morphological variability of attachment organs in monogeneans, for example, the morphological evolution of the opisthaptor (Morand *et al*., 2002; Vigon *et al*., 2011). Monogeneans attach to their host either through anterior attaching organs known as the prohaptor or more often by the posterior opisthaptor. The opisthaptor is primarily associated with attachment in order to remain on the host, while prohaptor developments may also be associated with feeding activities (Wright and Dechtiar, 1974). Parasites can attach physically; the opisthaptor allows the attachment onto hosts through the use of hooks, suckers, clamps, and/or chemical-bonding (using bio adhesives) (Buchmann and Lindenstrøm, 2002). According to Kearn (1994), secretion of adhesives might have served as early attachment of monogeneans, which are commonly found in present-day free-living platyhelminthes for temporary attachment to substrates. It is believed that the first monogeneans were small skin parasites that only made use of small marginal hooks to attach themselves to the host's epidermis (Kearn, 1994). Hooks aided in the attachment to host epidermal cells, limiting the force and size of each to avoid separation of the pierced epidermal cell membranes, restricting not only the size of the hooks but also on the overall size of the parasite. However, few present-day adult monogeneans are small enough to be sustained by marginal hooks alone, therefore this method of attachment only persists in oncomiracidia. The circular arrangement of 14 or 16 marginal hooklets (number varies in species) across the cup-shaped haptor aids in spreading the weight and the size of the parasite. The posterior position of the hook bearing haptor enables freedom for the anterior end for feeding (Kearn, 1994). The general hypothesis is that the development of larger monogeneans and their survival on more active hosts led to the appearance of one or two pairs of larger hooks (hamuli), providing a more stable anchorage by penetrating the tough, fibrous dermal layer of the skin, as well as the development of suckers. The incorporation of suckers into the haptor was a significant advance in the development of the monogeneans (Kearn, 1994). Suction as means of attachment to

the skin of the host has been proven efficient, judging by the fact that it is present among several monogeneans, along with other skin-parasitic invertebrates such as leeches and crustaceans. Kearn (1994) also made the statement that the hooks originally might have served as internal attachment sites within parasites, only later obtaining a secondary function for attachment to the host. The attachment of monogeneans such as head organs, anchors, suckers and clamps provided with muscle fibres help them to function efficiently and effectively.

Apart from the haptoral sclerites, the musculature systems of adult monogeneans also play an important role in host-locomotion, attachment, feeding and reproduction (Lim, 2008). Mair *et al*. (1998), identified three main muscle systems within adult flatworms; 1) somatic muscles used in locomotion and movement, 2) muscles functioning with attachment, and 3) muscles of the alimentary and reproductive tracts and copulatory organs. For the purpose of this study we only focused on the muscles associated with the attachment. Muscle fibres assist in the efficient and effective functioning of monogenean attachment organs such as anchors, suckers and clamps. The association between muscles and haptoral attachment have been noted in a number of monogeneans. In some cases muscles attach to the root of the anchors and accessory sclerites, assisting in the formation of suction at the haptor and facilitate the insertion of anchors or assist in the grasping of mechanisms. In other cases the musculature assists in the grasping of host tissue (Lim, 2008).

Monogeneans are typically soft-bodied organisms, and therefore very plastic in body shape. Their hard sclerotised sclerites are taxonomically important and morphologically the most informative structures for the separation of species; including marginal hooklets, followed by the hamuli and then the ventral bar (Garcia-Vasquez *et al*., 2012). These hard structures consist of scleroproteins (Lyons, 1966); assisting in differentiation among species and are considered to have functional, taxonomic and evolutionary significance (Ramasamy and Brennan, 2000). Sclerotised structures include the anchors, clamp sclerites (hamuli and marginal hooklets) and suckers, and the genital apertures. Marginal hooklets aid in the attachment of oncomiracidia to the

host, and as the parasite matures these marginal hooklets lose their function, since they are replaced by developing suckers. They are, however, still retained in the tissue and can often still be measured in flattened specimens. Since these structures are retained in the adult parasites and since their morphology is stable within a species, it makes marginal hooklets of polystomes important taxonomic characters (Du Preez and Maritz, 2006).

In order to adapt to their different hosts and environments, monogeneans have specialised in attachment organs and mode of attachment (Chisholm and Whittington, 1998), as well as attachment areas. This adaptation relates predominantly to morphological specialisation for posterior attachment by the haptor of the parasites (Cribb *et al*., 2002). Changes of habitat by monogeneans from fish to other hosts have also revealed a tendency to abandon the exposed ectoparasitic mode of life for an enclosed meso- or endoparasitic lifestyle. Poulin (2011) mentioned that an internal environment is in general more predictable than the external environment (since all conspecific hosts are virtually identical in construction and function with organs performing the same function or secreting the same chemicals). Adaptations in these more predictable conditions are likely to spread to other members of the population, for example, any behaviour increasing the chance of arriving at the correct site of infection would be favoured (Poulin, 2011). Some of the new habitats can still be considered as external such as the nasal cavities, while other habitats are truly internal, such as the oesophagus, the stomach and the urinary bladder (Euzet and Combes, 1998). The natures of the substrate to which these parasites attach, along with water currents, play an important role in the adaptation of attachment organs and associated structures. Chisholm and Whittington (1998) demonstrated that the complexity of the haptor can be related to the habitat of the parasite. Parasites that live in habitats exposed to strong water currents, such as the gills and dorsal skin surface, generally have more complex haptors compared to those in environments exposed to weaker or no water currents, such as the nasal fossae, urogenital system and body cavity. It also appears that different haptoral structures are important for attachment to the host at different stages in the development of the parasite. Monogeneans that are subjected to less disturbed

conditions (internal sites) have developed a need for simpler attachment organs (Euzet and Combes, 1998). The biology of *Polystoma* (infecting anurans) is an excellent example of such transition. When oncomiracidia attach to the gills of very young tadpoles, they mature into adult parasites known as neotenics and lay eggs, but die during metamorphosis of the host. When oncomiracidia attach to older tadpoles, they remain on the gills, undergoing very little to no growth, and migrate to the urinary bladder at metamorphosis where they mature into adults. Neotenic forms differ morphologically from parasites found in the urinary bladder and the question still remains; why species of *Polystoma* are capable of expressing two dissimilar sets of developmental genes (one on tadpole gills and one in adult urinary bladder) (Euzet and Combes, 1998)? While in most other genera of polystomatids the oncomiracidia directly invade the adult host.

In order for parasites to reproduce and perform optimally they need access to appropriate nutrients (Buchmann and Lindenstrøm, 2002). Species found on the gills or in the buccal cavity or urinary bladder, feed mainly on blood, epithelium and mucus; while skin parasites feed on epidermal cells. Analysis of species of *Polystomoides, Polystomoidella* and *Neopolystoma* polystomatids which infect chelonians, along with *Oculotrema,* infecting hippopotamus, has shown that this group have diverged nutritionally from related parasites. No haemoglobin was found in the gut caeca (Allen and Tinsley, 1989). Most monopisthocotyleans and many juvenile polyopisthocotyleans are exceedingly mobile and move by alternating attachment of the posterior haptor and the anterior region. Monogeneans move across surfaces in a leech-like fashion known as looping (appropriate and coordinated extension and contraction of the body musculature, somatic muscles) and it is possible that anterior attachment by polyopisthocotyleans makes more use of suction than adhesion (Whittington *et al*., 2000).

The origin of sclerites are not fully understood and variations may be controlled by phylogeny and local adaptation to a host or local environment (Vignon *et al*., 2011). This leads to the idea that ectoparasitic species that inhabit a mutual habitat on a host

should show similarities in attachment organs (Morand *et al*., 2002). Monogeneans are often considered unique in terms of specialisations that are exceptional in animal evolution (Tinsley, 2004). Differences between specimens from different hosts could be due to different fixation and different degrees of maturity, but hard parts such as genital and marginal hooks are unlikely to be affected by either. Parasites such as monogeneans have developed different strategies and possess various specialised organs adapted to their micro-environment within the hosts, thus ensuring their evolutionary success (Vignon *et al*., 2011). These specialised organs are well displayed among the Polystomatidae.

1.4 Polystomatidae:

Polystomatidae is an example of a monogenean group that has radiated on to semi-terrestrial vertebrates, infecting relative internal sites, mainly the urinary bladder. Polystomatidae is not one of the most diverse groups in comparison with the monogenean class as a whole, but polystomes are widely distributed across the globe, with the exception of Antarctica and desert areas. *Polystoma* is the most diverse among the 24 known polystomatid genera, with 74 nominal species representing about one-third of the total number of species described in the family. The genera presently known include *Concinnocotyla* Pichelin, 1991 from the skin and gills of the Australian lungfish *Neoceratodus forsteri* Krefft, 1870, *Diplorchis* Ozaki, 1931; *Eupolystoma* Kaw, 1950; *Kankana* Rahariviololoniaina, 2011; *Madapolystoma* Du Preez, 2010*; Mesopolystoma* Vaucher, 1981; *Metapolystoma* Combes, 1976; *Neodiplorchis* Yamaguti, 1963; *Neoriojatrema* Imkongwapang and Tandon, 2010; *Parapolystoma* Ozaki, 1935; *Parapseudopolystoma* Nasir and Fuentes Ambrano, 1983; *Polystoma* Zeder, 1800; *Protopolystoma* Bychowsky, 1957; *Pseudodiplorchis* Yamaguti, 1963; *Riojatrema* Lamothe-Argumento, 1964; *Sundapolystoma* Lim and Du Preez, 2001, and *Wetapolystoma* Gray, 1983 from the kidneys and urinary bladder of frogs; *Pseudopolystoma* Yamaguti, 1963 and *Sphyranura* Wright, 1879 from the gills and skin

of salamanders; *Nanopolystoma* Du Preez, *et al.*, 2008 from the bladder and phalodeum of caecileans; *Neopolystoma* Price, 1939; *Polystomoidella* Price, 1939 and *Polystomoides* Ward, 1917 from the eye, nostrils, mouth or urinary bladder of freshwater turtles and *Oculotrema* Stunkard, 1924 from the eye of the hippopotamus (Du Preez *et al*., 2008; Verneau *et al.*, 2009).

1.5 Selection of species to be studied

Phylogenetics is a dynamic discipline and aims to contribute to the understanding of the phenomenon of life (Wiley and Lieberman, 2011). Phylogenetic relationships within Platyhelminthes and Monogenea has been well studied (Bychowsky, 1957; Llewellyn, 1970; Malmberg, 1990; Rohde, 1990; Boeger and Kritsky, 1997), as well as for the Polystomatidae (Verneau *et al.,* 2002; Bentz *et al*., 2006; Badets *et al.*, 2011). Current phylogenetic trees are based on molecular evidence. However, available molecular trees for the Polystomatidae were obtained from only a selected few of the 24 polystome genera for which suitable material were available. We decided to conduct a study and to apply cladistics protocols based on 53 morphological characters for all 24 polystome genera and then to compare this tree with a tree based on molecular evidence. On the basis of the tree obtained (Chapter 4) and the material available, we selected a few species of polystomes. Genera that will be represented in the current study include *Protopolystoma*, *Polystoma*, *Eupolystoma*, *Polystomoides*, *Neopolystoma* and *Oculotrema*.

1.6 Life cycles of genera studied:

1.6.1 *Protopolystoma*:

Protopolystoma, like all other monogeneans, has a direct life cycle with no intermediate host involved (Figure 1.1). Egg production starts 3–4 months post-infection

at 22 °C (Tinsley and Owen, 1975; Tinsley and Jackson, 2002). Since *Protopolystoma xenopodis* has no uterus, eggs are continually expelled directly into the host's urinary bladder (Tinsley, 2004). When a frog urinates, eggs, together with urine, are expelled into the external environment. Infective oncomiracidia hatch from an opercular egg after approximately 22 days. The larva, also known as the oncomiracidium, is the infective stage and swims and actively searches for a potential host. Oncomiracidia can actively swim for up to 24 h and once contact has been made with a potential host, the oncomiracidium enters the cloaca and migrates to the kidneys (Thurston, 1964). Developing parasites establish inside ducts in the kidney where they attach and feed on blood. They develop within the kidneys for approximately 2–3 months, and subsequently migrate to the urinary bladder via the urinary duct, where they continue to develop and reach maturity after 3–4 months post infection.

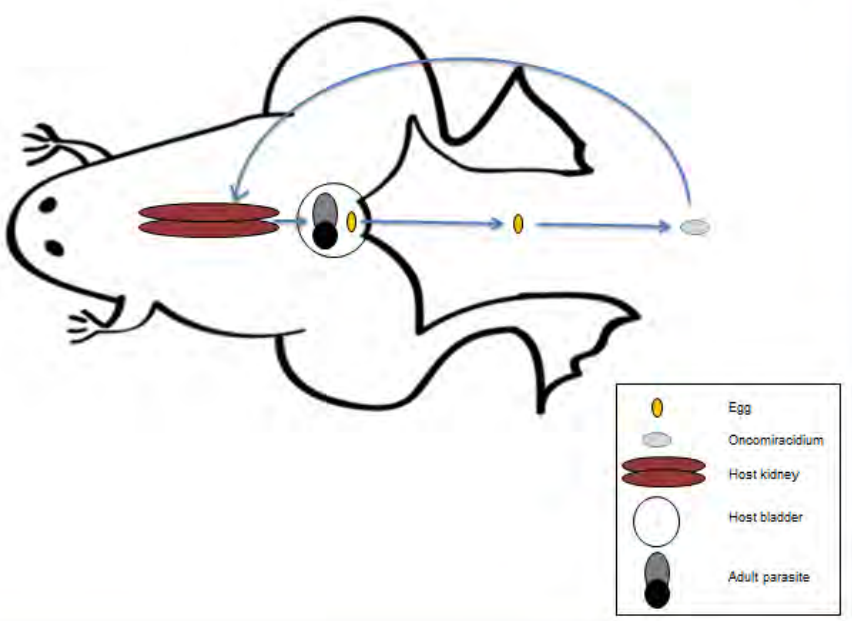


Figure 1.1: Life cycle of *Protopolystoma xenopodis*.

1.6.2 *Eupolystoma*:

Eupolystoma have two possible life cycles; internal and external. Parasites accumulate eggs in the uterus where they develop. Ciliated and unciliated oncomiracidia hatch immediately upon release within the bladder and emerge when stimulated by change in osmotic pressure, due to the influx of water into the urinary

bladder (Combes *et al*., 1973; Fournier and Combes, 1979). Ciliated oncomiracidia leave the host and swim into the external environment where they actively search for another or possibly the same host to infect (external cycle as in Figure 1.2 A). Unciliated oncomiracidia attach directly to the host's bladder wall alongside its parents (internal cycle - auto infection, as in Figure 1.2 B), (Combes *et al*., 1973; Tinsley, 1990). In the case of the external cycle; oncomiracidia enter the cloaca, in some species oncomiracidia migrate via the Mulerian ducts and the kidneys to the bladder, and in other species complete larval development takes place solely in the bladder, where they then establish and reach maturity (Tinsley, 1978a). Unciliated oncomiracidia attach beside parent parasites within the same host and reach maturity. The occurrence of auto infection is confirmed in a study by Du Preez *et al*. (2003), discovering both immature and mature forms of *Eupolystoma vanasi* within host individuals. Auto infection often results in large numbers of *Eupolystoma* commonly found within the bladders of frog hosts. As many as 2 000 *Eupolystoma anterorchis* individuals were found in a single *Amietophrynus pantherinus* host individual (Combes *et al*., 1973; Tinsley, 1973). Large numbers are accommodated by the huge and vascularised urinary bladders of toads.

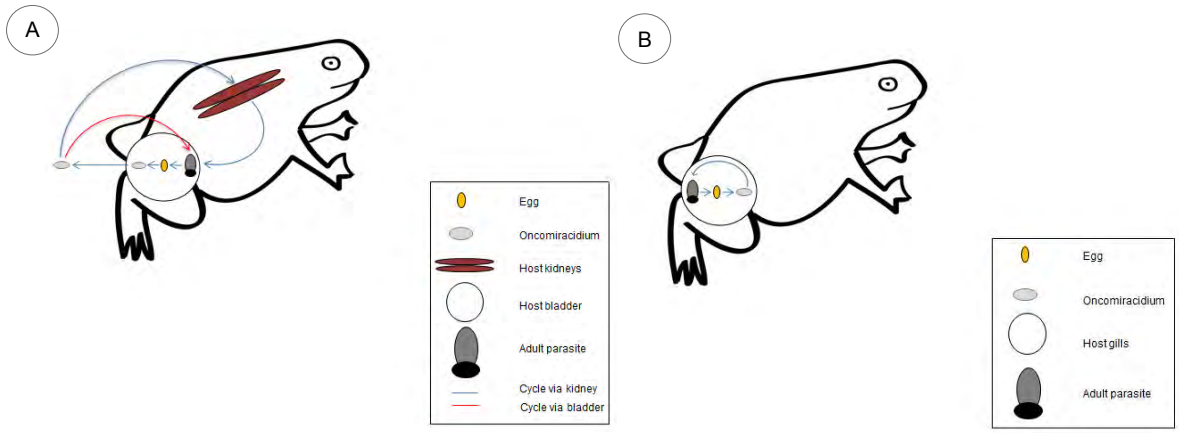


Figure 1.2: (A) External life cycle of *Eupolystoma*. **(B)** Internal life cycle of *Eupolystoma*.

1.6.3 *Polystoma*:

Some species of *Polystoma* and *Metapolystoma* differ from other polystomes by having a neotenic phase in the life cycle (Murith *et al*., 1977; Du Preez and Kok, 1998) (Figure 1.3). *Polystoma* species parasitise the tadpoles and adults of mesic anurans.

The life cycles of parasite and host are closely synchronised and parasites release eggs when frog hosts reproduce (Tinsley, 1978b). After hatching from an operculated egg (Llewellyn, 1957), oncomiracidia actively swim around in search of a suitable host tadpole. After spawning, frog eggs develop rapidly and after hatching, tadpoles remain in the area for the first few days. Since polystome eggs only hatch after a period of about 10–16 days, it brings the oncomiracidium in close proximity of potential host tadpoles. Once contact has been made with a tadpole, of the specific host species, the oncomiracidium will remain on the tadpole until it locates the spiracle, where it enters and subsequently establish on the internal gills (Du Preez *et al*., 1997). If an oncomiracidium makes contact with a non-host tadpole it will break contact and continue to swim in search of another host tadpole (Du Preez *et al*., 1997). In the specific host tadpole the oncomiracidium attach to the gills where it starts feeding on blood. If the tadpole happens to be a young tadpole in pre-metamorphosis, the oncomiracidium rapidly develops over a period of approximately 16 days into an egg producing neotenic parasite. The neotenic parasites' role is to reproduce and boost the parasite population. They have a short life span and die as soon as the front legs of the developing metamorph break through. If an oncomiracidium attaches to an older tadpole in pro-metamorphosis, it remains on the gills, undergoing very little development. The hamulus primordial start to develop into hamuli and when the front legs of the developing frogs break through, the parasites migrates to the outside of the host, over the surface of the tadpole and enters the cloaca. It then crawls to the urinary bladder where it attach and start to feed on blood (Williams, 1961, Combes, 1968). Within the urinary bladder it slowly develops and matures into an adult parasite that will start producing eggs during the next breeding season (Kok and du Preez, 1987).

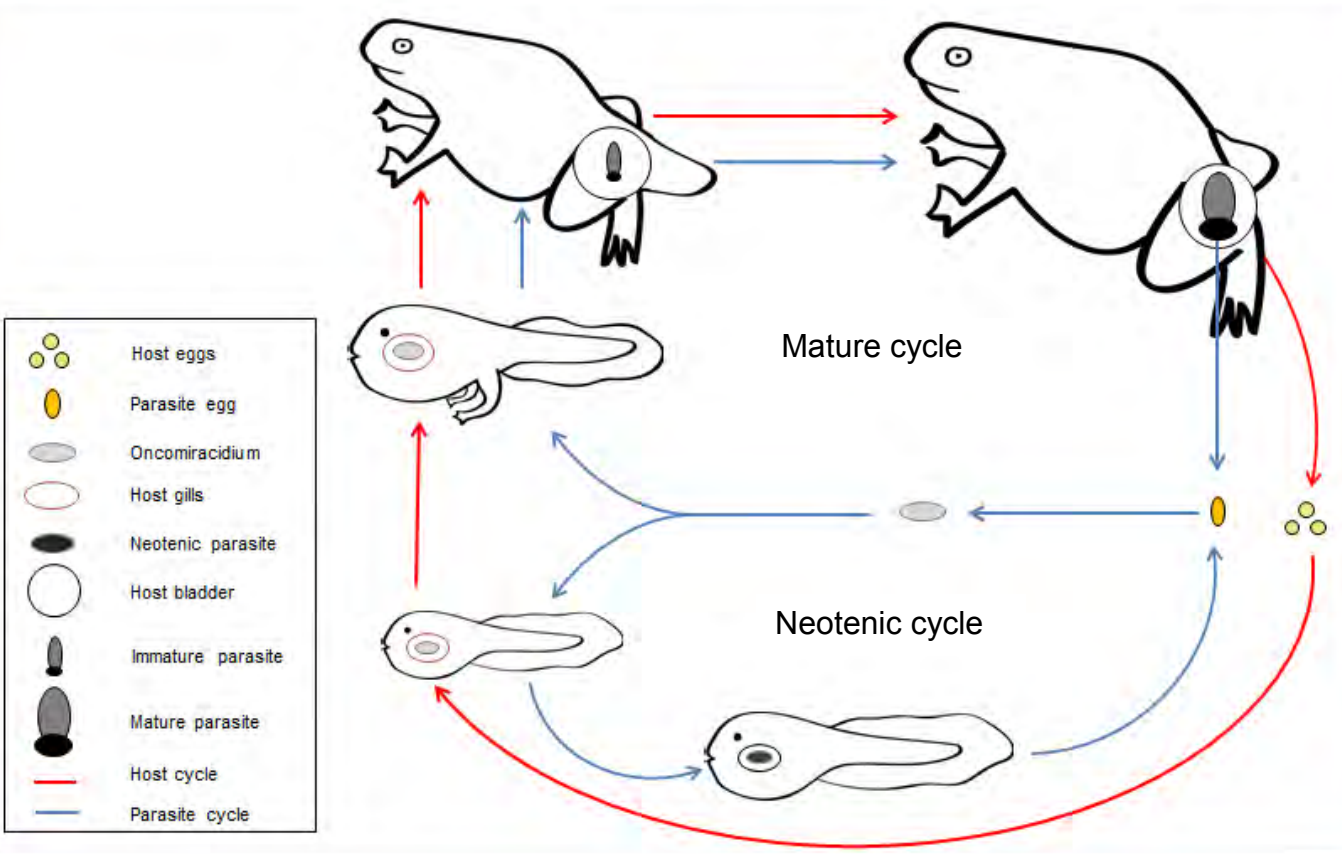


Figure 1.3: Life cycle of *Polystoma*.

1.6.4 *Neopolystoma* and *Polystomoides*:

Oncomiracidia hatch from operculated eggs and actively search for a potential host within the water body. Once a suitable host is found, depending on the site specificity of the species, the oncomiracidium either establish in the urinary bladder (Figure 1.4 A), the oral region (Figure 1.4 B), or on the eye (Figure 1.4 C). Oncomiracidia develop into mature parasites and start producing eggs. Eggs are expelled almost continuously without long delays due to host's association with water.

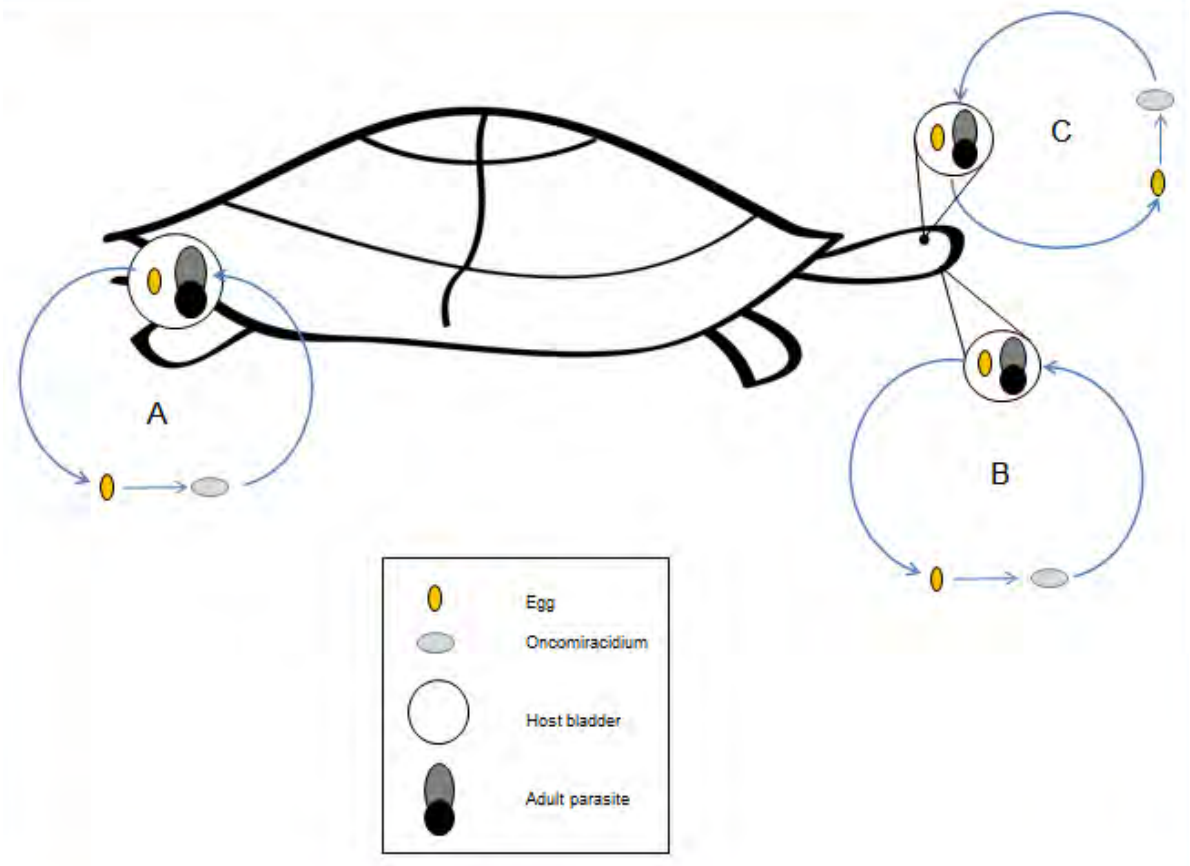


Figure 1.4: Life cycles of chelonian infecting polystomes: **(A)** Bladder (*Neopolystoma* sp., *Polystomoides* sp., and *Polystomoidella* sp.); **(B)** Oral (*Neopolystoma* sp. and *Polystomoides* sp.); **(C)** Eye (*Neopolystoma* sp.).

1.6.5 *Oculotrema*:

Oculotrema hippopotami is found under the eyelid where they attach to the surface of the hippopotamus eye. Eggs expelled into the external environment hatch after approximately 20 days at 30 ºC (Figure 1.5). Oncomiracidia actively search for potential new hosts or possibly the same host. It seems almost impossible for an oncomiracidium to find a new host after hatching within a vast natural water body, and when the host is found, to locate the eye before dying. On the contrary, high levels of infections (prevalence of > 90%) have been reported (Thurston, 1968; Du Preez and Moeng, 2004). Developing eggs have been found within the mucous of the hippopotamus eye and confirms the possibility of an internal life cycle. Not only do the eggs confirm this,

but also the different life stages, presence of mature and immature worms, on the same eye. Auto-infection and re-infection are therefore both likely to occur. Hippopotami are very social mammals and have a close physical relationship with offspring, enhancing chances of cross infection onto new hosts. In a study done by Thurston (1968), a high rate of infection in hippopotamus claves was also reported.

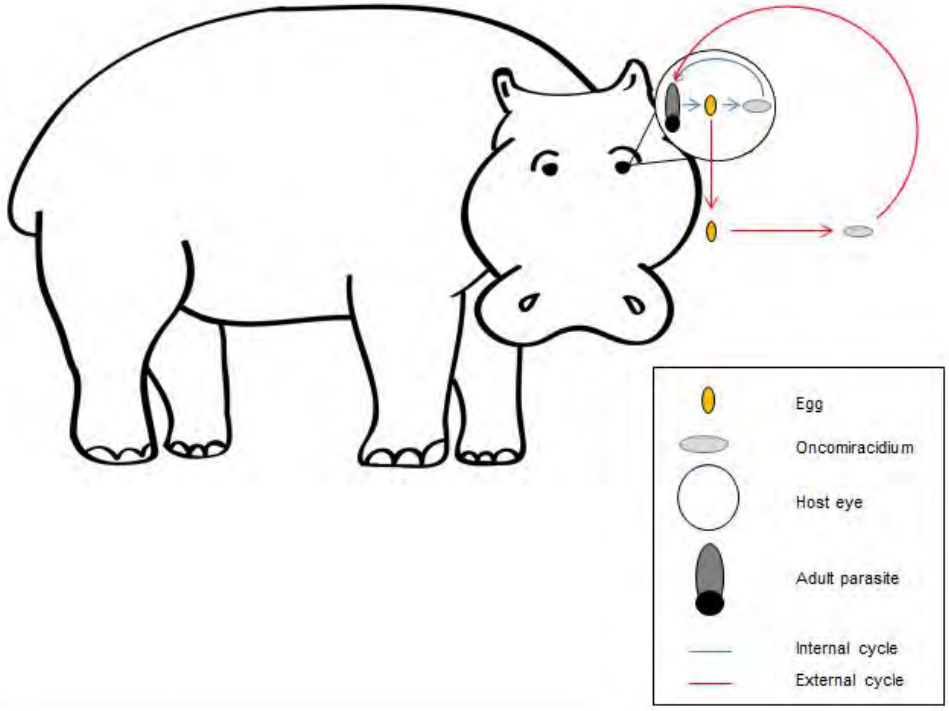


Figure 1.5: Life cycles of *Oculotrema hippopotami*.

1.7 The aims for this study were to:

- Present a phylogenetic hypothesis for 24 genera of the Polystomatidae family based on a cladistics study of 53 morphological character series.
- Study the differences in the morphology of haptoral suckers among the selected polystome genera.
- Study the mechanisms involved in the functioning of suckers in selected polystome genera.
- Study the correlation between haptoral morphology and the attachment site in the host.

1.8 The objectives for this study:

- Based on the findings of the phylogenetic study, a number of genera were selected in the study of attachment structures. Genera include: *Protopolystoma*, *Polystoma*, *Eupolystoma*, *Neopolystoma*, *Polystomoides* and *Oculotrema*.
- A variety of microscopy techniques were used to study the morphology and the functioning of haptoral suckers among the selected polystome genera. Light microscopy and scanning electron microscopy was used to study the external morphology. Histology followed by light microscopy, confocal microscopy and enzyme digestion techniques followed by scanning electron microscopy was used to study the internal morphology.

Chapter 2

Material and methods

CONTENTS

2.1 Collecting of material

2.1.1 ***Protopolystoma*** (Price, 1943) Macnae, Rock and Markowski, 1973

Wild *Xenopus laevis* were collected throughout the study using baited 20 ℓ bucket traps fitted with an inward directed funnel (Figure 2.1 A) and baited net traps (Figure 2.1 B). Traps were set at various sites, ponds and irrigation dams, in and around the city of Potchefstroom, North-West Province. Traps were baited using chicken and/or beef liver, left overnight, and retrieved the following morning. To prevent frogs from swallowing the bait, pieces of liver were placed inside a small gauze bag which was placed inside the trap.

Figure 2.1: **(A)** 20 ℓ bucket traps used to collect *Xenopus laevis*. **(B)** Net traps used to collect *Xenopus laevis*.

Captured *X. laevis* were individually screened for parasite eggs. Frogs were each placed in a 500 mℓ plastic tub which contained approximately 250 mℓ borehole water and maintained at a room temperature of 20 °C. After a period of approximately 24 h the frogs were transferred into clean water and the residual suspended debris were allowed to settle. The top water was progressively decanted and the remaining volume containing suspended debris was studied under a stereo microscope. A gentle rotating action was used through centripetal force to concentrate the sediment into the centre of

the dish. The presence of characteristic golden, shiny pyriform eggs (Figure 2.2) was used as an indication of a positive infection. Tubs with infected hosts were marked. Eggs, larval and adult stages of the life cycle of infected individuals were collected and subsequently prepared for microscopy. Eggs earmarked for incubation were transferred to Petri dishes containing distilled water and incubated at 24 °C. The incubation period for *P. xenopodis* was roughly between 22 and 25 days. Development of eggs were monitored using a stereo microscope and when fully formed oncomiracidia were observed moving within the eggs, Petri dishes were placed in direct sunlight for approximately 30 seconds, resulting in rapid hatching. Hatched oncomiracidia were studied live and pipetted, along with fully embryonated eggs at the point of hatching and empty egg shells and fixed in 70 % ethanol.

Figure 2.2: Presence of golden, shiny pyriform eggs under microscope.

In order to obtain mature parasites from the urinary bladder, frogs were euthanized by placing them in a 3 % ethyl-4-aminobenzoate (MS 222) solution (Sandoz), for approximately 15 minutes and dissected. The urinary bladder was inspected for the presence of parasites. The dark colour, as result of the blood pigments haematin in the gut channel, makes it easy to spot parasites, within the transparent

urinary bladder. The bladder was carefully removed and placed in a Petri dish containing a 0.03% saline solution after which it was cut open and parasites were removed and fixated in 10% neutral buffered formalin (NBF) for light microscopy or in Todd's fixative for scanning electron microscopy (SEM) studies. To fixate parasites that were still attached to the bladder wall, a piece of thin cotton string was used to tie off the bladder (Figure 2.3). Todd's fixative was then carefully injected into the bladder using a 1 mℓ syringe.

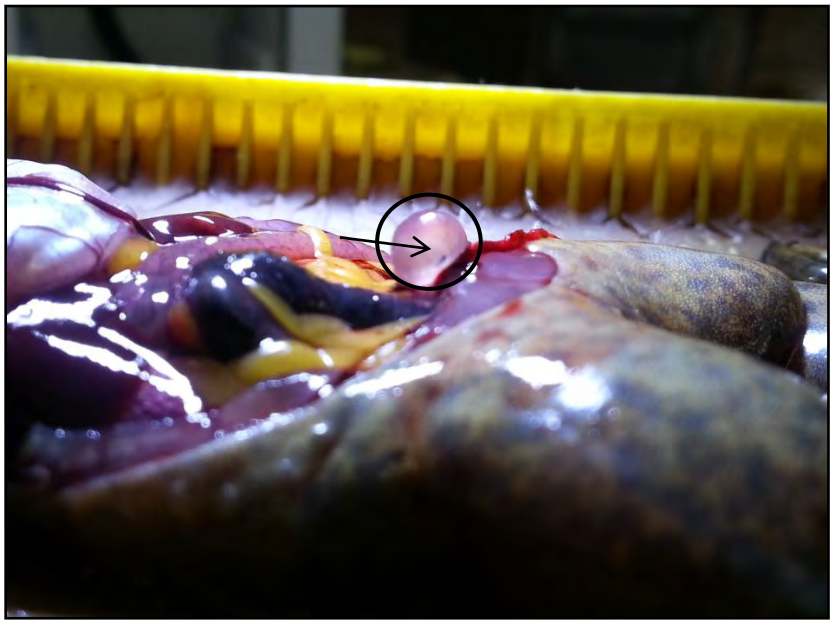


Figure 2.3: *Xenopus laevis* bladder (outlined) tied off with cotton string, with parasite inside, the position of parasite indicated on figure (**O**).

In order to study sclerites, oncomiracidia were mounted under cover slip in lactophenol to clear the specimens. Cover slips were secured using clear nail varnish. Marginal hooklets were studied using a Nikon E800 compound microscope while measurements were taken with the use of Nikon NIS Elements software.

2.1.2 ***Polystoma*** Kok and Van Wyk, 1986

Polystoma australis specimens from the African Amphibian Conservation Research Group polystome collection were used is this study. Specimens were collected in 1994 from *Semodactylus wealii*, at Ladybrand in the Eastern Free State Province, South

Africa. Specimens were fixed under coverslip pressure in 70 % EtOH or 10 % neutral buffered formalin.

2.1.3 *Eupolystoma* Du Preez *et al.,* 2003

Eupolystoma vanansi specimens in the African Amphibian Conservation Research Group polystome collection were used is this study. These specimens were collected in 2007 by Adri Delport from Red toads (*Schismaderma carens*) in the Malelane area in the Mpumalanga Province of South Africa. Specimens were fixed under coverslip pressure in 70 % EtOH or 10 % neutral buffered formalin. Permanent mounts were stained in acetocarmine or alum carmine and mounted in Canada balsam.

2.1.4 *Neopolystoma* (Stunkard, 1916) Price, 1939

Neopolystoma orbiculare specimens were collected from eight red-eared sliders (*Trachemys scripta elegans*), from the Fosseille River in Perpignan, France (Figure 2.4 A – B). Turtles were caught using baited crayfish traps (Figure 2.5 A – B). Traps were set overnight, using chicken and/or beef liver, and collected the following morning. Caught turtles were transported back to the University of Perpignan and subsequently placed in individual containers with a water depth of approximately 5 – 10 cm (depending on the size of the turtle) and left overnight.

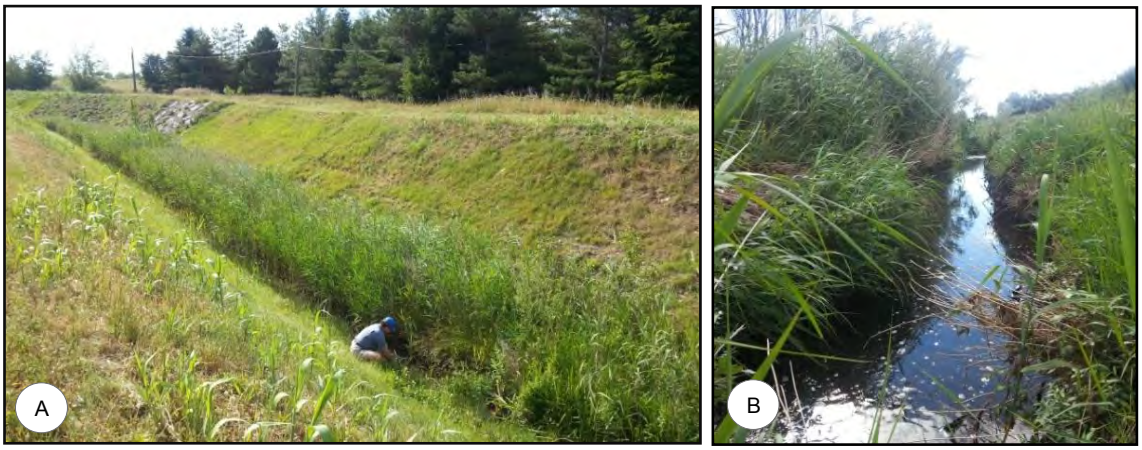


Figure 2.4: **(A – B)** Setting of traps in the Fosseille River, Perpignan, France.

Figure 2.5: **(A – B)** Crayfish traps used to collect *Trachemys scripta elegans*.

Water was screened for parasite eggs the following day. Turtles were transferred into clean water while the old water and residual suspended debris (faeces and parasite eggs, if present) were poured through a set of sieves of 500 µm and 100 µm, respectively (Figure 2.6). The coarse material was collected on the 500 µm sieve while fine debris and eggs were collected on the 100 µm sieve.

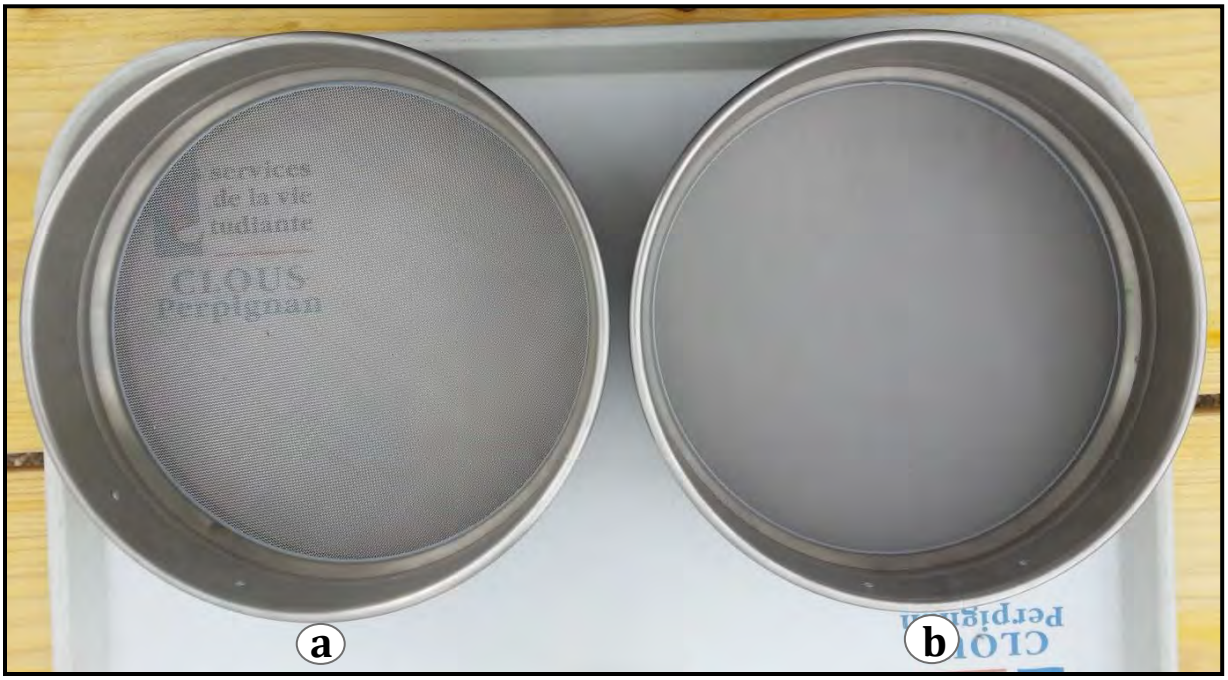


Figure 2.6: Sieves of 500 µm (a) and 100 µm (b).

The contents of the 100 µm sieve was then washed into a Petri dish and studied under the stereo microscope in search for characteristic golden, pear-shaped parasite eggs (Figure 2.2). Following screening, the uninfected turtles were released in the exact location where they were trapped. Infected turtles were kept to harvest eggs for incubation and were subsequently euthanized with a lethal injection of 1 mℓ sodium pentabarbitone (Euthapent) (pre-diluted in 9 mℓ of lukewarm water) and dissected. The cloaca together with the urinary bladder was carefully removed to search for bladder parasites. Parasites were washed in a Petri dish with water and a small drop of dishwashing liquid to remove most of the mucus and dirt before fixating them.

2.1.5 *Polystomoides* Ward, 1917

The same *Trachemys scripta elegans* specimens, from the Fosseille River in Perpignan, France, were used for the collection of *Polystomoides* spp. The head and neck of the turtle was carefully severed in order to search the eyes, nostrils and pharyngeal area for parasites. *Polystomoides* were found after thorough examinations of the mouth and pharyngeal pouches of the hosts. Nasal cavities were directly examined under a stereo microscope and subsequently flushed with the use of a pipette. All surfaces were also inspected under a stereo microscope for sub-adult polystomatids. In order to remove individual polystomatids from the oral mucosa and nasal cavities, washing into a holding dish with a pipette was required (Snyder and Clopton, 2005).

2.1.6 *Oculotrema* Stunkard, 1924

Oculotrema specimens in the African Amphibian Conservation Research Group polystome collection were used is this study. Specimens were obtained from hippopotami culled in a hippopotamus culling program in the Ndumo Game Reserve on the border of KwaZulu-Natal, South Africa (Du Preez and Moeng, 2004).

2.2 Preservation

In this study, material was preserved in a variety of fixatives, depending on the use after preservation. Specimens were fixed in 70 % ethanol or 10 % NBF for whole mounts, Todd's fixative for SEM studies or Bouin fixative for histological sectioning. Polystomatid monogeneans are quite large and muscular and do not relax in distilled water. Techniques that place pressure on the specimens during fixation is often times consuming and may distort the shape and size of the parasite. A different method of flat fixing was used; individual polystomatids were placed on a clean glass slide within a small drop of distilled water, the slide was gently but quickly heated from underneath with a lighter causing the specimen to relax and straighten, producing superior morphological specimens (Snyder and Clopton, 2005).

2.3 Histological sections

Material prepared for histological sectioning was fixed in Bouin fixative and stored in 70 % ethanol. For sectioning the material was further dehydrated in an ethanol series of 70 %, 80 %, 90 % and twice in 100 % for 10 - 15 min each. Dehydrated material was cleared in a Xylene-ethanol mixture for ten minutes and finally in two replacements of pure Xylene for 20 minutes each. Material was impregnated with paraffin wax at 60 °C for 24 h; impregnated material was embedded in paraffin wax with a melting point of 65 °C in a SLEE MPS/P2 Histocene embedding machine (Figure 2.7 A – B).

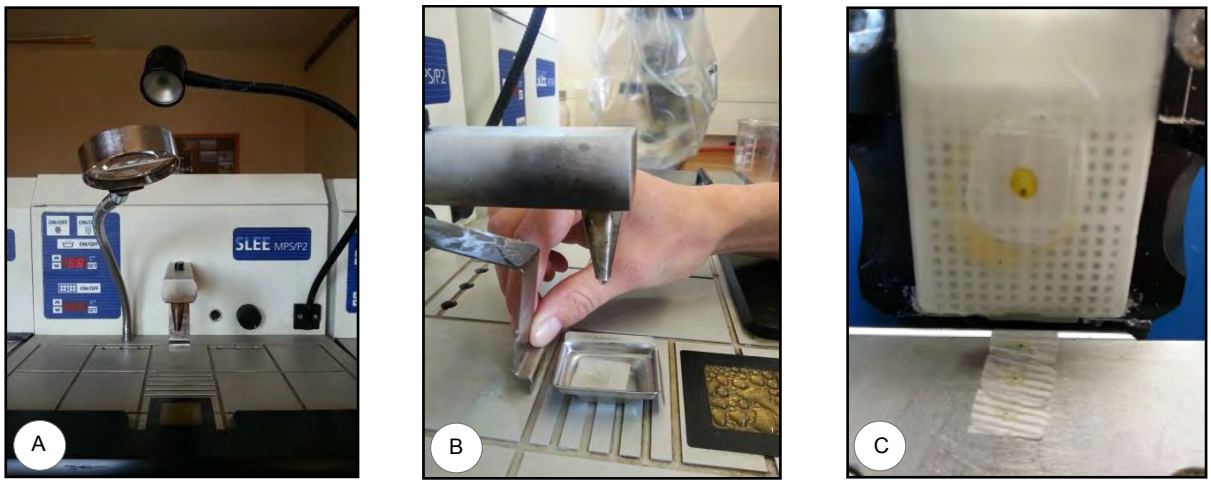


Figure 2.7: **(A - B)** SLEE MPS/P2 Histocene embedding machine. **(C)** *Xenopus laevis* urinary bladder with parasite, embedded in paraffin wax and mounted unto Reichert Jung motorised microtome.

Material was sectioned at 5 µm on a Reichert Yung motorised microtome (Figure 2.7 C). Wax sections were placed on a glass slide covered with an albumin adhesive solution, stretched on a stretching plate and dried over night at 40 °C in an oven. Sections were stained in routine Harris' Haematoxylin and Eosin and permanently mounted using Entellan (Figure 2.8 A – B).

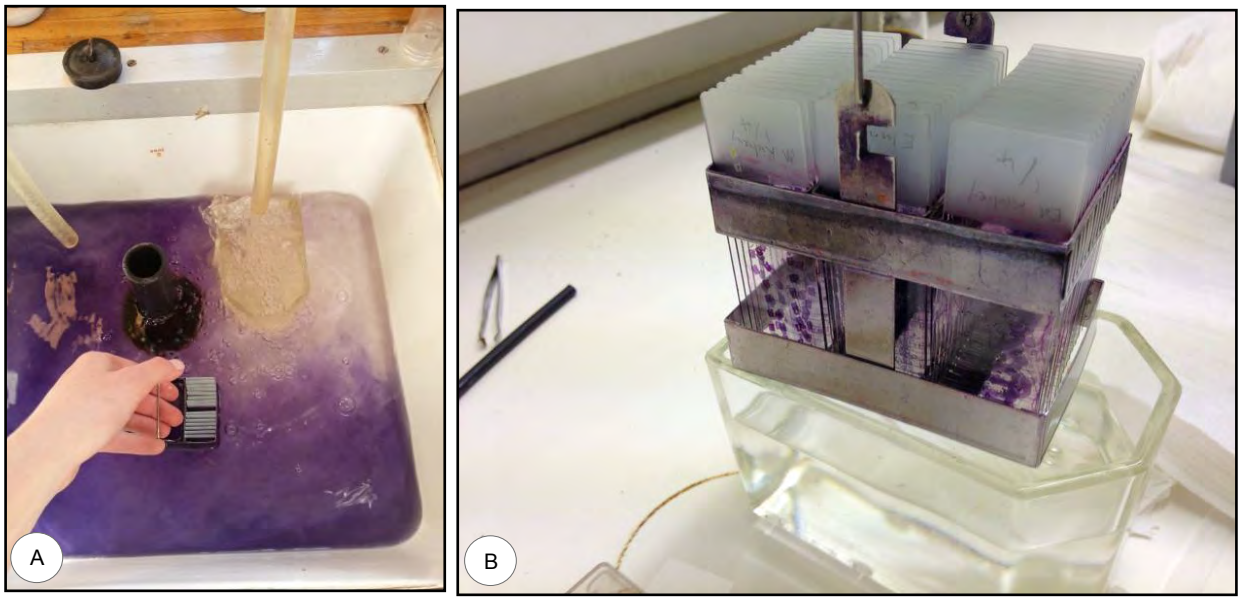


Figure 2.8: **(A)** Slides washed under running tap water after staining in commercial Haematoxylin for ten minutes. **(B)** Slides after completion of routine Harris' Haematoxylin and Eosin staining process.

2.4 Enzyme digestion

In order to study the harder skeletal structures embedded within the soft tissue, soft tissue was digested using an enzyme digestion technique; adapted from a technique described in Harris and Lazarus (1999). An enzyme solution was made up as follows:

A stock solution was made up of 25 mg Proteinase K powder mixed with 10 mℓ ultra-distilled water (concentration of 2,500 µg/m). A 50 mℓ digestion buffer ten times (75 mM Tris-HCL pH 8.0, 10 mM EDTA, 5% SDS) was prepared as follows:

$n_{(mol)} = (C_{mol/L})(V_L)$

$m_{(g)} = (MR_{g/M})(n_{mol})$

Tris-HCL:

n = (0.075 mol/L)(0.05 L)

= 0.00375 mol

m = (121.14 g/mol)(0.00375)

= 0.454 g

EDTA:

n = (0.05 L)(0.01 mol/L)

=0.0005 mol

m = (372.24 g/mol)(0.0005 mol)

= 0.186 g

SDS :

5% van 50ml = 2.5 g

A final concentration of 100 µg/mℓ Proteinase K was made up:

Concentration1	C_1	= 2,500 µg/mℓ
Volume1	V_1	= x
Concentration2	C_2	= 200 µg/mℓ
Volume2	V_2	= 2 mℓ

$C_1V_1 = C_2V_2$

$V_1 = C_2V_2 / C_1$

= (200 µg/ml)(2 ml) / (2 500 µg/ml)

= 0.16 ml

≈ 160 µl

Therefore, 2 mℓ final concentration of 200 µg/mℓ Proteinase K was made up by adding 160 µℓ stock solution Proteinase K to 1,840 µℓ of digestion buffer. Fixed individual parasites from *Polystomoides, Protopolystoma, Polystoma, Neopolystoma* and *Oculotrema* were placed in distilled water in a watch glass to rehydrate after the body was cut from the haptor using a new scalpel blade (Figure 2.9 A).

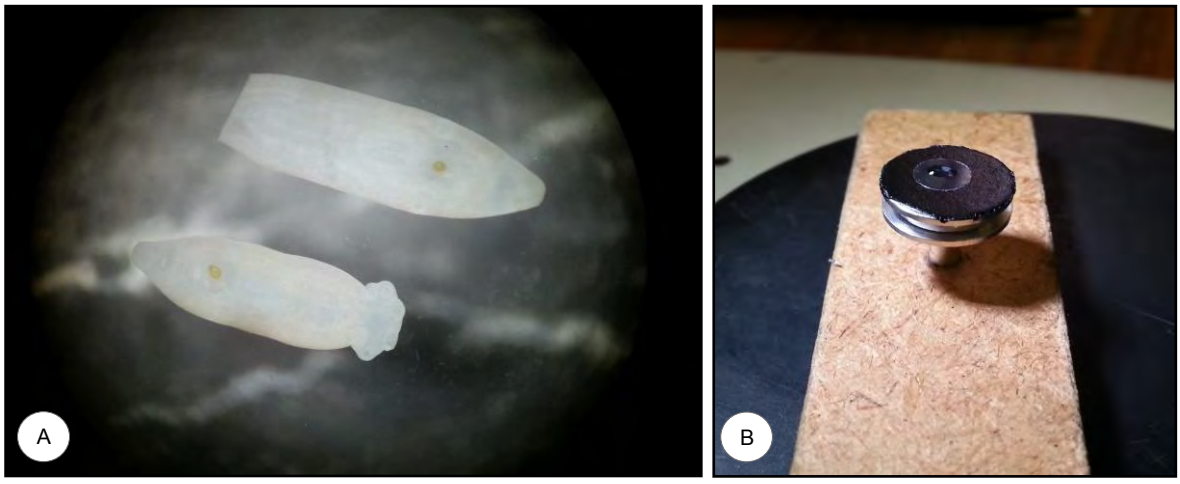


Figure 2.9: **(A)** *Polystomoides* specimen body cut from the haptor with new scalpel blade. **(B)** *Polystomoides* specimen placed on disc and mounted on aluminium stub for enzyme digestion.

The haptor was further cut into two or three sections with one to three suckers per section. Discs with a diameter of 5 mm were cut from an acetate sheet (overhead transparency) with a hole-punch and mounted on an aluminium stub using double-sided carbon tape (Figure 2.9 B). Haptor sections were individually transferred onto the disc on top of the stub, in order to monitor and regulate the digestion process and stop at different stages of digestion. Harris *et al*. (1999) also noted that varying degrees of dissociation could be archived through varying the period of digestion and in effect improving the visualisation of structures such as the dorsal and ventral bars in monogeneans. A drop of enzyme digestion solution was added to the specimen, which was then incubated at 50°C for up to 10 – 15 minutes at a time. After one or two incubation sessions, an equal volume of distilled water was added to the dried specimen and allowed to rehydrate for 2 – 5 minutes to remove excess salts. The fluid was removed using a small wedge cut from filter paper. The progress of digestion was monitored microscopically until lysis occurred. The incubation process was repeated seven to eight times. Remaining digestion buffer was carefully removed and replaced with distilled water. The water drop was carefully removed, and the specimens were allowed to air dry for later observation under SEM. The film disc was sputter coated with gold palladium mixture, and examined.

2.5 Scanning electron microscopy

Specimens were fixed Todd's fixative and/or 70% ethanol for SEM. Materials fixed in 70% ethanol for a minimum of 2 - 8 hours were dehydrated consecutively in an ethanol series; 80%, 90%, and twice in 100% for 15 minutes each. During this process the samples were not exposed to air. Materials fixed in Neutral Buffered Formalin (NBF) or Todd's Fixative were washed three times in 0.05 M cacodylate buffer for 15 minutes each and then washed three times in distilled water for 15 minutes each. Samples were then dehydrated in an ethanol series; 70%, 90% and twice in 100% for 15 minutes each. The samples were critical point dried (CPD), mounted on aluminium stubs with

the use of double sided carbon tape, sputter coated with gold palladium and examined with a FBI ESEM Quanta 200 scanning electron microscope.

2.6 Confocal microscopy

Methods for confocal microscopy by Yoon *et al*. (2013) and Garcia-Vasquez *et al*. (2012) followed in this study. Specimens were rinsed in distilled water for 24 h and then transferred to a solution of phalloidin-based stain prepared by adding 20 µℓ of a 10% Triton-X solution (product T9284, Sigma Aldrich, Poole, UK) to 200 µℓ 10% neutral buffered formalin. The phalloidin stock solution (kept at −20°C) was prepared by dissolving 300 U phalloidin (Alexa Fluor 488, Invitrogen, Paisley, UK) in 1.5 mℓ methanol where after a working solution, which must be made up fresh on each occasion as required, was made by adding 5 µℓ phalloidin stock solution to the 205 µℓ Triton X–NBF solution.

2.7 Phylogenetics

The characters:

In phylogenetics the data matrix is composed of a number of data columns, and there are at least two character states in a transformation series of morphological characters.

Poe and Wiens (2000) describe a couple of reasons why it is important to be explicit in character selection:

- It increases the objectivity of morphological systematic when criteria for character choices are explained, and confirms that the study provides an unbiased sample of polymorphic characters.
- It allows for the testing of the validity of character selection criteria and the properties of particular types of characters. Intraspecific variation is one of the main reasons many scientists tend to exclude certain characters, however, if there are any variations indicate what the nature and extent of the variations are.

Character states in this study are coded with numbers. The general convention is to code the presumed plesiomorphic character state as “0” and the derived, apomorphic state as “1” or more. Whether character changes from 0 – 1 or from 3 – 2, the former character (0 and 3) is apomorphic, and latter character (1 and 2) plesiomorphic. The latter example, 3 – 2, is known as a reversal. The best hypothesis is said to be the shortest, which reduces the chances of reversals and number of convergences needed to explain the evolutionary change and relationships. Evolutionary steps are indicated as short lines cutting vertically through an edge (speciation line) on a phylogenetic tree; Figure 2.10 indicates two evolutionary steps and evolutionary transformation series 0 → 1 → 2.

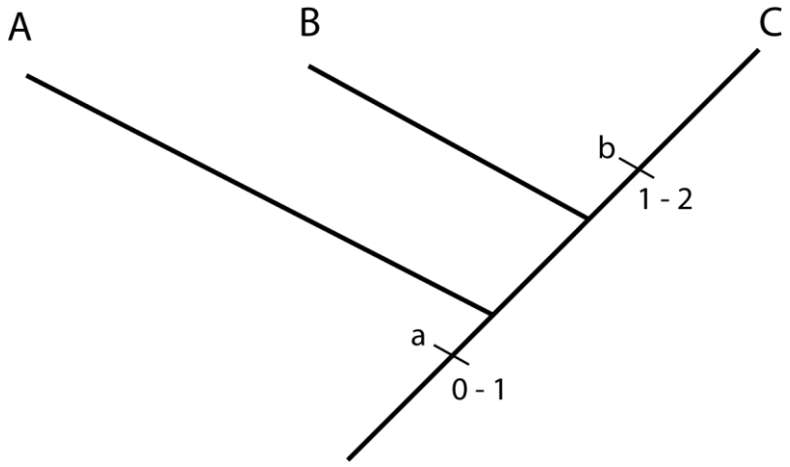


Figure 2.10: Phylogenetic tree with two hypothesised evolutionary steps (a and b).

Vague character statements such as long vs. short, or wide vs. narrow were avoided. The knowledge of variation, together with the gaps within variation, of taxa is helpful to assist and assign accurate qualitative character states that cater for such variations and gaps. According to Wiley and Lieberman (2011) defining characters in qualitative terms delivers positive results. Characters can be described in terms of **qualitative** (descriptive) or **quantitative** (numerical) data. Characters differ in terms of kind of identity and differences are expressed in codes; for example, wings are quantitatively different from arms but the degree to which quantitative data differ is described as qualitative data; two taxa may comprise of wings but differ in length and width (Thiele, 1993). Properties of variation can either be **continuous** or **discrete**. Continuous data consists of mathematical properties that can be measured or observed at any given time; for example the body length or width of an organism at a certain age. Whereas, discrete data is expressed as set-values (count data); for example the number segments of a trilobite. Discrete data can also be allocated as presence or absence data.

There is no definite consensus on a suitable coding method for quantitative data, since some types of continuous variation are difficult to characterise objectively or the accuracy of the hypothesis is reduced by the use of such characters. In the light of continuous variation, character states should be adapted in order to compensate for such variety. For example, if there is no discrete manner for coding colour, a numerical value can be assigned to shades of colour, or if there is no discrete manner for coding shape, use morphometric analysis. It is common to come across specimens that do not have complete character information. Not all the characteristics can be studied for all specimens, especially when making use of ancestral species of which very few samples have been obtained or information is known. **Missing data** should not be the only reason to exclude certain characters from the sample. Whenever possible, a broad range of taxa should be included into the analysis and if missing data entries are randomly distributed among taxa, or limited to a monophyletic group of taxa, inclusion of such characters is more likely to increase, rather than decrease it. It is important to

discuss character selection and to give operational criteria for rejecting characters (Poe and Wiens, 2000). *Concinnocotyla* is presented as a sistergroup, as well as the outgroup in the phylogenetic analysis, based upon the most primitive host.

The reason in determining the character states is discussed and the proposed transformation is indicated for each character. A data matrix (Appendix A, Table 7.1) was constructed with the coded characters listed below and the character numbers correspond with those in the table attached.

1. Host

Monogeneans are mainly ectoparasitic on fish, as in the case of our outgroup selection *Concinnocotyla* infecting the Australian lungfish; however, polystomes within the family Polystomatidae mainly parasitise aquatic and semi-aquatic forms of tetrapods; such as amphibians and chelonian reptiles. *Oculotrema* also radiated onto the sole mammal host, the hippopotamus (Williams, 1995).

- Dipnoi = 0
- Urodela = 1
- Anura = 2
- Gymnophiona = 3
- Chelonia = 4
- Mammalia = 5

Proposed transformation 0 → 1 → 2 → 3 → 4 → 5

2. Found external on host

Host-parasite relationships indicate that as amphibians became adapted to a terrestrial way of life, polystomes in turn adapted to the change in environments in order to survive environmental pressures such as dehydration and starvation, by moving from the external gill position to an internal infection site such as the urinary bladders of their selected hosts. Since water is a prime requirement for

infection, hosts which remain a certain degree of contact with aquatic environments were most likely to be targeted (Williams, 1995). *Concinnocotyla* was recorded on from the gill lamellae, outer opercula and lips of Australian lungfish (Pichelin *et al*., 1991). *Sphyranura* is also parasitic on the external gills of a salamander *Necturus.* The remaining genera are found in urinary bladders of frogs, cloaca and phalodeum of caecileans, on the eye, nostrils, mouth, cloaca or urinary bladder of freshwater turtles, and on the eye of the hippopotamus. Colonisation of new, enclosed sites provides protection and the opportunity for blood sucking from example vascular gills and bladder tissue.

- No = 0
- Yes = 1

Proposed transformation 1 → 0

3. Found in bladder

Amphibians became adapted to a terrestrial mode of life, and in the process the gills in the adult form were lost, eliminating potential habitat for polystome parasites. Polystomes started colonising new infection sites such as the soft, vascularised urinary bladders of their hosts (Williams, 1995). Toads, however, have large urinary bladders in order to store large volumes of water when they hibernate. These urinary bladders are also highly vascularised; allowing sufficient feeding opportunity for a multitude of parasites. *Concinnocotyla, Oculotrema* and *Sphyranura* are the only three genera that are not found within the bladder of their host.

- No = 0
- Yes = 1

Proposed transformation 0 → 1

4. Found in mouth

Only three genera, namely *Polystomoides, Neopolystoma* and *Concinnocotyla,* parasitise the oral cavity of the respective hosts. Host feeding and water currents entering the oral cavity are two environmental pressures that these parasites have overcome to successfully inhabit the specific site.

- No = 0
- Yes = 1

Proposed transformation 0 → 1

5. Found on eyes

Only two genera namely, *Neopolystoma* and *Oculotrema* infect the eyes of specific hosts.

- No = 0
- Yes = 1

Proposed transformation 0 → 1

6. Eyes visible in adult parasites

- No = 0
- Yes = 1

Proposed transformation 0 → 1

7. Food

Species found on or in the gills, buccal cavity, and urinary bladder feed mainly on blood, epithelium and mucus, while skin parasites feed on epidermal cells. Present analysis of species of *Polystomoides, Polystomoidella* and *Neopolystoma;* polystomatids which infect chelonian reptiles, along with

Oculotrema, has shown that this group have diverged nutritionally from related parasites.

- Blood = 0
- Mucus = 1

Proposed transformation 1 → 0

8. Mouth

- Terminal = 0
- Subterminal = 1

Proposed transformation 0 → 1

9. Cephalic lobes

- No = 0
- Yes = 1

Proposed transformation 0 → 1

10. Internal organs (Figure 2.11)

- Through body = 0
- Anterior 2/3 = 1

Proposed transformation 0 → 1

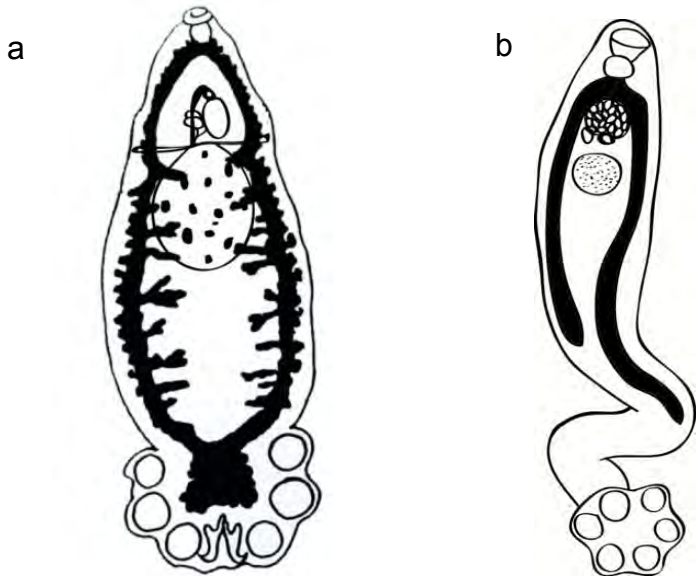


Figure 2.11: (a) Diagrammatic representation of internal organs through more than 2/3 of the body. **(b)** Diagrammatic representation of internal organs only in 2/3 of the body.

11. Gut confluent posteriorly

- No = 0
- Yes = 1

Proposed transformation 0 → 1

12. Gut extends into opisthaptor (Figure 2.12)

- No = 0
- Yes = 1

Proposed transformation 0 → 1

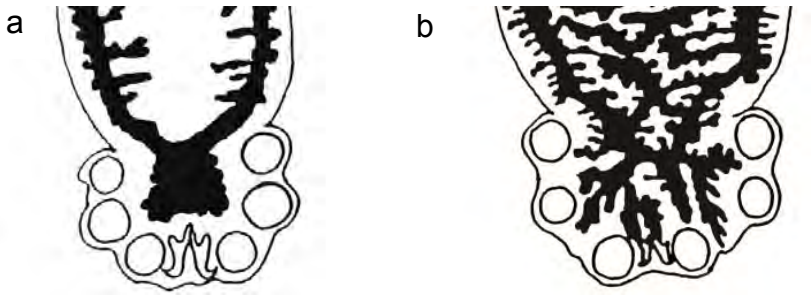


Figure 2.12: (a and b) Diagrammatic representation of gut caeca extending into the region of the haptor.

13. Gut caecums of equal length (Figure 2.13)

Oculotrema is the sole genera with gut caeca of unequal length.

- Yes = 0
- No = 1

Proposed transformation 0 → 1

Figure 2.13: Diagrammatic representation of gut caecum of unequal length.

14. Medial diverticula absent

- No = 0
- Yes = 1

Proposed transformation 0 → 1

15. Medial diverticula small (Figure 2.14)

- No = 0
- Yes = 1

Proposed transformation 0 → 1

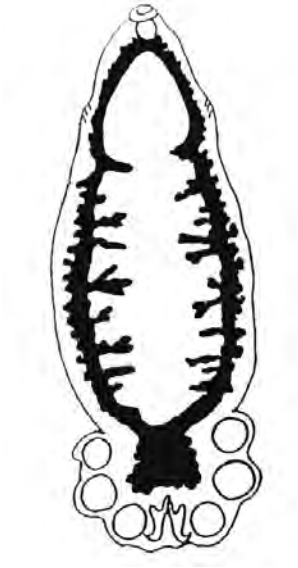


Figure 2.14: Diagrammatic representation of short medial diverticula.

16. Medial diverticula extensive (Figure 2.15)

- No = 0
- Yes = 1

Proposed transformation 0 → 1

Figure 2.15: Diagrammatic representation of extensive diverticula.

17. Medial diverticula network (Figure 2.16)

- No = 0
- Yes = 1

Proposed transformation 0 → 1

Figure 2.16: Diagrammatic representation of a network of diverticula.

18. Lateral diverticula

- No = 0
- Yes = 1

Proposed transformation 0 → 1

19. Anastomosis mostly present (Figure 2.17)

- No = 0
- Yes = 1

Proposed transformation 0 → 1

Figure 2.17: Diagrammatic representation of gut anastomosis.

20. Genitointestinal canal present

- No = 0
- Yes = 1

Proposed transformation 0 → 1

21. Haptor

Development of the haptor, adaptations such as muscular and sclerotised cups, could be correlated with the occupancy of new enclosed attachment sites, as well as the relatively permanent attachment to gill lamellae that are faced with environmental pressures such as respiratory water currents (Williams, 1995). *Sphyranura* is the only polyopisthocotyleans that contain two muscular adhesive organs on the haptor.

- 2 Lobes = 0
- 1 Lobe = 1

Proposed transformation 0 → 1

22. Hamuli present

Protopolystoma and *Polystomoides* are the only two genera that contain two pairs of hamuli. The outgroup consists of a single pair of hamuli between marginal hooklets 1 and 2, rather than between 2 and 3, as in other polystomes (Pichelin *et al.*, 1991).

- Absent = 0
- 1 Pair = 1
- 2 Pairs = 2

Proposed transformation 0 → 1 → 2

23. Number of suckers

The function of marginal hooklets as attachment organs have been replaced by suckers. Within ancestral species marginal hooklets was not only associated with larval stages, but was an adult characteristic. All the genera, except one, have three pairs of suckers. *Sphyranura* is unique in only having a single pair of suckers, and according to Williams (1995) represents a derived feature due to neotenic evolution rather than a primitive characteristic.

- None = 0
- 1 Pair = 1
- 3 Pairs = 2

Proposed transformation 0 → 1 → 2

24. Hamulus incision (Figure 2.18)

- Absent = 0
- Medium = 1
- Deep = 2
- No hamulus = 3

Proposed transformation 0 → 1 → 2 → 3

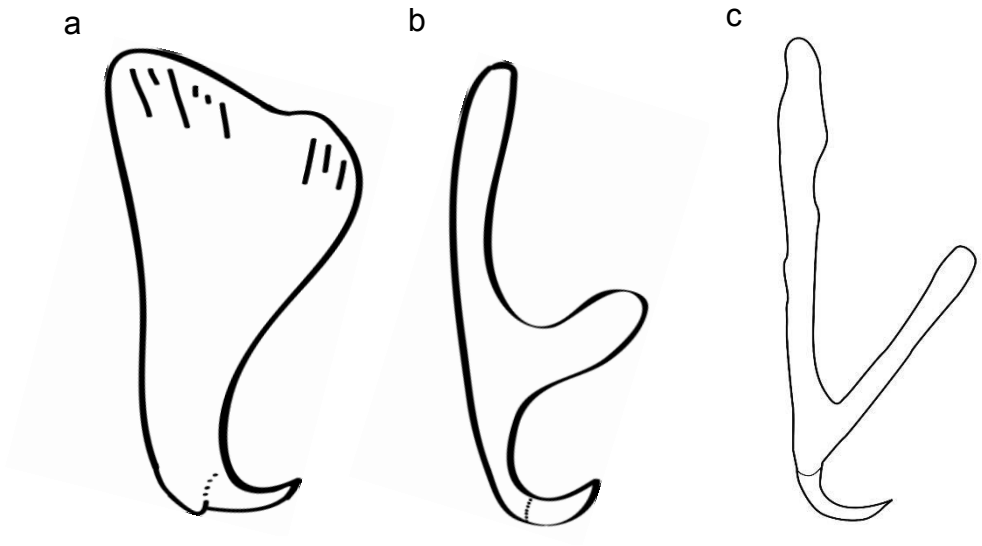


Figure 2.18: Diagrammatic representations of the variations in hamuli incisions. **a,** absent; **b**, medium, **c**, deep.

25. Suckers with skeleton

Concinnocotyla differs from other polystome genera - contains a complex network of sclerits within suckers. The chelonian (*Neopolystoma*, *Polystomoidella* and *Polystomoides*), single mammalian (*Oculotrema*) and single caecilian (*Nanopolystoma*) polystomes also consist of skeletal structures within their sucker cups.

- No = 0
- Skeletal elements present = 1
- Complex = 2

Proposed transformation 0 → 1 → 2

26. Sucker symmetry

Concinnocotyla differs from other polystome genera: haptoral suckers bilaterally symmetrical rather than radially symmetrical.

- Radial = 0

- Bilateral = 1

Proposed transformation 0 → 1

27. Testis position 1

- Anterior = 0
- Middle = 1
- Posterior = 2

Proposed transformation 0 → 1 → 2

28. Testis position 2

- Median = 0
- Lateral = 1

Proposed transformation 0 → 1

29. Testis compact or diffuse

- Compact = 0
- Diffuse = 1

Proposed transformation 0 → 1

30. Number of testis

- 1 = 0
- 2 = 1
- Many = 2

Proposed transformation 0 → 1 → 2

31. Copulatory organ

- None = 0
- Simple = 1
- Complex = 2

Proposed transformation 0 → 1 → 2

32. Genital spines

- No = 0
- 1 Set = 1
- 2 Sets = 2
- 1 or 2 Sets = 3

Proposed transformation 0 → 1 → 2 → 3

33. Number of genital spines

- None = 0
- 1 - 10 = 1
- 11 - 20 = 2
- >20 = 3

Proposed transformation 0 → 1 → 2 → 3

34. Ovary position

- Anterior = 0
- Middle = 1
- Posterior = 2

Proposed transformation 0 → 1 → 2 → 3

35. Ovary

- Germinal tissue = 0
- Proper ovary = 1

Proposed transformation 0 → 1

36. Vitellaria

- Diffuse = 0
- Compact = 1

Proposed transformation 0 → 1

37. Ovary pretesticular

- No = 0
- Yes = 1

Proposed transformation 0 → 1

38. Vitellaria

- Spread = 0
- Lateral fields = 1
- Lateral compact = 2

Proposed transformation 0 → 1 → 2

39. Vaginae present

Protopolystoma do not possess vaginae and is therefore seen as a neotenic genus, since it resembles the characters of the neotenic form of *Polystoma integerrimum* (Williams, 1995). *Sphyranura* possess paired vaginae that do not open to the exterior of the parasite, believed to be vestigial remnants.

- No = 0
- Yes = 1

Proposed transformation 0 → 1

40. Uterus present

Parasites containing a uterus have the advantage to:

- Rapidly form egg capsules during spawning,
- Accumulation and storage of eggs within the uterus before amphibians spawn, and
- Opportunity for many eggs to be deposited immediately after.

Neodiplorchis and Pseudodiplorchis both infect spadefoot toads in desert areas, these hosts have a limited spawning period and transmission time for parasites are also very limited (Williams, 1995). Fully developed larvae of *Pseudodiplorchis* hatch immediately, as soon as eggs are deposited, entre the nostrils of adults toads, and migrate to the lungs, later the juvenile worms pass through the toad's alimentary tract to the urinary bladder.

- Absent = 0
- Tubular = 1
- Sacciform = 2

Proposed transformation 0 → 1 → 2

41. Uterus extends posteriorly

- Absent = 0
- No = 1
- Yes = 2

Proposed transformation 0 → 1 → 2

42. Eggs in utero

No specimen of *Protopolystoma, Neopolystoma* or *Polystomoides* has been reported with more than one developing egg. All the species of *Eupolystoma* and *Metapolystoma* normally contain more than 100 intra-uterine eggs.

- 0 = 0
- <10 = 1
- 11 - 20 = 2
- 21 - 50 = 3
- >50 = 4

Proposed transformation 0 → 1 → 2 → 3 → 4

43. Eggs fusiform

- No = 0
- Yes = 1

Proposed transformation 0 → 1

44. Egg shape oval

Egg shape varies and, according to Schmidt and Roberts (1985), the shape is determined by the oötype walls. Within the Polystomatidae, the shape of the egg varies from oval-round (Timmers and Lewis, 1979; Lim and Du Preez,

2001), pear shaped (Verneau *et al*., 2009), elliptical (Du Preez and Lim, 2000; Platt, 2000; Verneau et al., 2009), spindle shaped (Du Preez and Morrison, 2012) to fusiform, or diamond shaped.

- No = 0
- Yes = 1

Proposed transformation 0 → 1

45. Eggs yellow-tan

The colour of polystome eggs varies from whitish, in the case of the thin shelled type of eggs produced by the species of *Eupolystoma* and *Madapolystoma,* to a dark, shiny, golden colour of those produced by *Oculotrema.*

- Yes = 0
- No = 1

Proposed transformation 0 → 1

46. Eggs operculated

- Yes = 0
- No = 1

Proposed transformation 0 → 1

47. Neotenic

It has been suggested that the neotenic form is the ancestral form of the bladder parasite. The presence of a neotenic stage in the life cycle is considered to be a pleisiomorphic character. Neotenic forms have only been reported for *Polystoma* and *Metapolystoma*. *Protopolystoma* do not possess a neotenic form; however,

the species itself may represent a neotenic form and is therefore an apomorphic character.

- No = 0
- Yes = 1

Proposed transformation 0 → 1

48. Internal cycle

- No = 0
- Yes = 1

Proposed transformation 0 → 1

49. Vivipary

When an embryo develops inside of the maternal body and lead to giving birth to a fully developed embryo, as opposed to laying eggs.

- No = 0
- Yes = 1

Proposed transformation 0 → 1

50. Ovovivipary

Ovoviviparity is the production of an egg with persistent membranes which hatches in the maternal body. Ovoviviparity is of advantage for polystomatid monogeneans (Tinsley, 1983) autoinfection.

- No = 0
- Yes = 1

Proposed transformation 0 → 1

51. Ciliated cells

- None = 0
- 53 = 1
- 55 = 2
- 59 = 3
- 64 = 4

Proposed transformation 0 → 1 → 2 → 3 → 4

52. Number of marginal hooklets

Marginal hooklets are essentially larval features, and aid in the attachment of oncomiracidia to the host, and as the parasite matures these marginal hooklets lose their function, since they are replaced by developing suckers. They are, however, still retained in the tissue.

- 14 = 0
- 16 = 1

Proposed transformation 0 → 1

Chapter 3

Hosts

CONTENTS

3.1 Protopolystoma

HOST: *Xenopus laevis* Daudin, 1802 (Figure 3.1).

Common name : African clawed frog
Family : Pipidae
Host for : *Protopolystoma xenopodis* Bychowsky, 1957

Figure 3.1: *Xenopus laevis*

The Common Platanna or Clawed frog is a fully aquatic species, occupying nearly every kind of water body in sub-Saharan Africa; including lakes, rivers, swamps, artificial irrigation ditches, waterholes and reservoirs (Tinsley and Kobel, 1996; Tinsley and Jackson, 1997; Conradie *et al*., 2006). Breeding generally occurs between September and February; thousands of eggs are laid one by one and hatch within two to three days. Breeding is not restricted to a single brood, both male and females can breed more than once a year. They differ considerably in size with males being

generally smaller than females, sometimes up to two thirds of the female's body size. The average length of adults vary between 35 mm and 130 mm, with a maximum length of 147 mm (Du Preez *et al.*, 2009). *Xenopus* being primarily aquatic allows to serve as host, or intermediate host, for several parasites. *Xenopus* is host to a rich assemblage of more than 25 parasite genera, from seven invertebrate groups (Tinsley, 1996). These parasites are relatively distinct from those in other anurans, shown by their isolated taxonomic position (Tinsley, 1996). Parasites that infect *Xenopus* firstly benefit from the fact that large numbers of frogs are often confined to relatively small areas of suitable habitat, predominantly when water levels drop during dry seasons. Secondly, from the fact that *Xenopus* is involved in a complex web of interactions (Tinsley, 1971) and adapts well to new water bodies; due to their flexibility in diet (generalist predators) and habitat type (Measey, 1998).

3.2 Polystoma

The genus *Polystoma* is the most diverse among Polystomatidae parasites (Bentz *et al*., 2001). Species within the genus *Polystoma* has a worldwide distribution, with the largest distribution in Africa. Anuran hosts have different geographical distributions, contributing to a geographical isolation of associated species and sequentially contributes to the strict host specificity, a key feature among polystomatid parasites (Du Preez and Kok, 1997). For this study *Polystoma australis* from the host, *Semodactylus wealii,* was used.

HOST: *Semodactylus wealii* Boulenger, 1882 (Figure 3.2).

Common name : Rattling Frog
Family : Hyperoliidae
Host for : *Polystoma australis* Kok and Van Wyk, 1986

Figure 3.2: S*emodactylus wealii*

Semodactylus wealii inhabit the grassland biome of South Africa; especially well-vegetated areas nearby pans and wetlands. Breeding takes place from September to the end of February in temporary or permanent water bodies. Males and females join some distance away from water and move together, in amplexus, to it. Between 100 and 500 eggs are individually laid in shallow water onto submerged vegetation, (Conradie *et al*., 2006; Du Preez *et al*., 2009). Metamorphosis is only reached after eight weeks. *Semodactylus wealii* are middle sized frogs, ranging between 35-40 mm, with a maximum length of 44 mm (Du Preez *et al.*, 2009). These frogs are adapted to climb grass stems with their fingers which are arranged into partially opposing pairs, for optimal grasping.

3.3 *Eupolystoma*

There are currently only five known species within the genus *Eupolystoma.* These species consist of: 1) *Eupolystoma anterorchis* from *Amietophrynus pantherinus, 2) Eupolystoma vanasi* from *Schismaderma carens, 3) Eupolystoma alluaudi* from *Ametia regularis, Ametia gutturalis, Nectophrynoides malcomi* and *Pyxicephalus adspersus* (from Africa), 4) *Eupolystoma chauhani* parasitic from an unidentified *Bufo* sp., and 5) *Eupolystoma rajai* from an unidentified *Rana* sp., (both unidentified host species are reported from India) (Delport, 2007). For this study information on one host, *Schismaderma carens,* will be provided.

HOST: *Schismaderma carens* Smith, 1884 (Figure 3.3).

Common name : Red toad
Family : Bufonidae
Host for : *Eupolystoma vanasi* Du Preez, Tinsley and De Sa, 2003

Figure 3.3: *Schismaderma carens*

Schismaderma carens readily adapt to human habitation and are widespread in savannah and grassland biomes of eastern and southern Africa (Conradie *et al*., 2006; Du Preez *et al*., 2009). Breeding is stimulated by heavy rain during summer and mating assemblages takes place intermittently during early September to March. Breeding occurs in fairly deep water bodies such as pools or dams. Toads lay approximately 20,000 eggs per amplectant pair, in double strings, and thread among submerged vegetation (Du Preez *et al*., 2009). *Schismaderma carens* share the common stubby, robust form of other toads, but have a bit more slender body shape, with a maximum body length of 92 mm (Du Preez *et al*., 2003, Du Preez *et al*., 2009). They wander to forage and hibernate a substantial distance from water bodies. Foraging and seeking of retreats under tree trunks, under the eaves of houses or in similar habitats, takes place until May.

3.4 Neopolystoma and *Polystomoides*

HOST: *Trachemys scripta elegans* Wied, 1839 (Figure 3.4).

Common name : Red-eared slider
Family : Emydidae
Host for : *Neopolystoma* spp. and *Polystomoides* spp.

Figure 3.4: *Trachemys scripta elegans*.

Trachemys scripta elegans have become one of the most popular turtles in the pet trade. Their natural range is limited to the United States, but has been widely introduced into other locations, largely due to commercial export. It has a bad reputation because of its ecological versatility and ability to rapidly invade and adapt to natural ecosystems in different countries. They live in a wide variety of freshwater habitats, but prefer calm waters with a muddy bottom, plenty vegetation and plenty basking spots. Typical habitats would include lakes, riparian zones, ponds, ditches, water courses, rivers, swamps and wetlands. *Trachemys scripta elegans* are medium sized turtles; with female carapaces reaching up to 280 mm in length, while males are slightly smaller, with a carapace length of 250 mm (Bonin *et al*., 2006). When male plastrons reach 90 to 120 mm and female plastrons 200 mm, they are ready to start mating. Courtship takes place in spring and summer and nesting from April to July, with 2 to 23 eggs per clutch. Up to five clutches can be laid in a single season and eggs hatch after approximately 60 to 80 days, depending on environmental conditions (Bonin *et al*., 2006). They have an omnivorous diet; forages on aquatic vegetation, small fish and snails, tadpoles, insect larvae and crayfish. They vary greatly in colour with mainly green and yellow-striped bodies and a characteristic red band behind the eye. Stripes on its chin are narrow and

each costal has a yellow transverse band (Bonin *et al*., 2006). They are diurnal (sleep during night time) and spend most of their day, especially during hot summer days, basking on rocks and logs, or stack themselves on top of one another when basking space is limited. In extremely hot and dry weather conditions they tend to estivate. The name “slider” is derived from the act of sliding off basking spots on their plastron.

3.5 Oculotrema

HOST: *Hippopotamus amphibius* Linnaeus (Figure 3.5).

Common name : Hippopotamus
Family : Hippopotamidae
Host for : *Oculotrema hippopotami* Stunkard, 1924

Figure 3.5: Hippopotamus.

Hippopotami are social animals and are found in groups, ranging from 6 to 15 animals per group. Grooming among adult animals, particularly when the partner is lying

in a horizontal position is common and often followed by the groomer resting its head on the partners back; a general expression of sociability. They weigh between 2000 kg and 3000 kg, approximately 150 cm in height and 350 to 450 cm in length. Hippopotami forage during the night and feed on grass, up to 130 kg per night, and sometimes forage kilometres from water, making use of consistent trails (Cillié, 1987). They spend most of their time during the day resting half submerged under water, or basking on sandbanks next to water bodies. The head region is adapted in such a way that the eyes, ears and nostrils are situated at the top of the head causing the body to be submerged and unexposed while still being able to see, hear and breath. Eyes, with a diameter of about 28 mm, are located in deep recesses on either side of the braincase and are capable of retracting to the depth of about 25 mm into the orbits. Mucus secreting cells ensure that the eyeball and the membranes are always lubricated. Adult hippopotami are capable of residing underwater for 5 – 6 minutes before surfacing to breathe. Hippopotami prefer habitats with still to slow moving waters of approximately one to one and a half meter in depth. Breeding is not restricted to certain periods of the year and birth is given to a single calf, under water, after a gestation period of 7 to 8 months. Newly born hippopotami are fairly small and can vary in weight, from 25 – 55 kg. Hippopotami are known for their close association with their young, not only their own offspring, and it is common to find a mother followed by a string of smaller animals. Mothers tend to lick, cuddle and scrape their young with their lower teeth.

Chapter 4

Results

Chapter 4.1

Polystomatid phylogeny

4.1.1 Phylogenetic tree

The phylogenetic tree obtained based on morphological characters are presented in Figure 4.1.1. Note that polystomes described from frogs group together while the chelonian, caecilian and mammalian polystomes forms a monophyletic group. *Sphyranura* occupies a basal position.

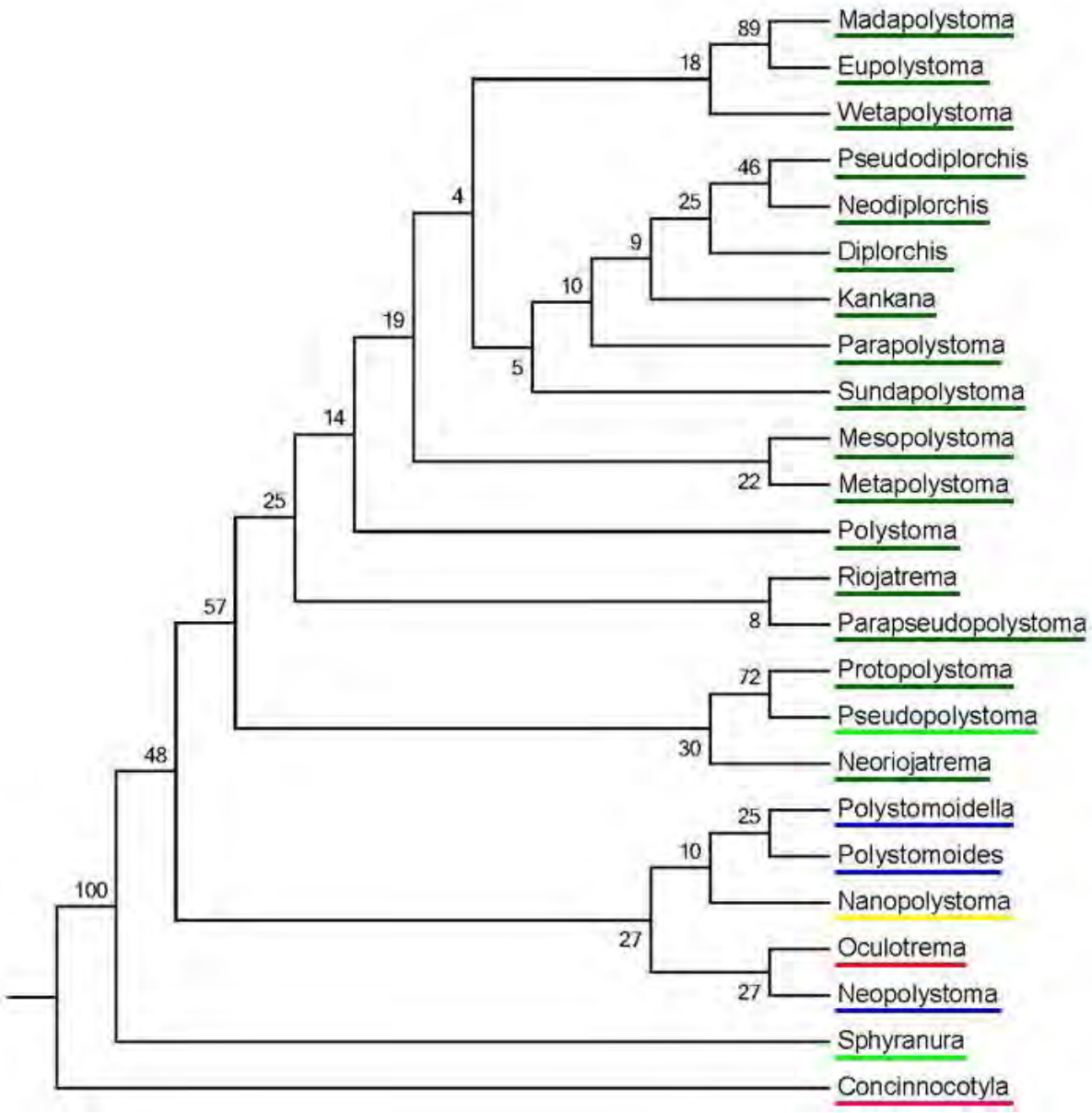


Figure 4.1.1: Phylogenetic tree obtained based on morphological characteristics. Colors indicate host group; Dark green = Anurans; Light green = Caudates; Yellow = Caecilians; Blue = Chelonians; Red = Mammal and Pink = Dipnoi as outgroup

4.1.2 Parasite selection

Based on the most parsimonious tree obtained (Figure 4.1.1.) and the availability of parasite material in the collection of the supervisor, we selected representatives of six polystome genera to study their attachment organs in-depth. We selected representatives in order to have representatives of the more primitive (i.e. *Protopolystoma*) to the more advanced. Polystome genera selected include: *Protopolystoma*, *Polystoma*, *Eupolystoma*, *Neopolystoma*, *Polystomoides* and *Oculotrema*.

Chapter 4.2

Protopolystoma

4.2.1 Mature parasite

The body of *Protopolystoma* is pyriform, narrowing posteriorly (Figure 4.2.1). Anterior positioned mouth is sub-terminal. The upper lip protrudes over the mouth (Figure 4.2.2 A). The haptor is clearly defined from the rest of the body; a large disc, armed with six suckers and two pairs of hamuli. Only one pair of hamuli develops into large falciform hamuli which are situated between the posterior most sucker pair. The hamuli consist of a base with a curved, sharp hook, and a bipedal root system referred to as the “handle” for the medial root and the “guard”. Prominent muscle bundles are attached to both the handle and the guard. The second pair of hamuli does not undergo any significant changes and resembles an oversized marginal hooklet. The 16 marginal hooklets from the oncomiracidium are retained in the mature parasite, but are no longer functional. Marginal hooklet pairs 1 and 2 are retained posterior-most between sucker pair 1, hooklets 3, 4 and 5 are retained at bases of the three pairs of suckers and marginal hooklet pairs 6–8 are retained anterior in haptor between anterior-most suckers. Highly branched intestinal caeca are situated posterior to mouth and pharynx; forming irregular medial and lateral diverticula along with inner intestinal anastomoses. Intestinal caeca do not join posteriorly and intestinal caeca do not extend into the haptoral region (Figure 4.2.1). Intestinal organs are situated medially, between the bifurcate intestinal caeca, in the anterior half of the body. *Protopolystoma* resembles the neotenic adult form of *Polystoma*; uterus and vagina is absent; only an oötype is present – usually containing only a single egg.

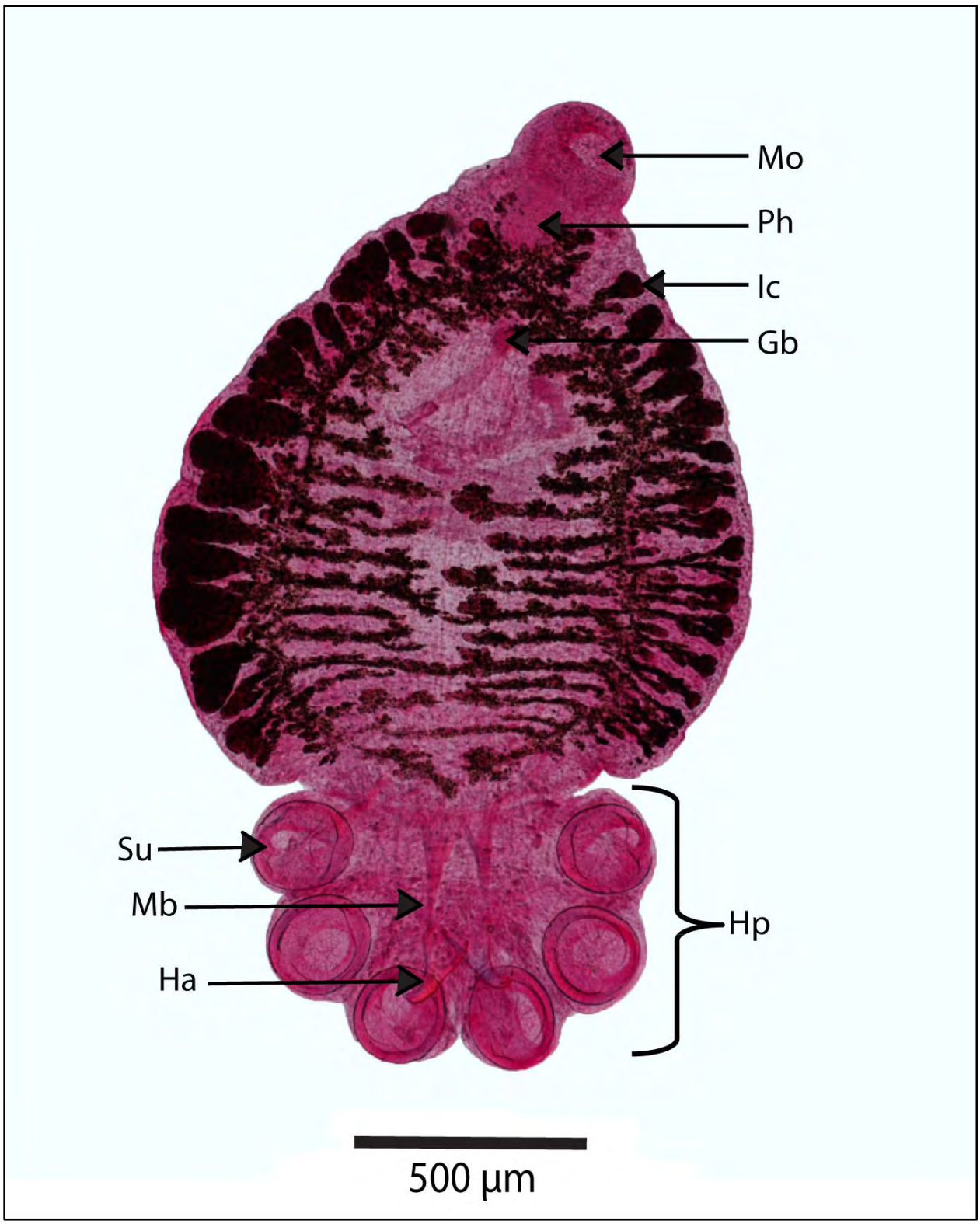


Figure 4.2.1: Light micrograph of a whole mount of *Protopolystoma xenopodis.* Annotations: Gb, genital bulb; Ha, hamuli; Hp, haptor; Ic, intestinal caecum; Mo, mouth; Mb, muscle-bundle; Ph, pharynx; Su, suckers.

The oral region is well supplied with sensory structures (sensillae). The haptor is very flexible, to the extent that whenever a parasite is removed from the bladder and placed in a Petri dish, the haptor folds over and suckers attach readily to the body proper. A wedge-shaped infolding in the anterior margin of the haptor between sucker pair 3 was observed (Figure 4.2.2 B and Figure 4.2.8 A). The bulging in the middle of the body proper is as result of an egg in the oötype (Figure 4.2.2 B). A manuscript on the morphology of *Protopolystoma xenopodis* is currently *in* press and is attached as Appendix B.

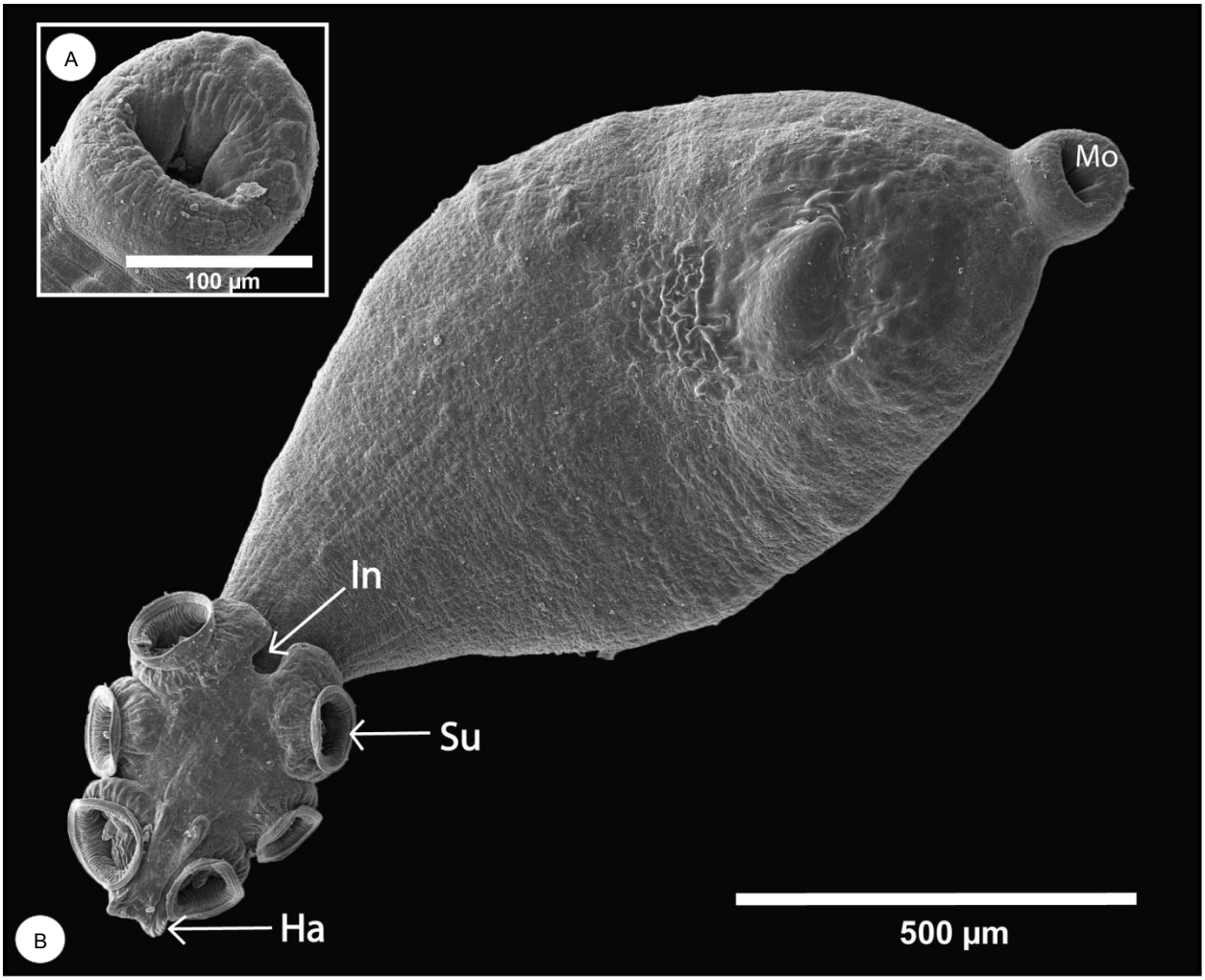


Figure 4.2.2: Scanning electron micrographs of **(A)** ventral mouth opening; **(B)** Mature *Protopolystoma xenopodis* specimen. The ventral view show the mouth opening (Mo) in the anterior of the parasite and the haptor armed with suckers (← Su), hamuli (← Ha) and a wedge shaped infolding (← In) between the third sucker pair at the anterior margin of the haptor.

4.2.2 Attachment structures

Oncomiracidia hatch after an incubation period of 22 days and upon hatching swim actively seeking a suitable host. After contact has been established with a potential host, the oncomiracidium will stay on the surface and move around until it locates the cloaca where it enters and then migrates to the kidneys where it develops and attaches to the kidney wall and start to feed on blood (Figure 4.2.4 A–D). The haptor is directed ventrally, concave, and elongated longitudinally (Figure 4.2.3 A–B). Its length is nearly one third of the total body length. The sclerites are mostly withdrawn (Figure 4.2.3 B), when the oncomiracidium is not attached to its host, however, they may sometimes protrude as shown in Figure 4.2.5 B.

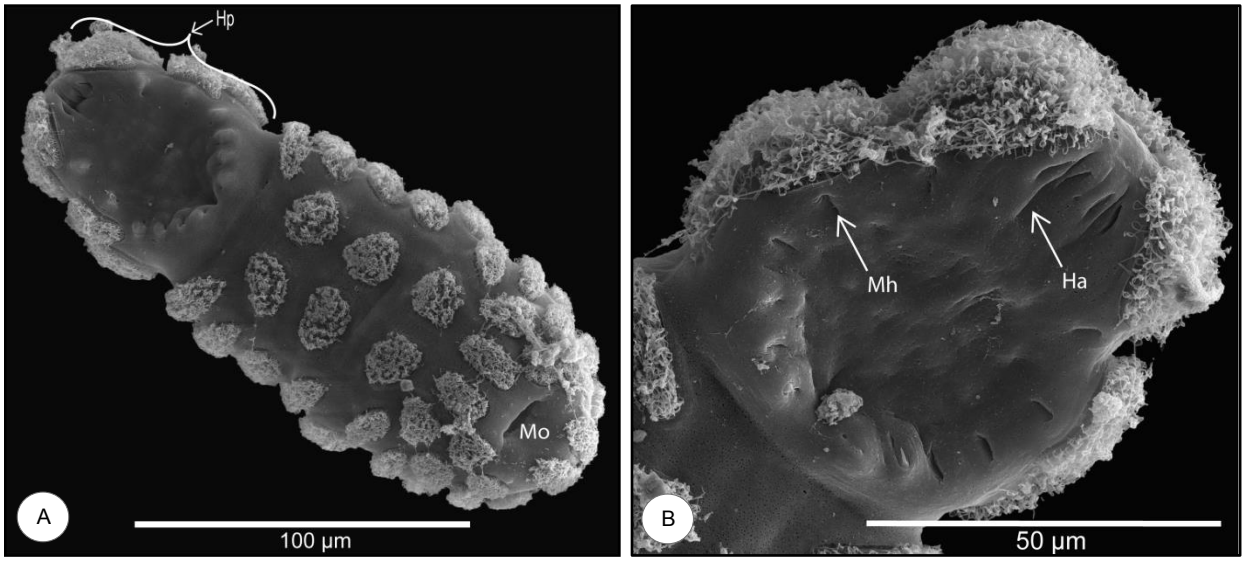


Figure 4.2.3: (A) Scanning electron micrograph of a ventral view of a *Protopolystoma xenopodis* oncomiracidium with posterior haptor (Hp) and anteriorly placed mouth opening (Mo). **(B)** Scanning electron micrograph of the haptor of a *Protopolystoma xenopodis* oncomiracidium, showing 16 retracted marginal hooklets (Mh) and two pairs of large primordial hamuli (Ha).

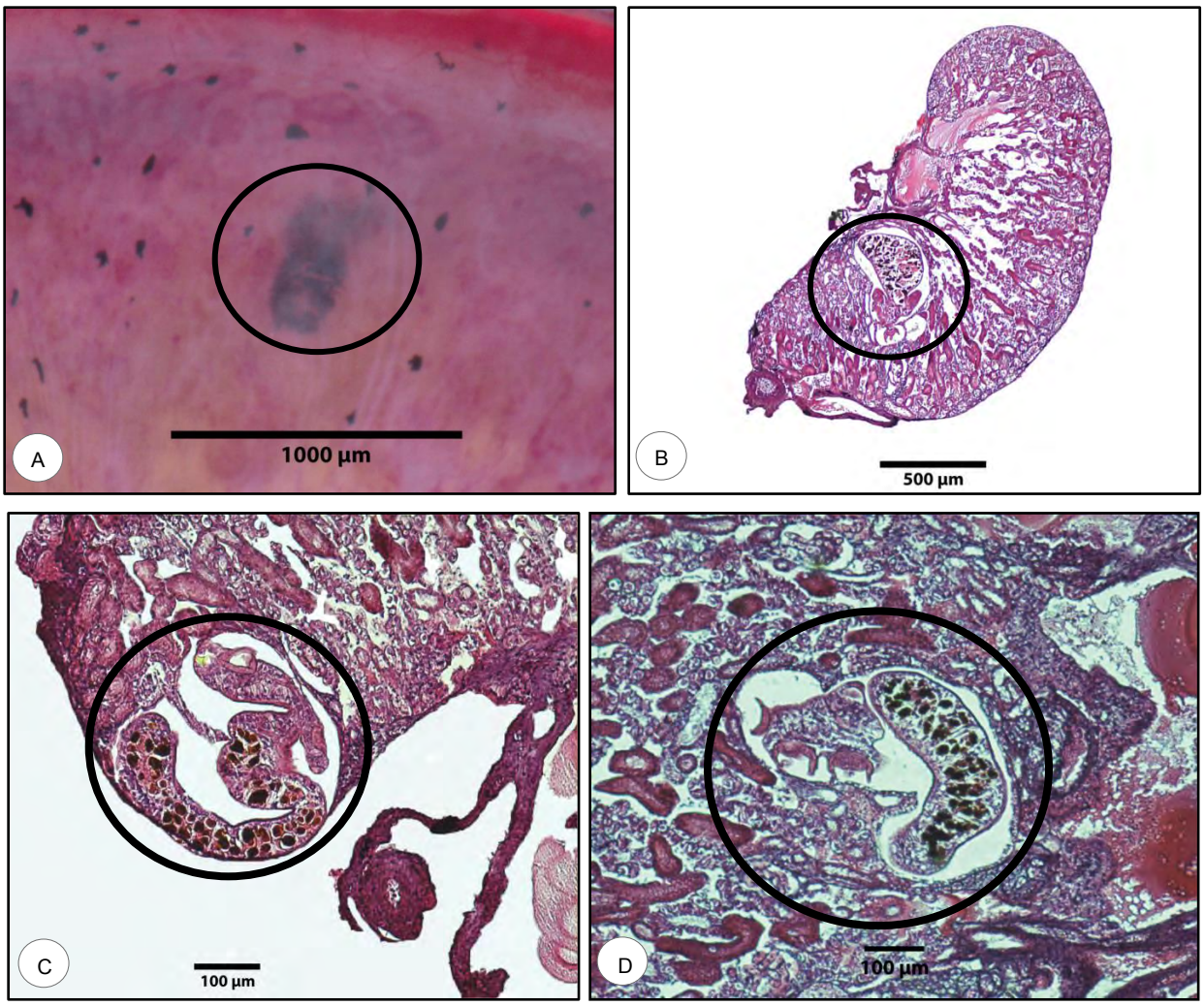


Figure 4.2.4: **(A)** Light micrograph of an immature *Protopolystoma xenopodis* within kidney of *Xenopus laevis*. **(B – D)** Light micrographs of histological cross sections through the kidney of host, *Xenopus laevis*, with immature *Protopolystoma xenopodis* inside. The position of the parasite is indicated on the figures (O).

Marginal hooklets are 13–14 µm in length, arranged in a circle alongside four hamulus primordia (Figure 4.2.5 A). Primordial hamuli with a total length of approximately 28–30 µm protrude posteriorly from the centre of the haptor. The second smaller pair of hamuli lies between the posterior and posterior lateral hooklets (hooklets 1 and 2); these small hamuli are thin, gracile structures, approximately 19–20 µm in length.

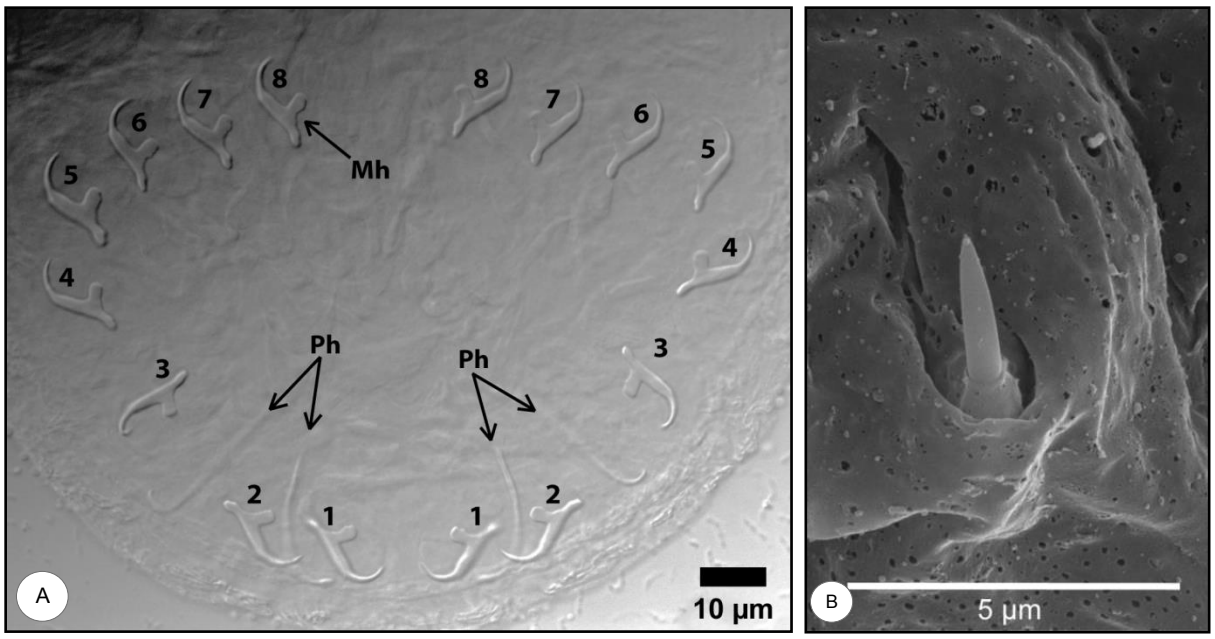


Figure 4.2.5: (A) Light micrograph of a lactophenol cleared haptor of a *Protopolystoma xenopodis* oncomiracidium. Haptor contains 20 sclerites; two pairs of eight (1 – 8) marginal hooklets (← Mh) and two pairs of primordial hamuli (← Ph). **(B)** Scanning electron micrograph of an emerging marginal hooklet.

Progressively the attachment role of the marginal hooklets is taken over by the six suckers and the two pairs of developing hamuli. Suckers develop in the position of marginal hooklet pairs 3, 4 and 5 which are retained inside the base of the respective suckers. Distal parts of the sucker, together with sucker cups are enclosed within a tegument (Figure 4.2.9 A–B). Between the tegument layers radial muscle fibres and ring muscles are situated. Parenchyma tissue is situated on the borders of the sucker cups. At the base of each sucker, a bulge of parenchyma tissue protrudes and finger-like projections are observed (Figure 4.2.10 C). These most likely have a sensory function. Marginal hooklets are embedded in the base of each sucker; bundles of muscle fibres are attached to base of the sucker cup. The functioning mechanism of the sucker is based on the vacuum principle; the sucker is pressed firmly against the host tissue; muscles attached to the central base of the sucker then contracts creating a vacuum inside the sucker; the host tissue is then draw into the sucker; ring muscles positioned along the outer periphery of the suckers then contract closing in around the bud of host tissue drawn into the sucker.

Only one pair of hamuli develop into large falciform haptoral hooks, approximately 150–175 µm in length (Figure 4.2.6 A, Figure 4.2.10 A and D). Bundles of muscle fibres from the area between the haptor and body proper to the hamuli are attached to the hamuli handle and guard (Figure 4.2.6 B, Figure 4.2.8 B). These muscles manoeuvre the hamuli to swing out and slam the hooks or blades into the host tissue. The mechanism involves the contracting of muscle fibres connected to handle, together with the relaxation of muscle fibres connected to the handle of the hamulus. To withdraw the hamuli muscle, fibres attached to the guard relax while those attached to the handle contracts (Figure 4.2.7).

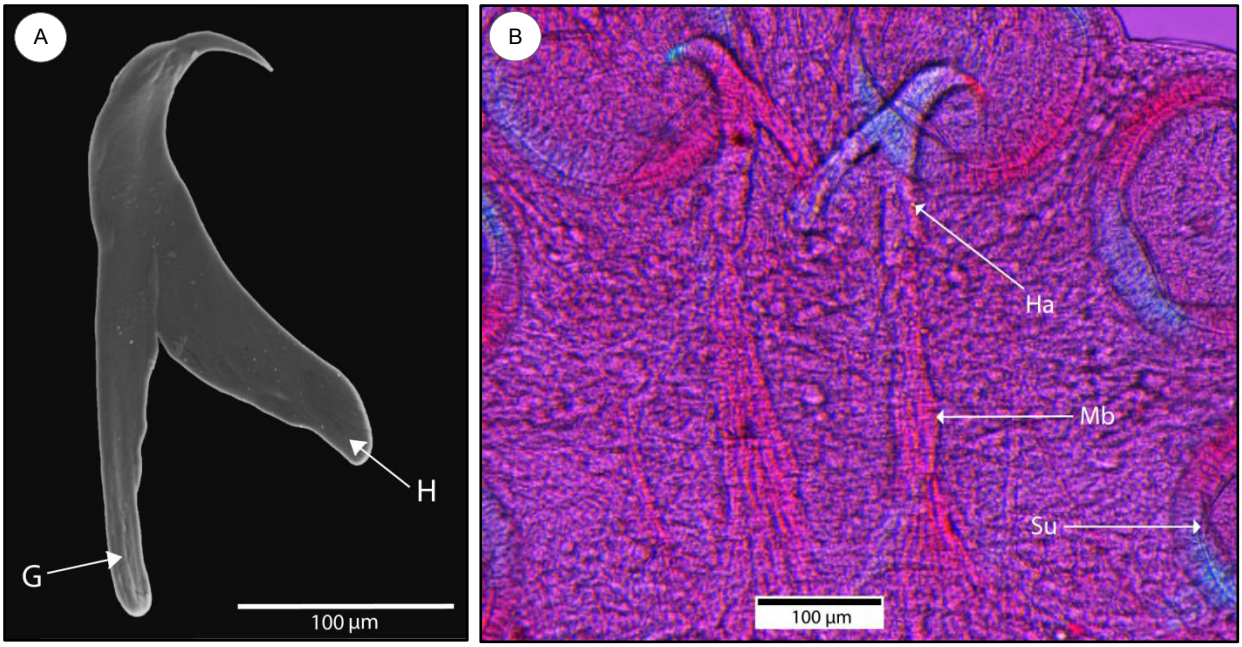


Figure 4.2.6: (A) Scanning electron micrograph of the highly developed hamuli**,** with very subtle spilt between guard (G) and handle (H), revealed through partial digested with proteinase K enzyme digestion solution. **(B)** 3D light micrograph of a lactophenol cleared *Protopolystoma xenopodis* specimen, showing suckers (Su) and extensive muscle bands (Mb) attached to handle of hamuli (Ha). **Note that this is a 3D image. Please use the anaglyph glasses provided on the inside back cover.**

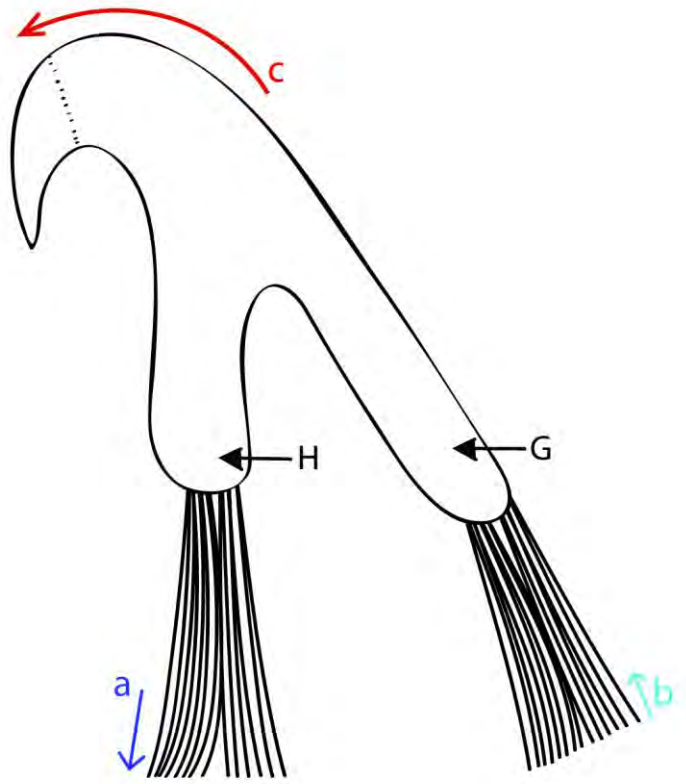


Figure 4.2.7: Drawing to illustrate the mechanism of *Protopolystoma* hamuli. Muscle fibres attached to the guard (G) contracts while those attached to the handle (H) relax causing the hamulus to swing outward (c) and penetrate into host tissue.

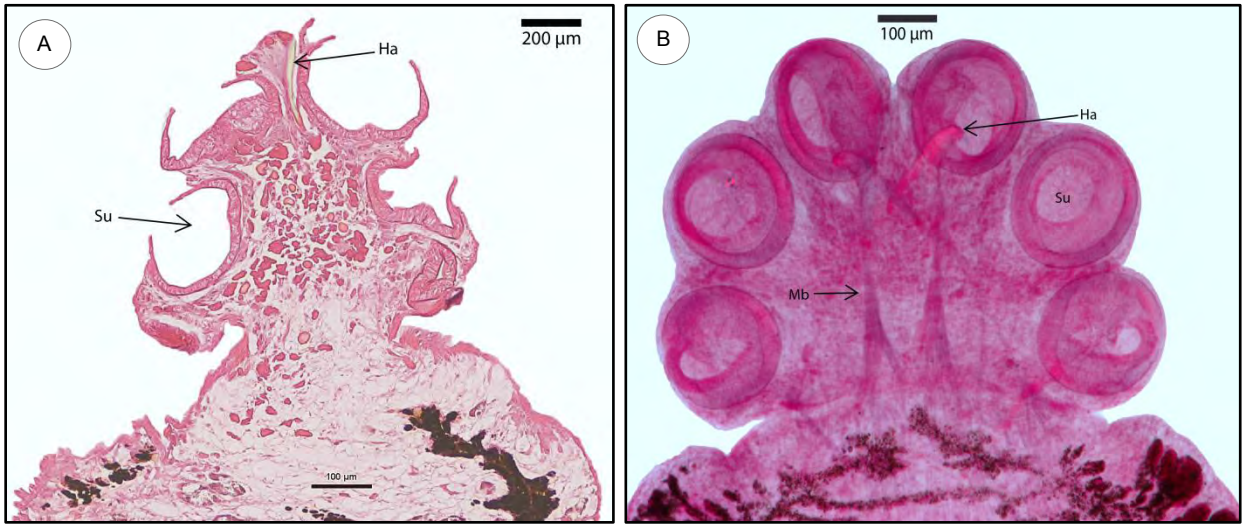


Figure 4.2.8: (A) Light micrograph of a histological section through the haptor of a *Protopolystoma xenopodis* haptor with six ventral suckers (Su) and hamuli (← Ha). **(B)** Light micrograph of an adult *Protopolystoma xenopidis'* haptor with six ventral suckers (Su) and hamuli (← Ha). Muscle bands (←Mb) are evident, running from hamuli and suckers to the centre of the anterior part of the body.

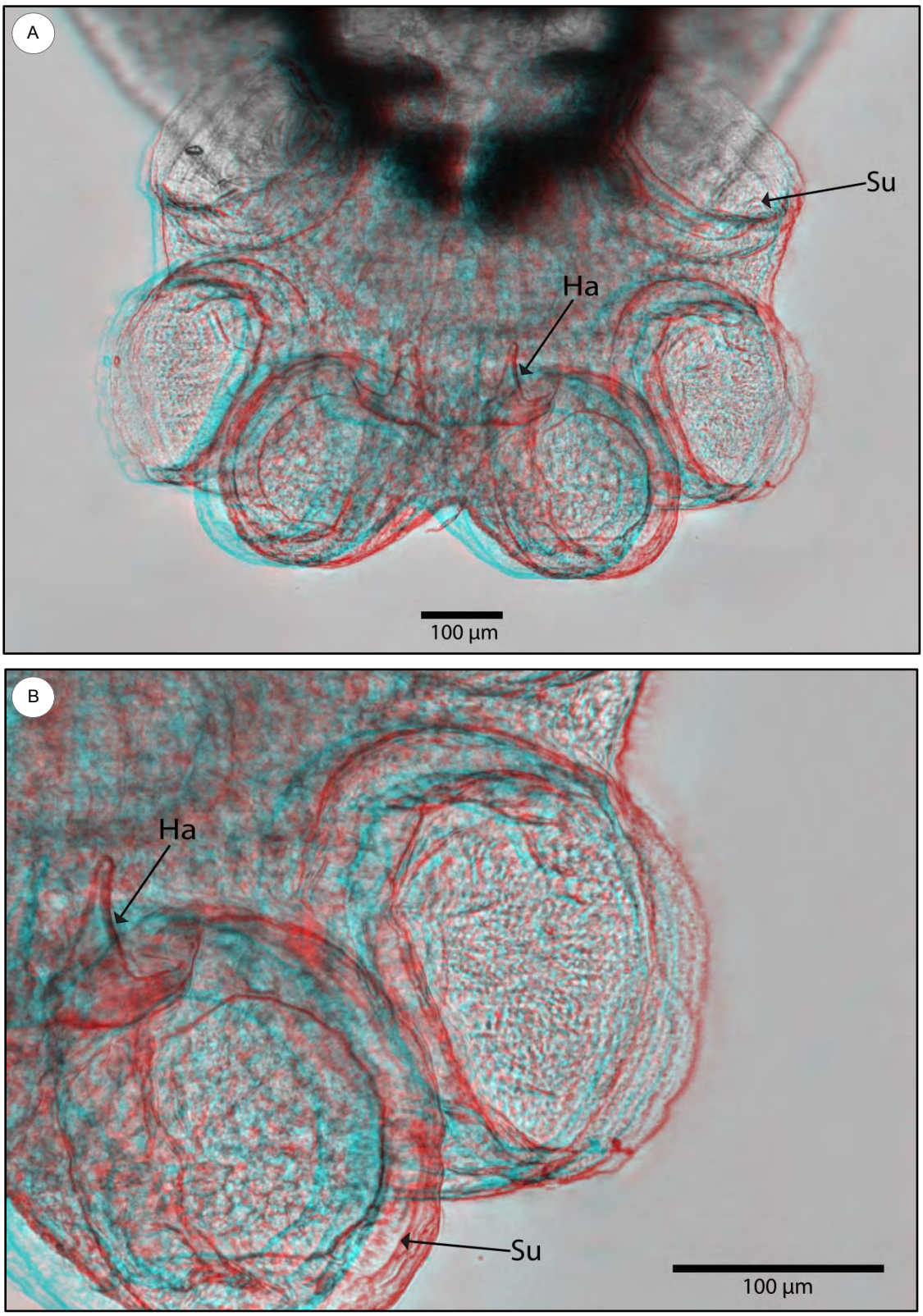


Figure 4.2.9: 3D light micrographs of a lactophenol cleared specimen of *Protopolystoma* haptor. **(A)** Haptor with six muscular sucker cups (Su), pair of hamuli (Ha) and muscle bands Mb) running from hamuli root to body. **(B)** Close-up of sucker. **Note that these are 3D images. Please use the anaglyph glasses provided on the inside back cover.**

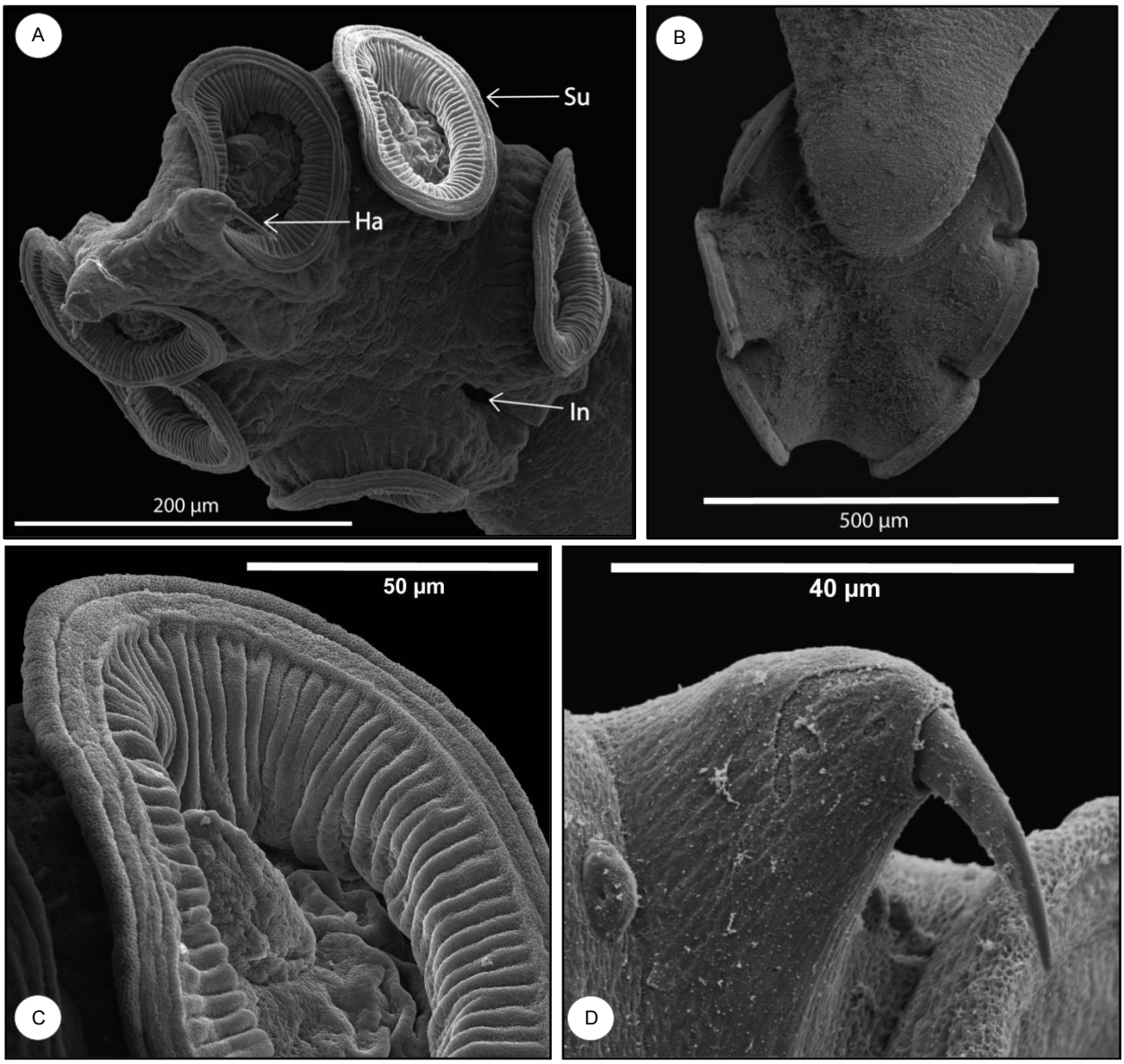


Figure 4.2.10: Scanning electron micrographs of mature *Protopolystoma xenopodis* specimens. **(A)** Haptor with six ventral suckers (← Su), hamuli (← Ha) and a wedge shaped infolding (← In) between the third sucker pair. **(B)** Scanning electron micrograph of the dorsal view of the haptor. **(C)** Scanning electron micrograph of a single sucker, showing the musculature of the sucker. **(D)** Scanning electron micrograph of a hamulus point.

Suckers, as observed in *Protopolystoma,* provide a very firm and secure attachment and the thin and soft urinary bladder lining (Figure 4.2.11 A) is effectively sucked into the sucker cups. The moment a sucker detaches the bladder tissue straightens and no damage is caused to the bladder lining. In order to illustrate how the host tissue is drawn into the sucker cups, we fixed a parasite *in situ* and only after

proper fixation we pulled off the parasite revealing the attachment site. The sucker imprints in the form of six buds are clearly visible (Figure 4.2.11 B–C).

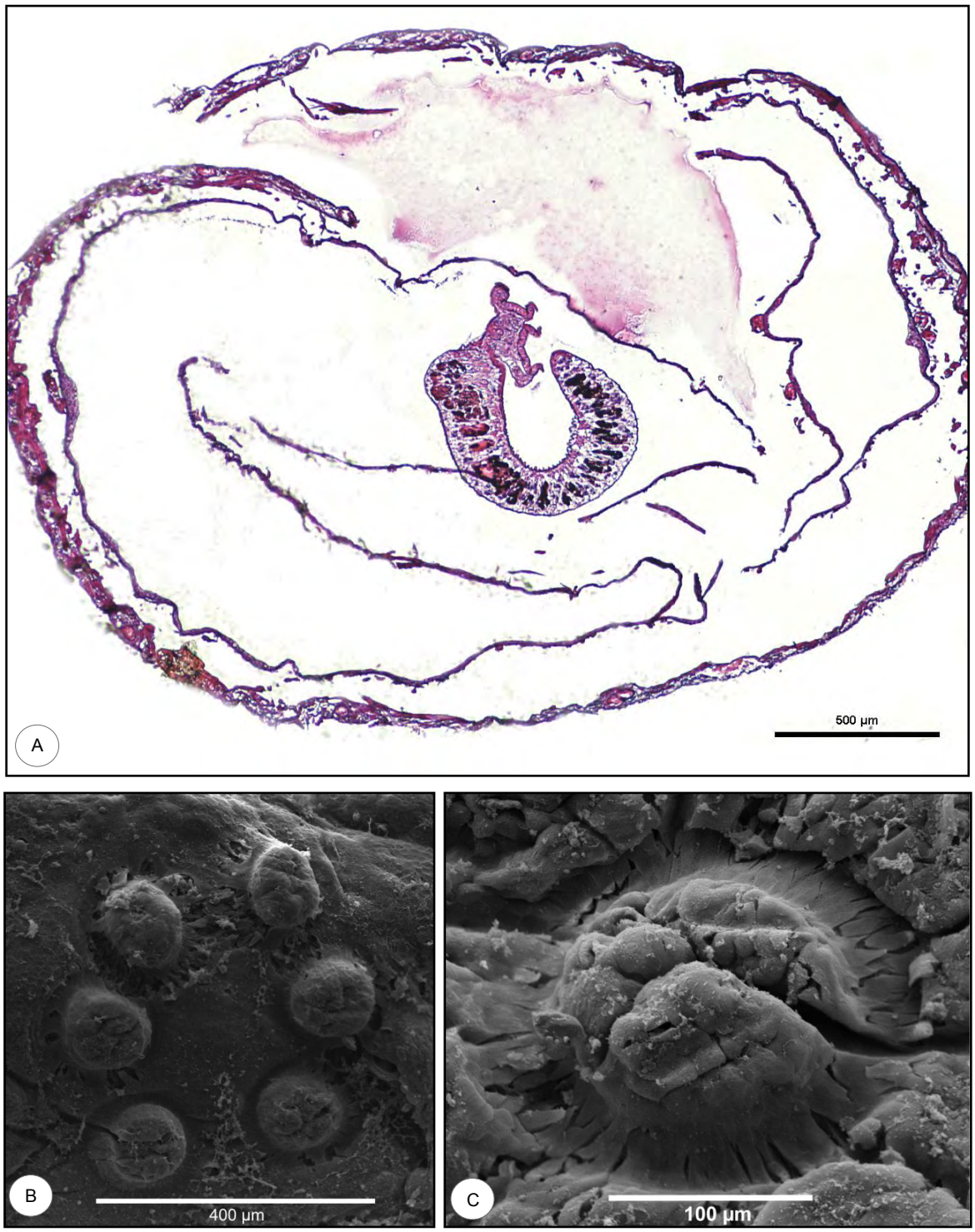


Figure 4.2.11: (A) Light micrograph of a histological cross section through the bladder of host, *Xenopus laevis*, with a mature *Protopolystoma xenopodis* inside. **(B – C)** Scanning electron micrographs of buds made by the parasite's suckers on the host's bladder surface (B) and high magnification of a single bud (C).

Chapter 4.3

Polystoma

4.3.1 Mature parasites

Polystoma has a moderately large, cylindrical body, tapered anteriorly and with a dish shaped haptor posteriorly (Figure 4.3.1, Figure 4.3.2). The haptor consists of six suckers along the periphery of the haptor and posterior-most one pair of hamuli. The mouth, commonly referred to as the prohaptor, is situated at the anterior end of the parasite and surrounded by a prominent false oral sucker. Intestinal caeca are confluent posteriorly, extending into the haptor where caeca forms a haptoral anastomosis. Lateral and medial diverticulae of the gut caeca are present and vary in size and shape for different species. Medial intestinal anastomoses are present in some species (Figure 4.3.1). The ovary is large and packed with developing oocytes. Vaginae are present antero-laterally. The uterus is tubular and confined in the pre-ovarian region and holds up to eight eggs. The uterus joins the seminal vesicle in the genital atrium where a cirrus, armed with about eight genital spines, is present. A single follicular testis is situated ventrally between the intestinal caeca, post-ovarian.

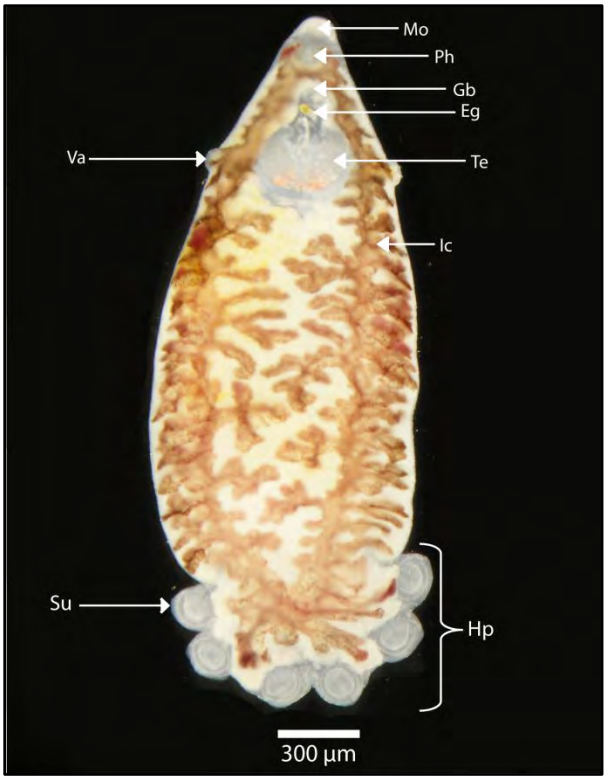


Figure 4.3.1: Light micrograph of a whole mount of *Polystoma claudecombesi*. Annotations: Eg, egg; Gb, genital bulb; Hp, haptor; Ic, intestinal caecum; Mo, mouth; Ph, pharynx; Su, suckers; Te, testis; Va, vaginae.

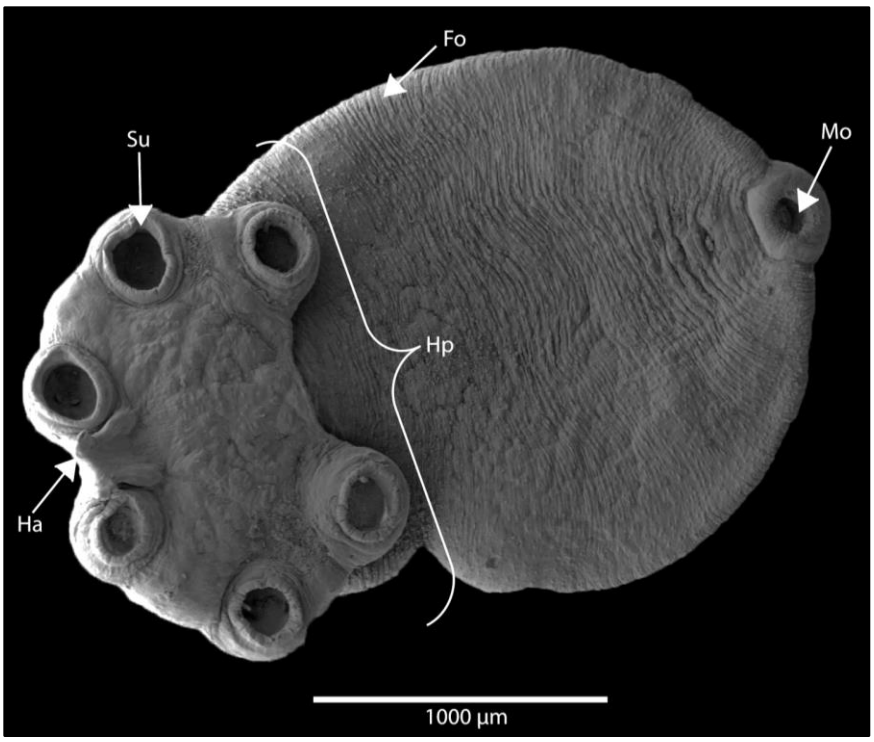


Figure 4.3.2: Scanning electron micrograph of ventral view of an adult *Polystoma australis*, showing the mouth opening (Mo) at the anterior of the parasite and the haptor (Hp), armed with six suckers (Su) and a pair of hamuli (Ha) at the posterior.

4.3.2 Attachment structures

Attachment of oncomiracidia to the gill surface is achieved through the cup-shaped haptor; directed ventrally, and armed with 16 marginal hooklets (Figure 4.3.3). Marginal hooks are withdrawn, but when the parasite attaches to a host they are slammed into the host's tissue and in the process secure a firm grip. On a young tadpole the oncomiracidium develop rapidly into a neotenic form with six haptoral suckers (Figure 4.3.4). As parasites mature, the 16 marginal hooklets are no longer functional. Marginal hooklets do not disappear, but are retained and imbedded in the parasite tissue. Marginal hooklet pairs 1 and 2 are retained posterior-most between sucker pair 1. Marginal hooklets 3 – 5 are retained at bases of the three pairs of suckers and marginal hooklet pairs 6 – 8 are retained anterior in haptor between anterior-most suckers. Muscular sucker cups develop along the periphery of the haptor (Figure 4.3.8). There is little size difference between anterior, median and posterior suckers. Suckers are flexible and made up of a series of musculature, muscle fibres running from the body wall and longitudinal muscles within the sucker cups (Figure 4.3.5 A–D).

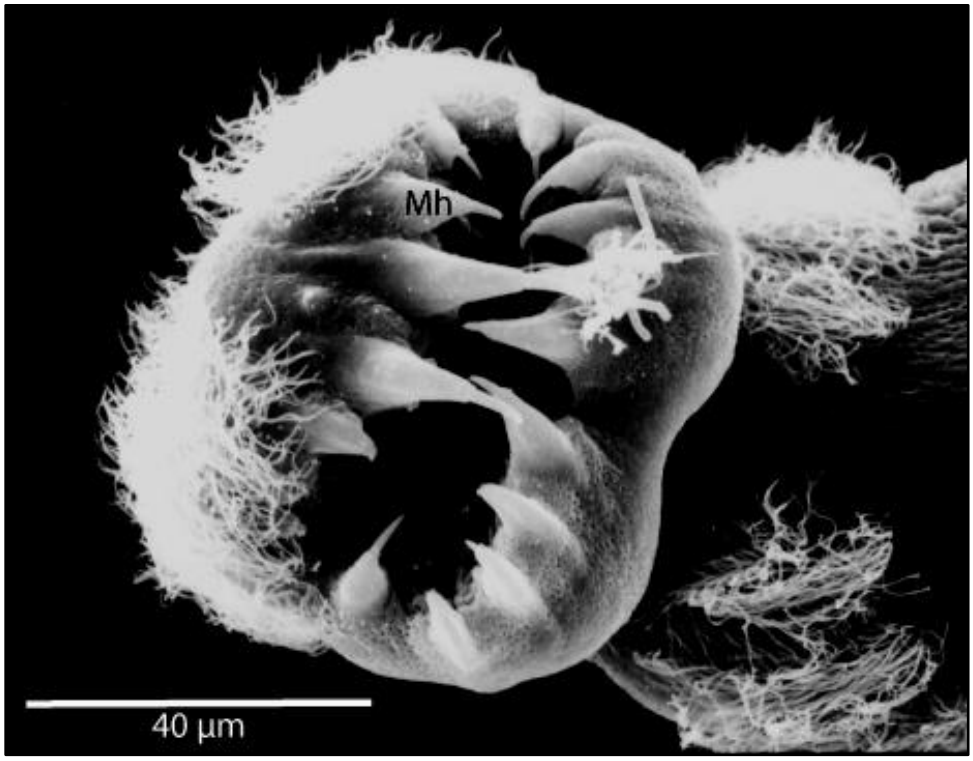


Figure 4.3.3: Scanning electron micrograph of the haptor of a *Polystoma australis* oncomiracidium; showing the marginal hooklets (Mh) protruding from the haptor.

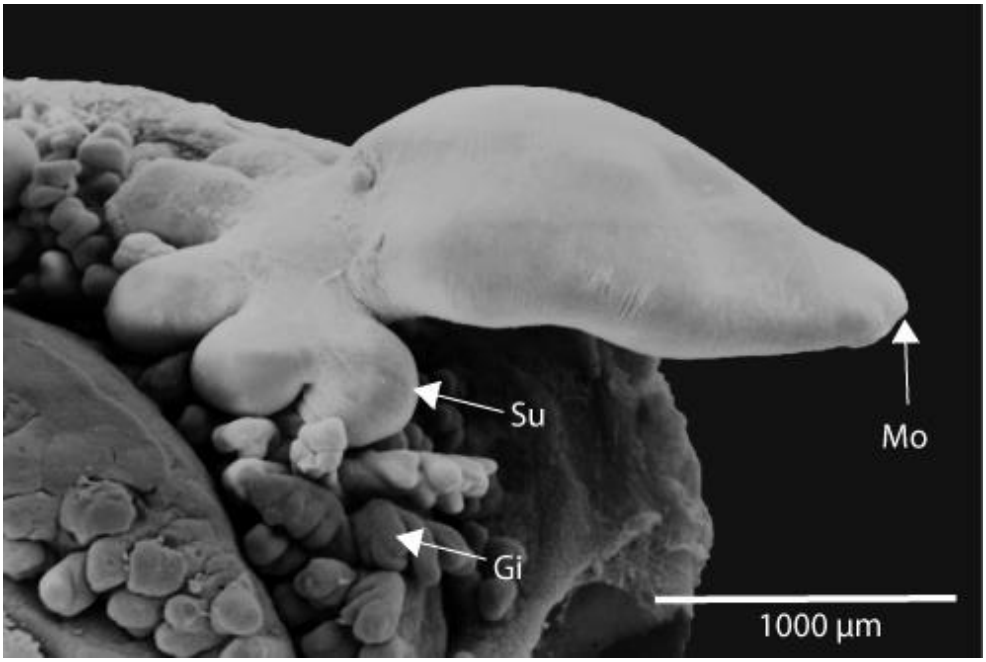


Figure 4.3.4: Scanning electron microscope of a neotenic *Polystoma australis* parasite attached to gills (Gi) of a *Semnodactylus wealii* host tadpole. Note how the six haptoral suckers (Su) attach to the gill filaments. The mouth (Mo) is thus free to feed.

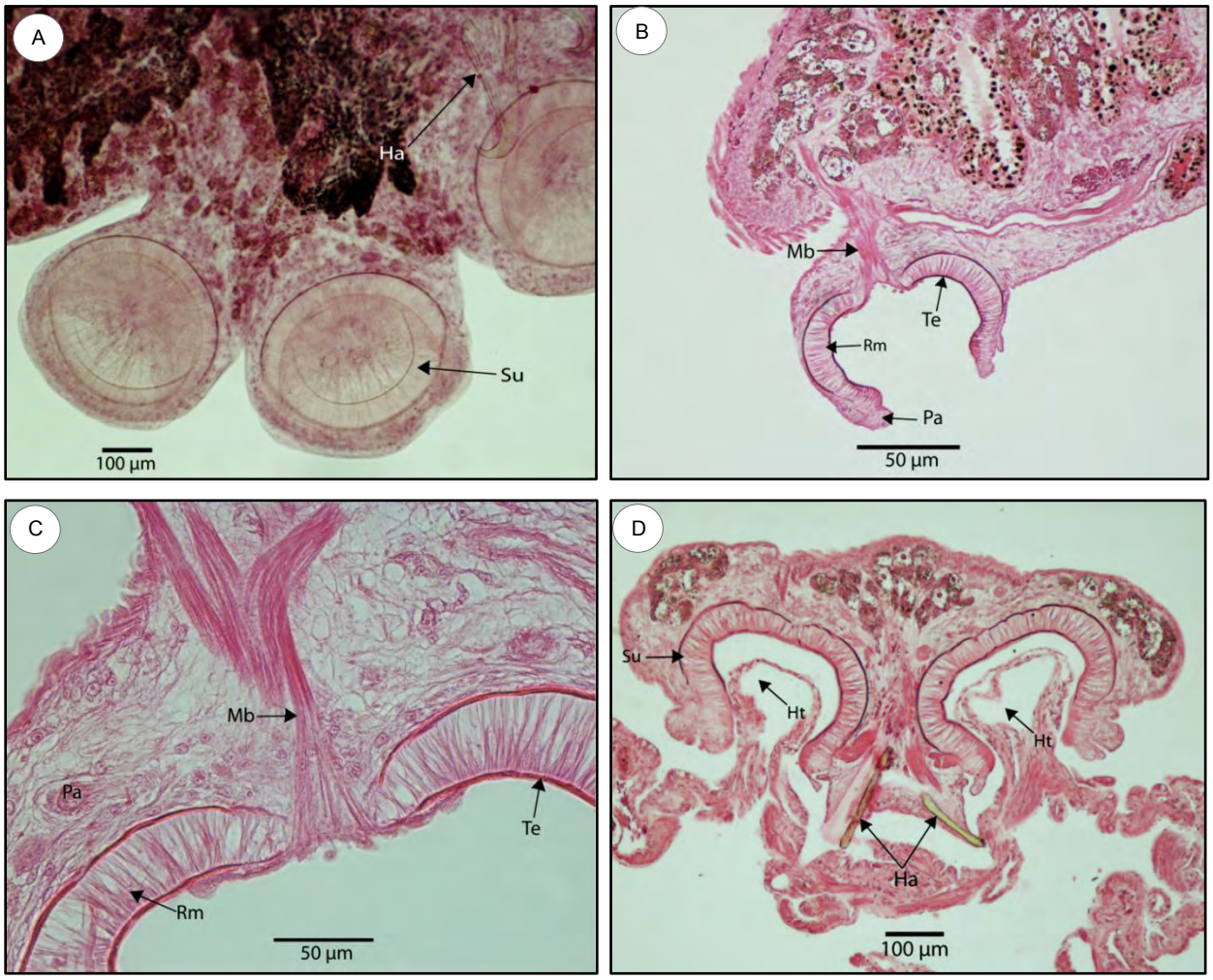


Figure 4.3.5: (A) Light micrograph showing ventral suckers (Su) and hamuli (← Ha) of an adult *Polystoma australis*. **(B – C)** Light micrographs of histological sections through suckers of adult *P. australis* haptor. Muscle bundles (Mb) are evident, originating at the base of the sucker, with radial muscle fibres (Rm) between tegument and parenchyma (Pa) surrounding sucker cups. **(D)** Light micrograph of histological section through the two anterior most suckers (Su) of *Polystoma australis*, with hamuli (← Ha) situated in between two suckers. Host tissue (Ht) is evidently drawn into each suckers during attachment to bladder wall.

Sucker cups are surrounded by a tegument lining and comprise of a series of radial muscle fibres in between (Figure 4.3.5 C). Muscle bands extending from the body proper attach to an inner disc at base of each sucker; when muscle bands contract, the disc is consequently pulled within the base, causing the outer ridges of sucker cups to draw closer to one another, locking host tissue in the cavity and securing an effective grip (Figure 4.3.5 D, Figure 4.3.7 A–B). Sucker function is based on the vacuum principle; host tissue is drawn into the cavity of the cup shaped sucker (Figure 4.3.6 A)

and through the contraction and relaxation of muscle system, host tissue is drawn and locked in.

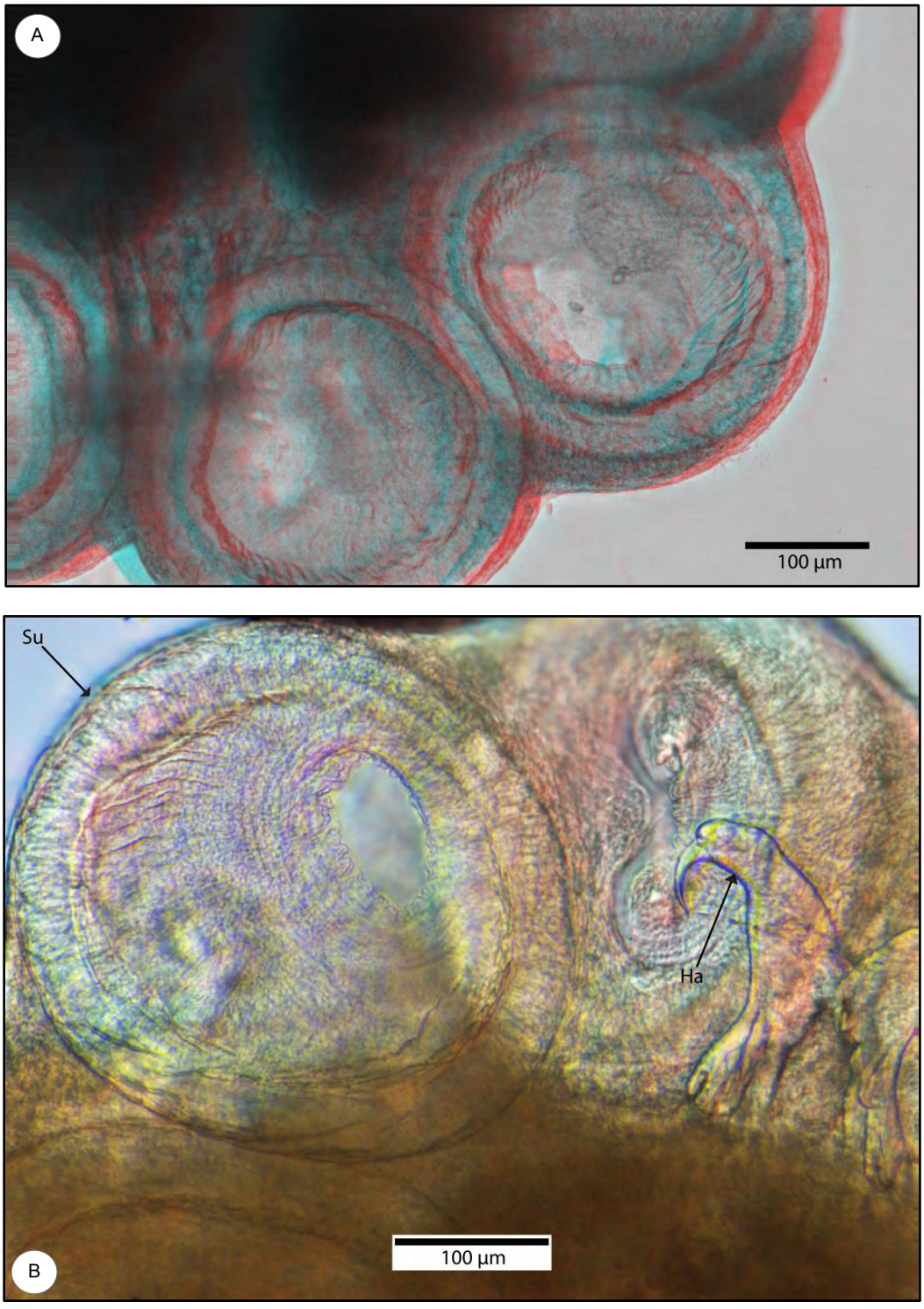


Figure 4.3.6: 3D light micrographs of a lactophenol cleared specimen of *Polystoma* haptor. **(A)** A close-up of haptor. **(B)** A close-up of the posterior of *Polystoma* haptor, showing sucker cups (Su) and hamuli (Ha). **Note that these are 3D images. Please use the anaglyph glasses provided on the inside back cover.**

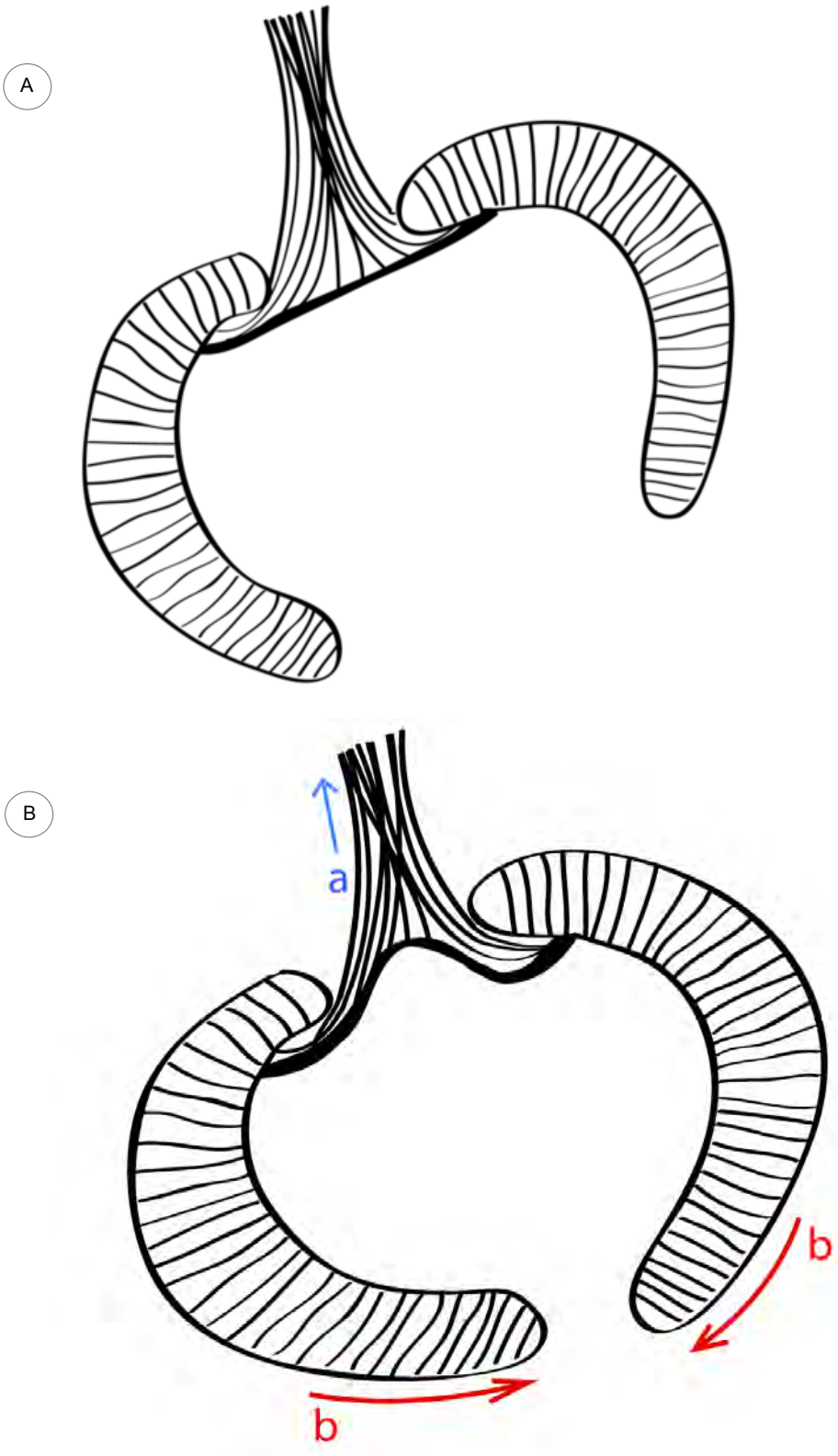


Figure 4.3.7: Drawing showing the mechanism of muscular sucker cups. **(A)** Relaxed state of muscular sucker. **(B)** Showing the contraction of muscles (a), extending from the body proper, causing the outer ridges of sucker cups to draw closer to one another (b).

The hamulus primordial develop into a pair of large hooks or anchors known as hamuli which are positioned between the posterior-most pair of suckers where the hamuli are deeply imbedded into the haptoral tissue (Figure 4.3.6 B). When attached to a host only the blade of the hamulus protrude to the outside and is slammed into the host's tissue. A bundle of muscle fibres extends from each root, respectively the handle the guard of the hamulus, to the body proper. The extension of muscle fibres connected to the guard together with the retraction of muscle fibres connected to the handle causes hamuli to swing forward (Figure 4.3.9).

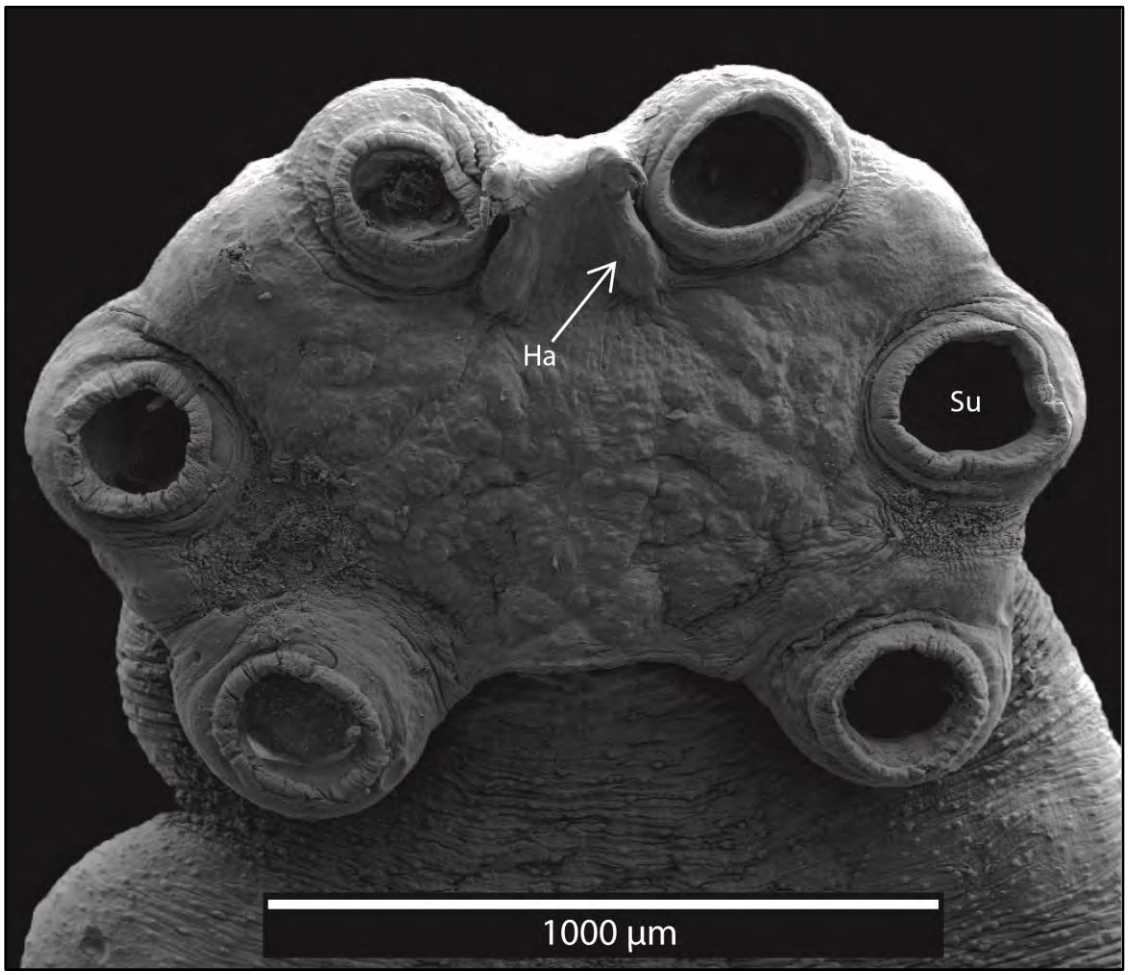


Figure 4.3.8: Scanning electron micrograph showing the full haptor, with six ventral suckers (Su) and one pair of hamuli (← Ha) of adult *Polystoma australis*.

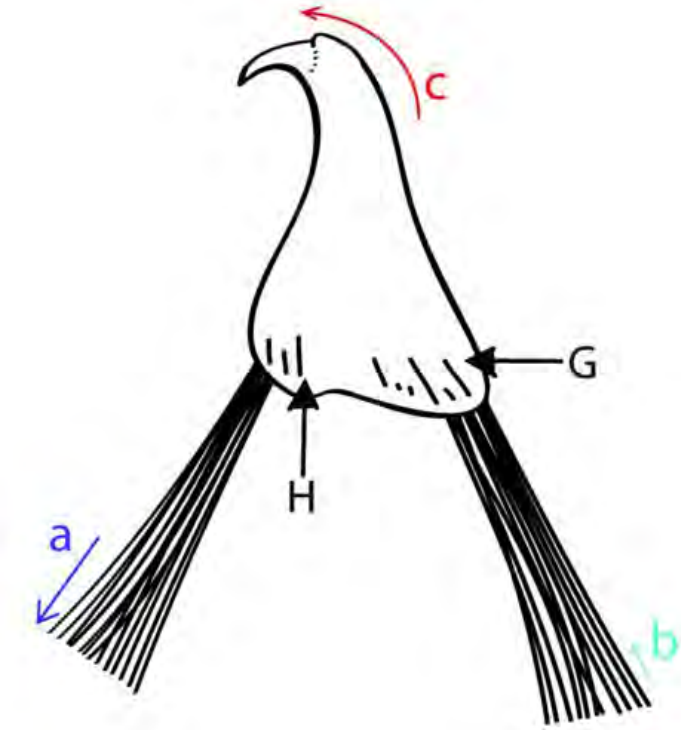


Figure 4.3.9: Drawing to illustrate the mechanism of *Polystoma* hamuli. Muscle fibres attached to the guard (G) contracts (b) while those attached to the handle (H) relax (b) causing the hamulus to swing outward (c) and penetrate into host tissue.

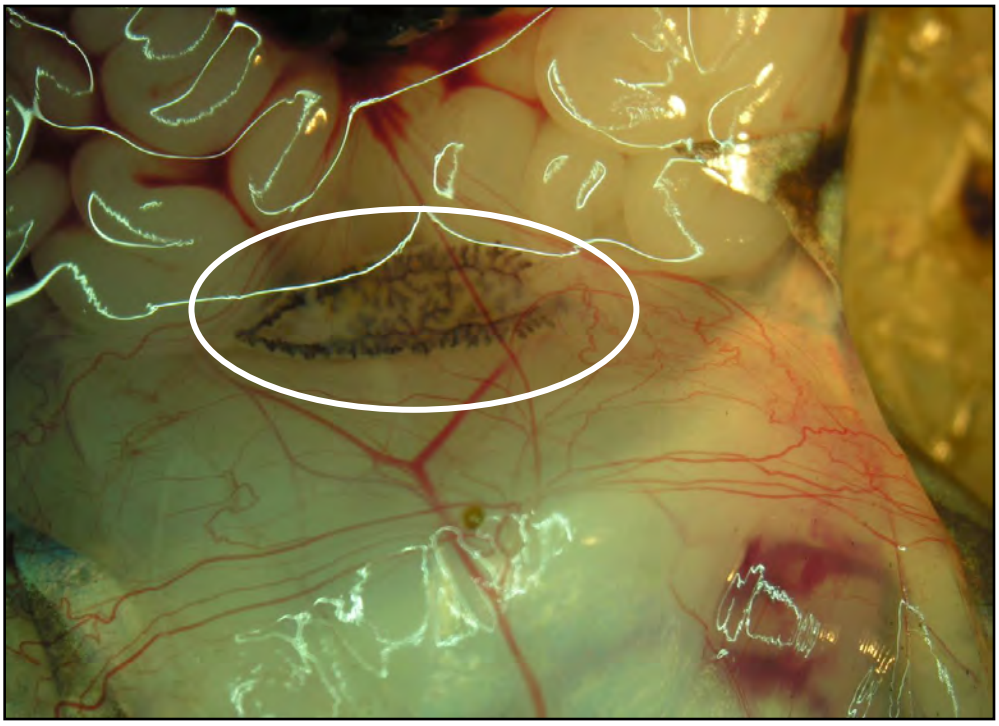


Figure 4.3.10: *Polystoma* within bladder of frog host (O).

Chapter 4.4

Eupolystoma

4.4.1 Mature parasites

Unlike most other polystomes, *Eupolystoma* has an internal cycle and oncomiracidia hatch as the fully embryonated eggs are released, or in some cases inside the maternal body before being released (Figure 4.4.3 A). These infective stages then either attach next to their parents or leave the host to seek other hosts that entered the water body to breed. Oncomiracidia do not infect tadpoles, but directly infect the adult breeding frogs via the cloaca and establish in the urinary bladder (Figure 4.5.1). The internal cycle as observed in *Eupolystoma* provides the opportunity for a massive build-up of a population (Figure 4.4.1). Tinsley (1978a) reported as many as 2 000 specimens of *Eupolystoma anterorchis* from a single *Amietophrynus pantherinus* host individual. The general external body features for *Eupolystoma* is similar as described for *Polystoma,* except that the parasite is a bit smaller and has no hamuli (Figure 4.4.2).

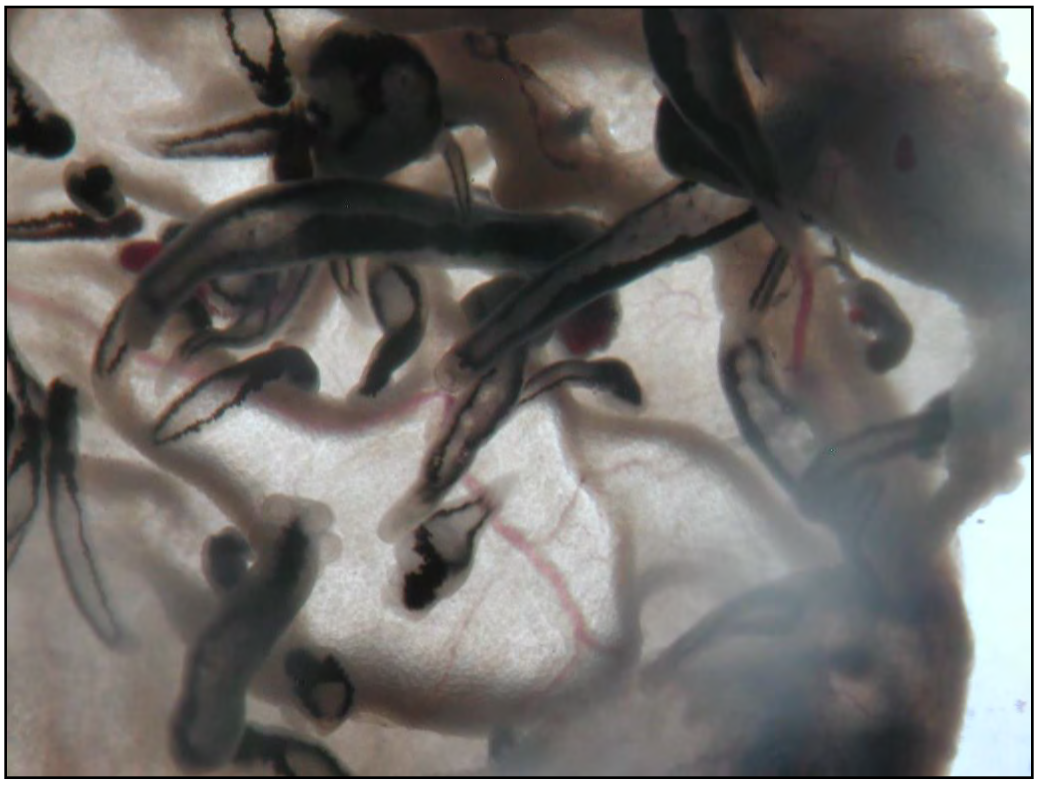


Figure 4.4.1: Light micrograph of *Eupolystoma vanasi* within the bladder of the host.

The gut caeca extends into the haptor and are confluent posteriorly to form a haptoral anastomosis. Lateral and medial diverticula of the caeca are present, but are small and pre-haptoral anastomoses are absent (Figure 4.4.2). The small testis is posteriorly positioned. The ovary is small, pre-testicular and posteriorly positioned in the body. Vitellaria are confined to two lateral extracaecal fields. The uterus is tubular and extensive and extends the full length of the body proper and occupies most of the intercaecal space. It contains a large number of eggs in various stages of development from unembryonated to fully developed and ready to hatch. Situated vitellarium develops laterally in two distinct fields of follicles.

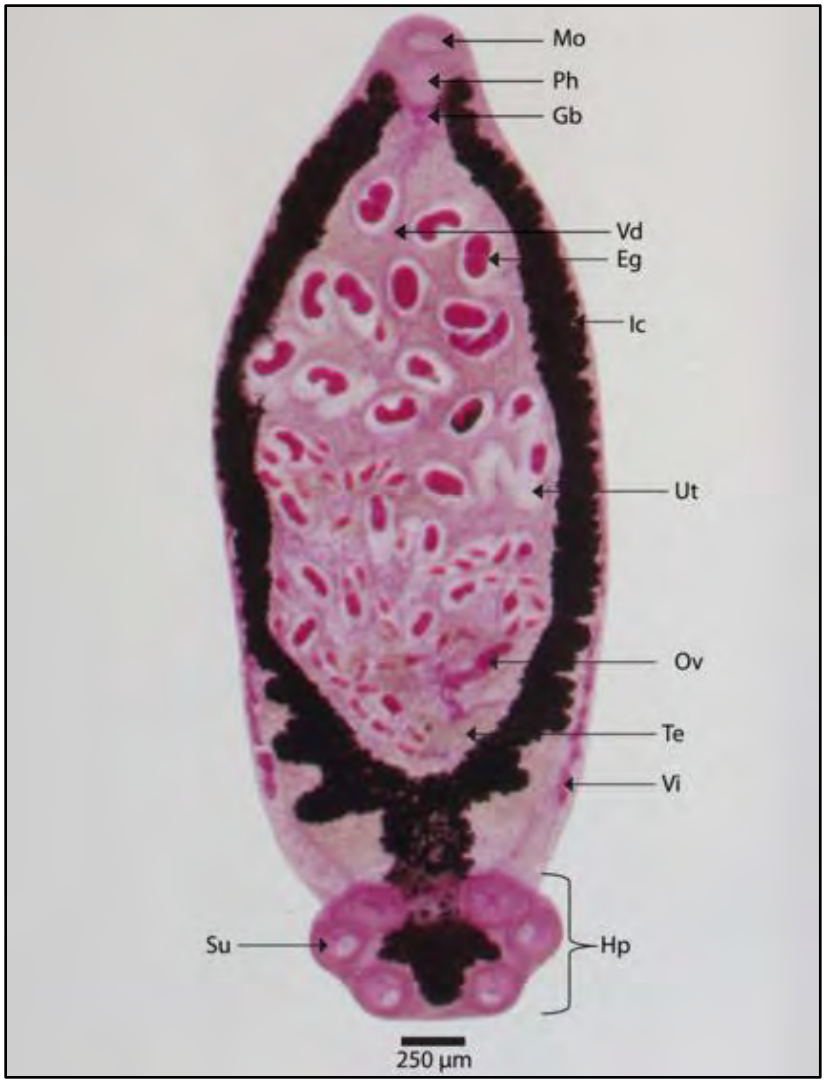


Figure 4.4.2: Light micrograph of a whole mount of E*upolystoma vanasi*. Annotations: Eg, egg; Gb, genital bulb; Hp, haptor; Ic, intestinal caecum; Mo, mouth; Ov, ovary; Ph, pharynx; Su, sucker; Te, testis; Ut, uterus; Vd, vas deferens; Vi, vitellaria.

4.4.2 Attachment structures

The placements of marginal hooklets are the same as for polystomes in general. The haptors of oncomiracidia are oval, elongated longitudinally, and their length is nearly one third of the total body length. The haptor is cup-shaped, directed ventrally, and bears a total of 16 marginal hooklets arranged in a circle (Figure 4.4.3 A). As the parasite matures the 16 marginal hooklets are no longer visible and submerged at the base of large muscular cups (Figure 4.4.3 B).

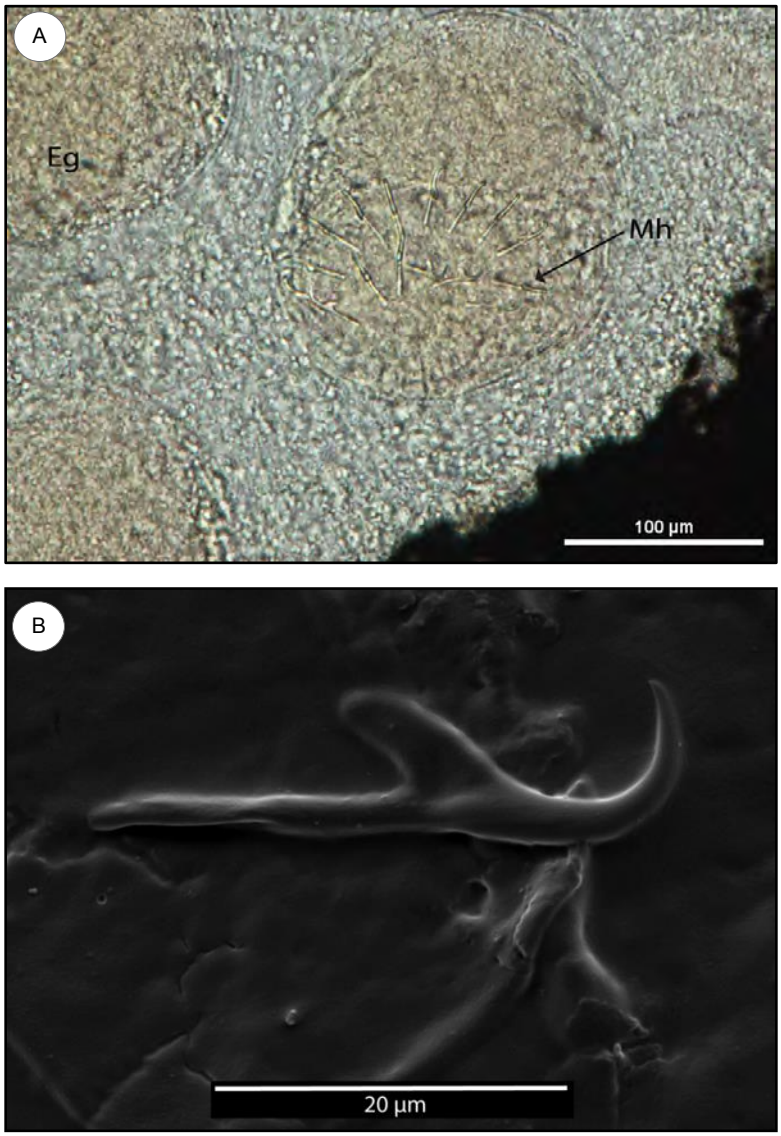


Figure 4.4.3: (A) Light micrograph of a lactophenol cleared *Eupolystoma* specimen, showing oncomiracidium inside maternal body. **(B)** Scanning electron micrograph showing a partially digested sucker, digested with proteinase K enzyme digestion solution, with a single marginal hooklet embedded in sucker tissue. Annotations: Eg, egg; Mh, marginal hooklet.

Haptors of *Eupolystoma* are of the simplest in terms of structure and form. They do not possess any additional hooks or hamuli beside the 16 marginal hooklets found in the larval stage used for attachment. They do not possess any skeletal elements or any form of reinforcements within the sucker. Haptor solely consist of musculature (Figure 4.4.5 A–B). Sucker cups are surrounded by a tegument lining and comprise of a series of radial muscle fibres in between muscle fibres (Figure 4.4.4 A–D).

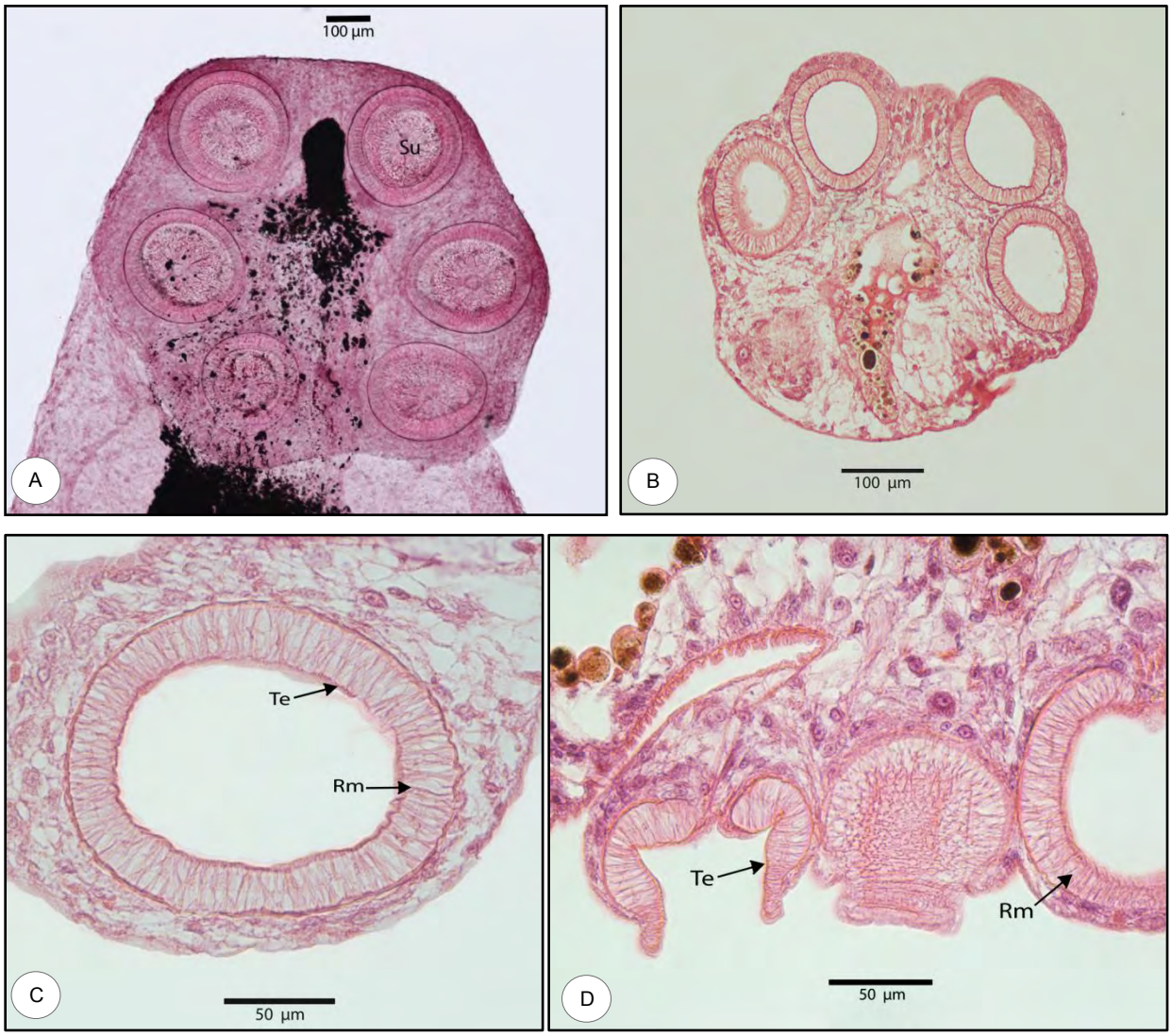


Figure 4.4.4: Light micrograph images of *Eupolystoma vanasi*. **(A)** Ventral view of the haptor. **(B)** Histological section through the haptor. **(C – D)** Histological sections through suckers; showing the radial muscle fibres (Rm) between the teguments (Te) of suckers.

Firm attachment is secured through host tissue being drawn into the sucker; muscle bundles extending from the body proper attach to an inner central disc at base of each sucker; when muscle fibres contract, the central disc is consequently pulled within the base, causing a vacuum and the outer ridges of sucker cups to close tight around the bud of host tissue securing a firm grip, same as in Figure 4.3.8. x

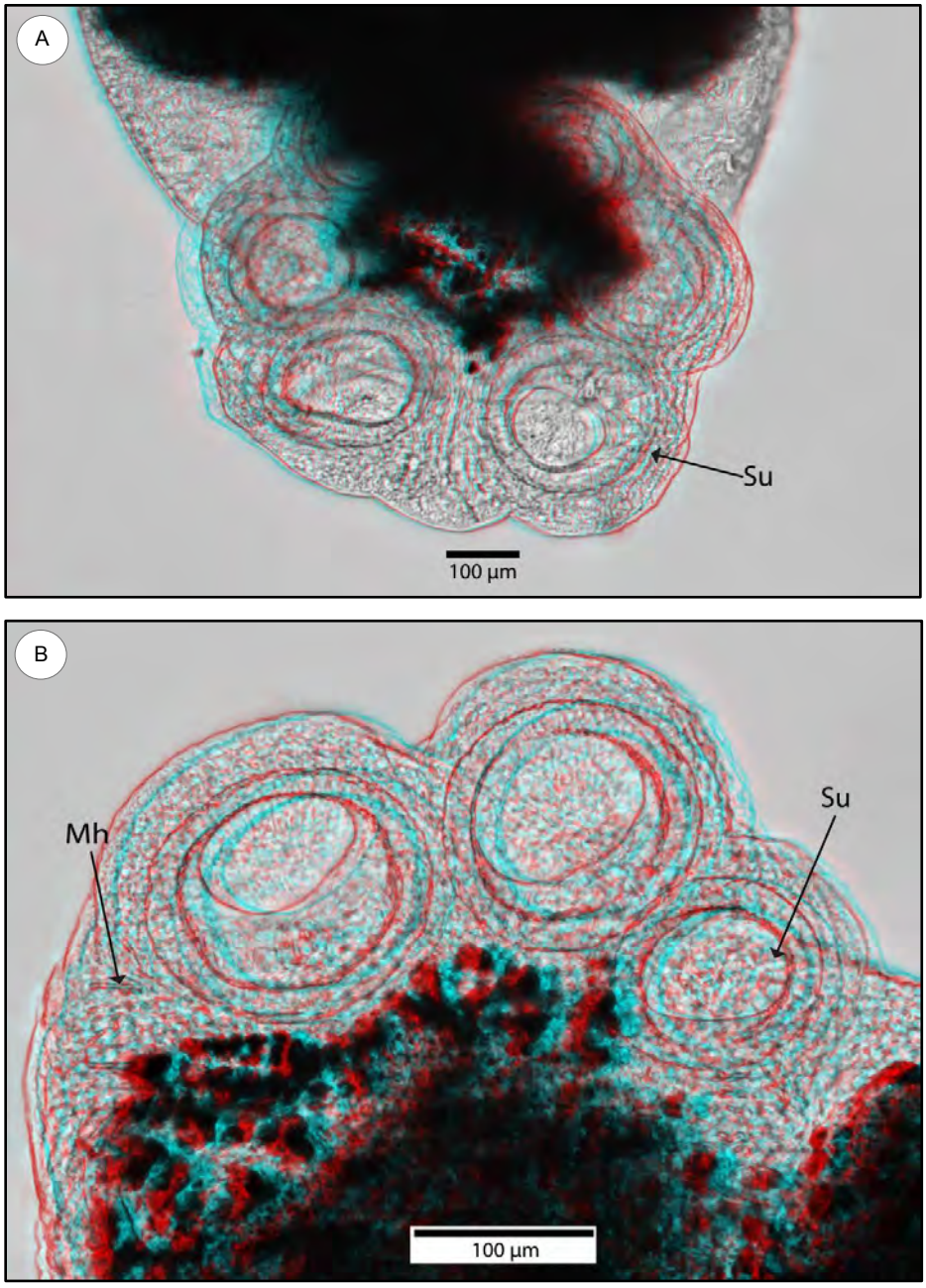


Figure 4.4.5: (A) 3D light micrograph of a lactophenol cleared *Eupolystoma* haptor with six suckers (Su). **(B)** 3D light micrograph of a lactophenol cleared *Eupolystoma* haptor with suckers (Su), showing some of the marginal hooklets (Mh) between the posterior most sucker pair. **Note that these are 3D images. Please use the anaglyph glasses provided on the inside back cover.**

Chapter 4.5

Neopolystoma

4.5.1 Mature parasites

Although the general body shape of terrapin polystomes are very similar to that of anuran polystomes, the internal morphology differ slightly. The mouth is sub-terminal and ventral. The body is elongated, cylindrical and narrows slightly posterior to the haptor (Figure 4.5.2, Figure 4.5.3 B). Haptor armed with six haptoral suckers, and hamuli are totally absent. The intestine consists of two intestinal caeca that may extend all the way down and slightly into the haptor, but do not fuse posteriorly. Lateral and medial diverticula are absent. The medial testis may be spherical or a network (Figure 4.5.2) and positioned in the middle of the body. From the testis the vas deferens extends anteriorly and opens to form a seminal vesicle that opens in the armed genital opening (Figure 4.5.3 A). The ovary is pretesticular and fairly small. The uterus is absent. Soon after an egg is fully formed within the oötype, the egg is expelled from the body. Vitellaria extend throughout most of the body except in the medial section and the oral region (Figure 4.5.2).

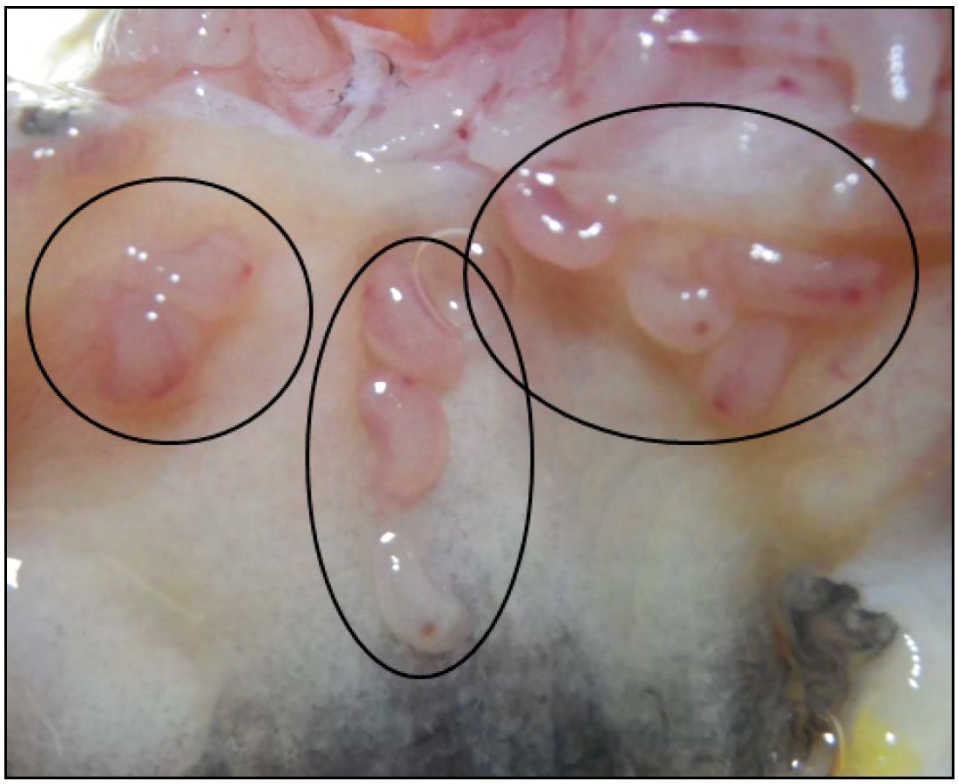


Figure 4.5.1: Light micrograph showing several mature specimens of N*eopolystoma* sp. attached to the bladder of its host.

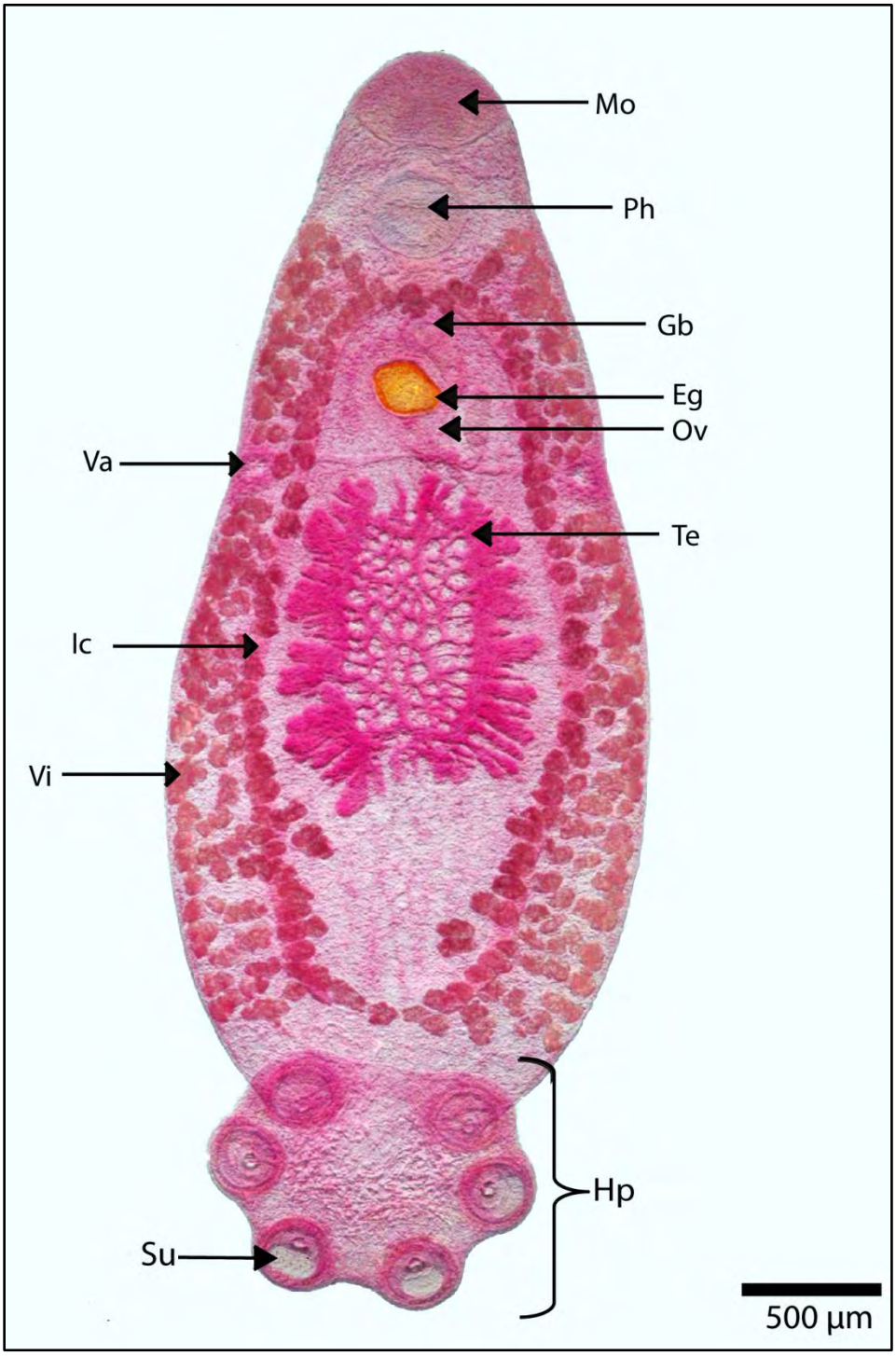


Figure 4.5.2: Light micrograph of a whole mount of *Neopolystoma* sp. Annotations: Eg, egg; Gb, genital bulb; Hp, haptor; Ic, intestinal caecum; Mo, mouth; Ov, ovary; Ph, pharynx; Su, suckers; Te, testis; Va, vagina; Vi, vitelline follicles.

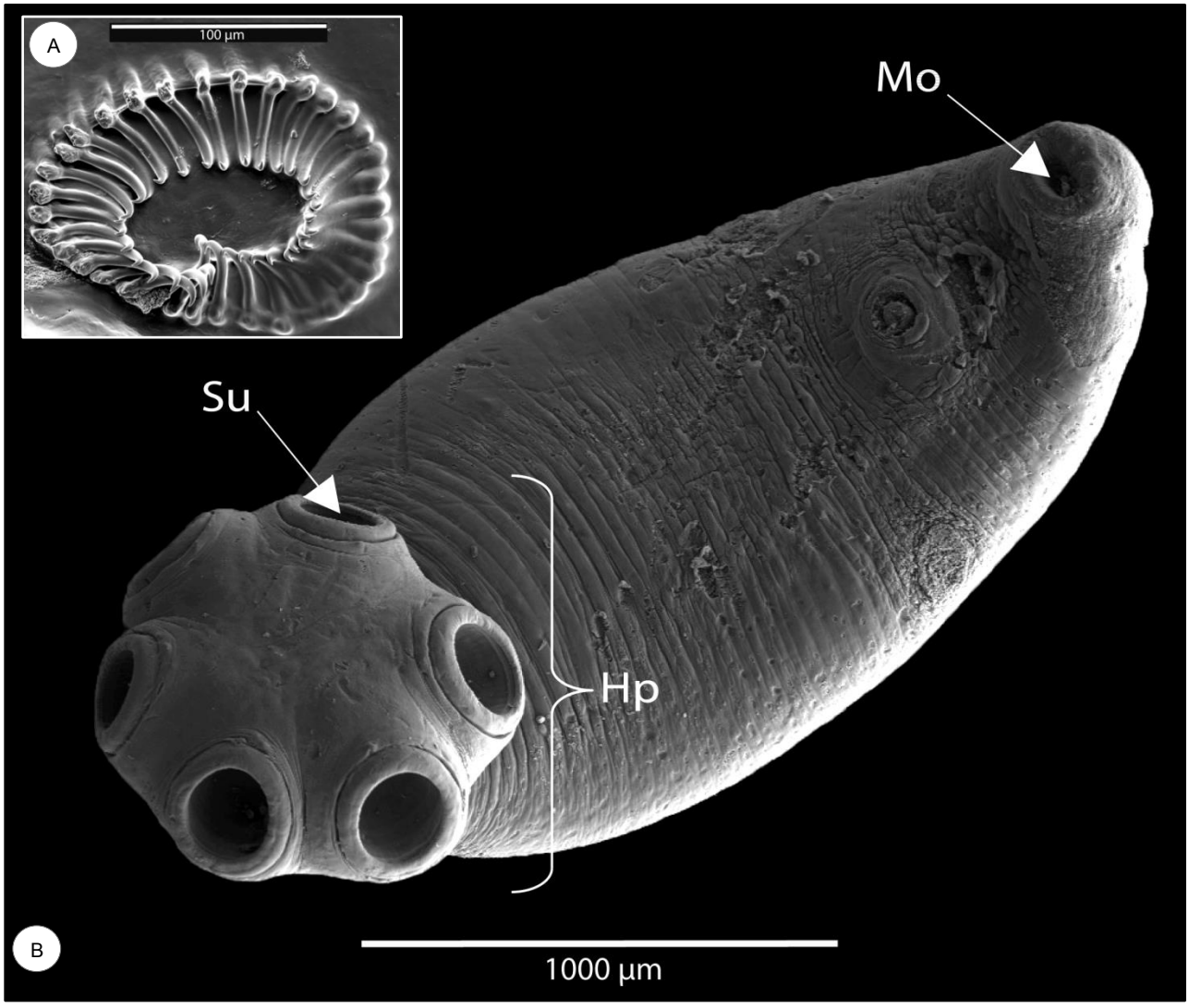


Figure 4.5.3: Scanning electron micrographs of *Neopolystoma* sp. **(A)** Genital crown of spines revealed through partial digested with proteinase K enzyme digestion solution. **(B)** Ventral view of a fully developed parasite. Note the lack of hamuli, the placement of the suckers (Su) on the haptor (Hp), the ventral mouth (Mo) at the anterior end.

4.5.2 Attachment structures

Hamuli are totally absent, but the haptor is fitted with six muscular cups, each with a complex skeletal structure (Figure 4.5.5 A–D, Figure 4.5.8). The suckers are positioned along the periphery of the haptor and directed ventro-laterally. Suckers are imbedded deep into the body of the haptor (Figure 4.5.4 A–B, Figure 4.5.7 A–B). Based

on morphology the sucker can be divided in three zones. The first zone also known as the central zone is the area deep in at the base of the sucker. It consists of a firm skeletal funnel at the base of the sucker (Figure 4.5.5 A and D); the sucker wall and the muscle fibres attached to the sucker wall and the skeletal funnel. Muscle fibres are attached to this funnel. The second zone is also known as the intermitted zone. The key element of the intermitted zone is a ring of skeletal elements resembling an in line bracelet consisting of a series of interconnected blocks (Figure 4.5.5 C–D, Figure 4.5.6 A–B). These blocks have a number of foramens and tubes through them (Figure 4.5.6 A–F, Figure 4.5.7).

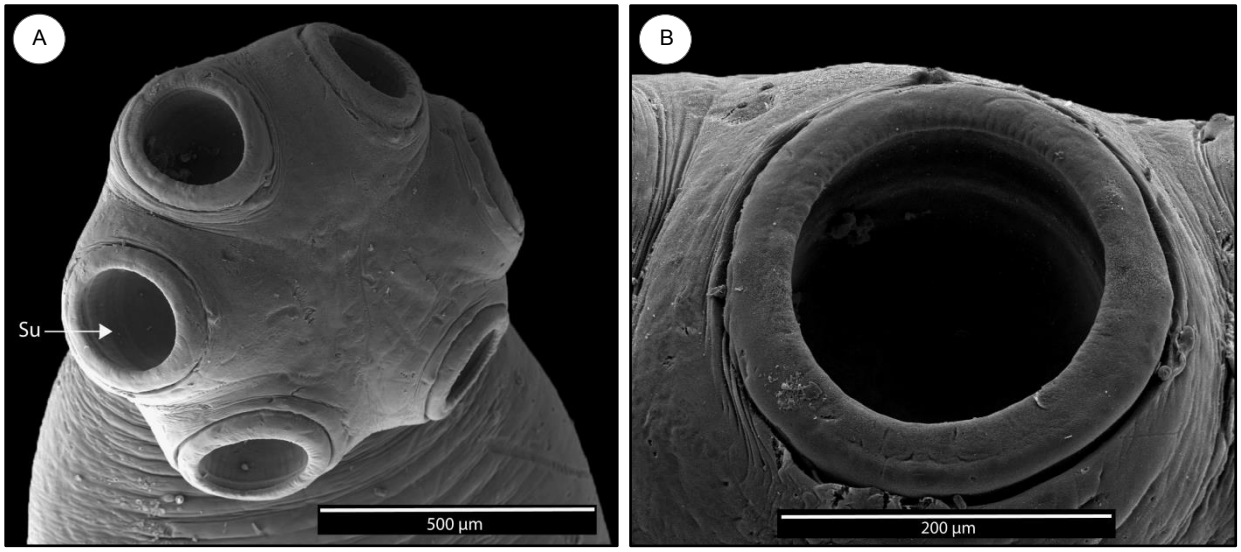


Figure 4.5.4: Scanning electron micrographs of *Eupolystoma* sp. **(A)** Haptor with suckers directed ventro-laterally. **(B)** Close-up of a single sucker showing how deep the sucker is set into the body of the haptor.

The intermitted zone is a continuous skeletal lining in the centre of the sucker cup. This is made up of skeletal segments and resembles a ring of “bricks” (Figure 4.5.6 F). This section can further be divided into two subsections: upper and lower skeletal lining (4.5.5 A and D). The lower lining is made up of solid skeletal segments, joined at irregular intervals (Figure 4.5.5 D).

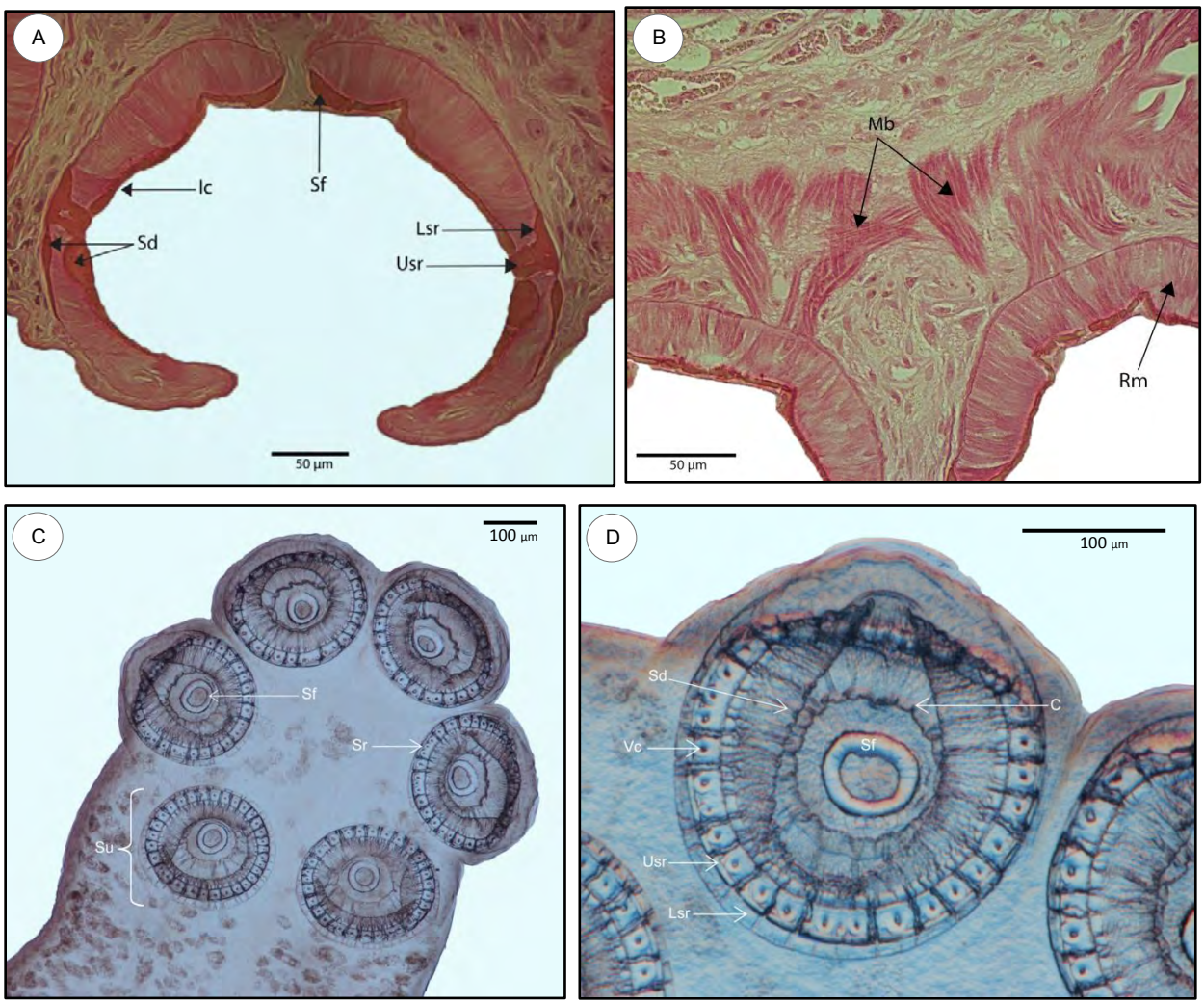


Figure 4.5.5: (A) Light micrograph of a histological sagittal section through the center of a sucker cup of *Neopolystoma* sp.; showing the skeletal funnel (Sf), inner costi (Ic), inner and outer chitinous digiti (Sd) and upper (Usr) and lower skeletal ring (Lsr). **(B)** Light micrograph of a histological section through the middle of a sucker cup of *Neopolystoma;* showing the radial muscle fibres (Rm) and muscle bands (Mb). **(C)** Light micrograph of *Neopolystoma'*s haptor armed with six suckers (Su) each with a skeletal funnel (Sf) at the base and a skeletal ring (Sr) dividing each cup into two. **(D)** Light micrograph of a close-up of *Neopolystoma'*s haptor, showing a single sucker (Su). At the base of the sucker is a skeletal funnel (Sf) which is connected to the skeletal ring by a series of costae (C). The skeletal ring is divided into two sections: lower skeletal ring (Lsr) and upper skeletal ring (Usr). The lower skeletal ring is a skeletal lining under the upper skeletal ring, which is made up of a series of skeletal segments. Skeletal segments have various canals, connecting them either to one another (horizontal canals) or a pathway for muscle fibres, vertical canals (Vc). Skeletal digiti (Sd) extent from skeletal ring to the end of the sucker lining.

The upper lining consists of segments that contain perforations, which appear as circles in the equatorial groove and are joined on both sides at regular intervals. Perforations or canals either connect segments to one another through a ring muscle (horizontal canals) or act as a pathway for muscle fibres (vertical canals) (Figure 4.5.6 C–F). Skeletal chitinous digiti extend from the central zone to the end of the sucker lining, known as the peripheral zone.

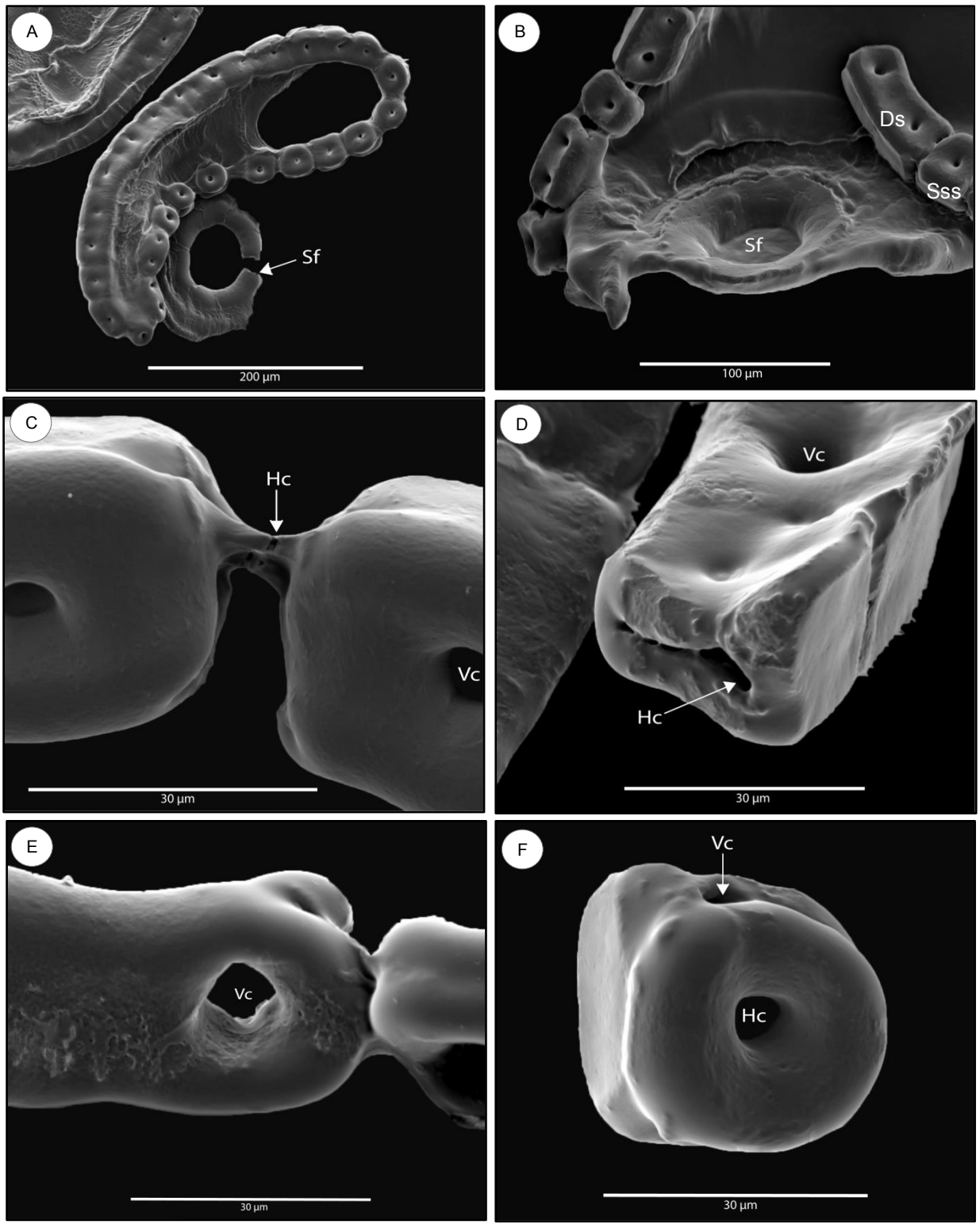


Figure 4.5.6: Scanning electron micrographs of sucker digested with Proteinase K enzyme digestion solution for 80min. **(A)** Single sucker. **(B)** Single sucker, showing skeletal funnel (Sf) still partially attached by tissue and costae to skeletal ring, made up of a series of single skeletal segments (Sss) and double segments (Ds). **(C)** Dorsal view of two skeletal segments connected through a horizontal connecting canal (Hc) opening on lateral sides of each segment. There is another canal (Vc), running vertically through the skeletal segment. **(D)** Lateral view interconnecting chain, showing horizontal canal (Hc), together with vertical canal (Vc) through skeletal segments. **(E)** Dorsal view of connecting skeletal segments, showing vertical canal (Vc). **(F)** Dorsal view of a single skeletal segment, showing both horizontal (Hc) and vertical canal (Vc).

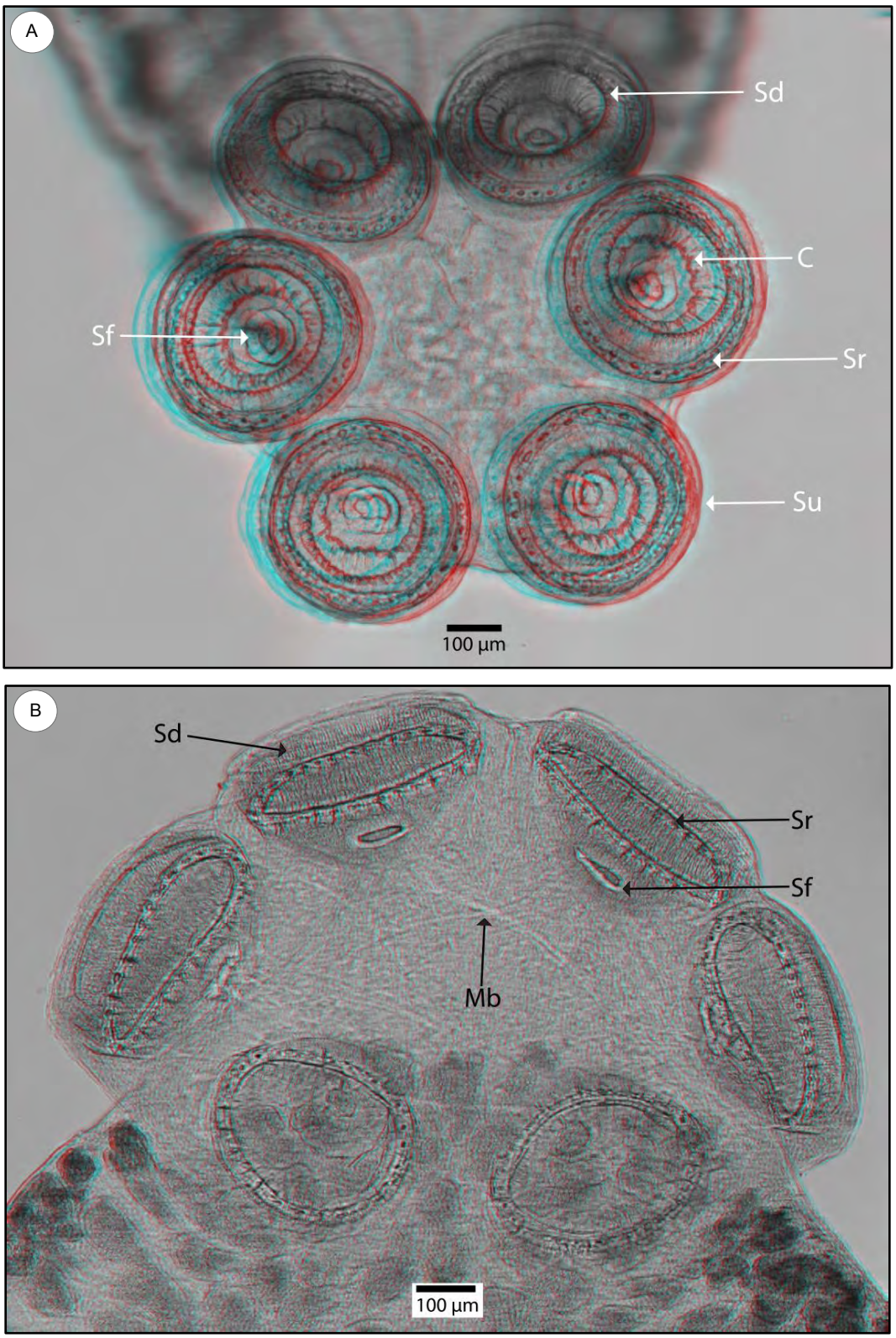


Figure 4.5.7: **(A – B)** 3D Light micrographs of a lactophenol cleared *Neopolystoma* sp. specimen. Haptor armed with six sucker cups (Su) and at the base of each sucker is a skeletal funnel (Sf) which is connected to the skeletal ring (Sr) by a series of costae (C). Skeletal digiti (Sd) extent from skeletal ring to the end of the sucker lining. Muscle bands (Mb) are evident running from each sucker across the centre of the haptor towards the body wall. **Note that these are 3D images. Please use the anaglyph glasses provided on the inside back cover.**

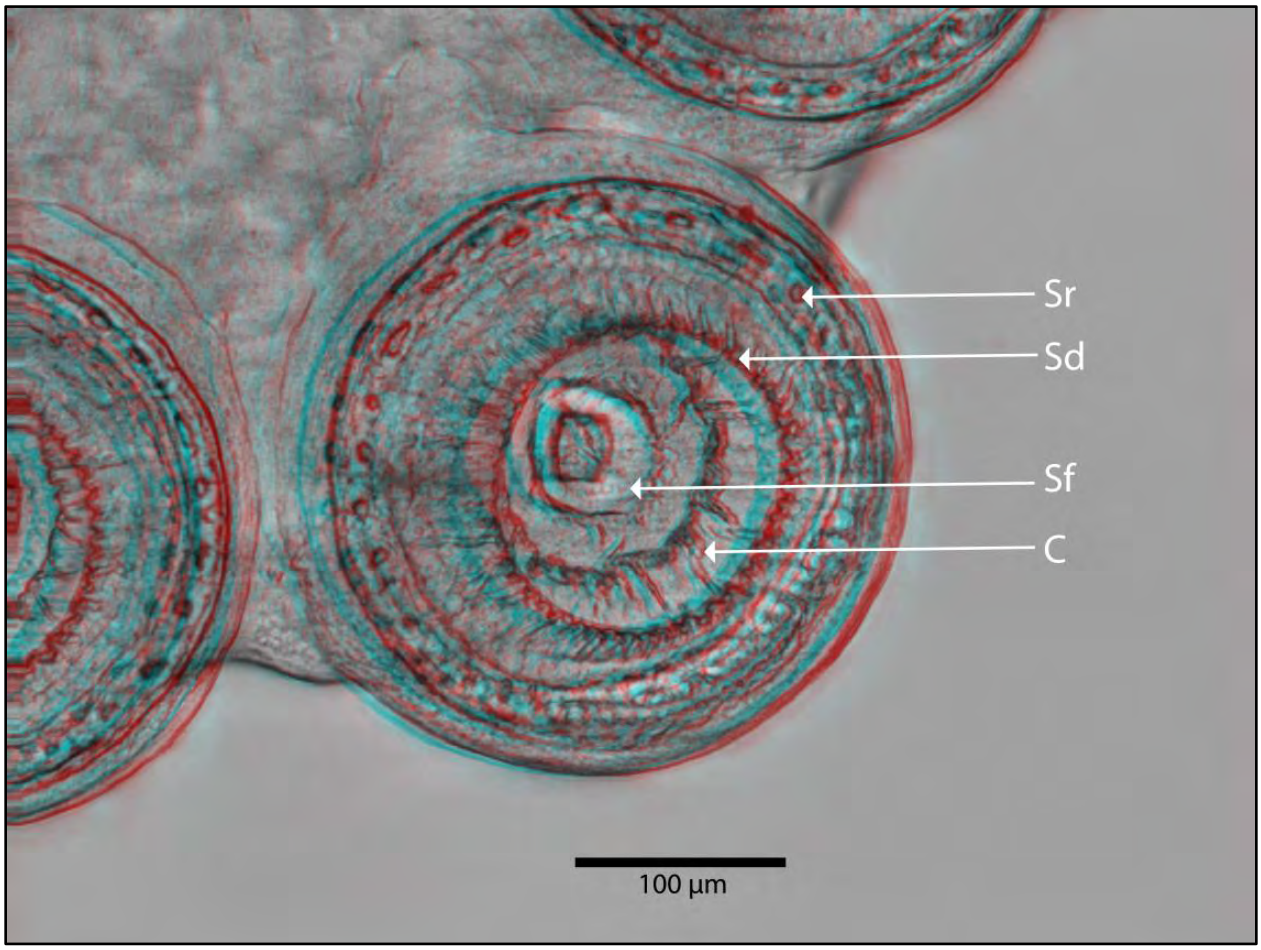


Figure 4.5.8: 3D Light micrograph of a lactophenol cleared *Neopolystoma* sp. haptor. At the base of the sucker is a skeletal funnel (Sf) which is connected to the skeletal ring (Sr) by a series of costae (C). Skeletal digiti (Sd) extent from skeletal ring to the end of the sucker lining. **Note that this is a 3D image. Please use the anaglyph glasses provided on the inside back cover.**

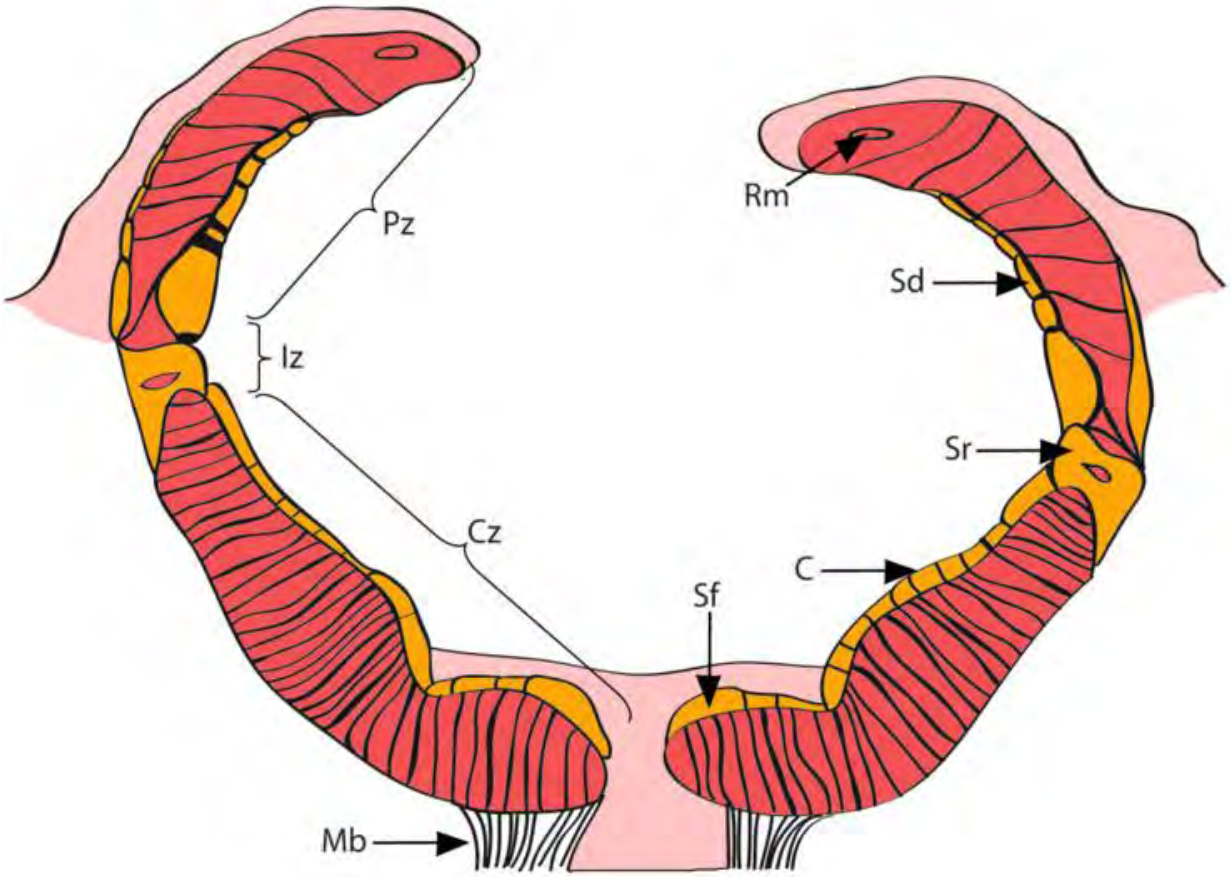


Figure 4.5.9: Drawing of the skeletal complex showing the three zones. Abbreviations: C, costae; Cz, central zone; Iz, intermediate zone; Pz, peripheral zone; Rm, ring muscle; Sd, skeletal digitae, Sf, skeletal funnel; Sr, skeletal ring.

Based on the morphology of the sucker we hypothesise that the attachment mechanism of the sucker is as follows (Figure 4.5.9 and Figure 4.5.10): The suckers are pressed firmly against the host tissue. The skeletal funnel at the base of the sucker is pulled back creating a vacuum within the sucker and host tissue is drawn into the sucker. Longitudinal muscles throughout the sucker (Figure 4.5.11); together with the muscles running through the skeletal ring, and a ring of muscle fibres along the outer periphery of the sucker contracts and produce a very firm clamp on the host tissue. This attachment is so firm that it is very difficult to pull free an attached *Neopolystoma*.

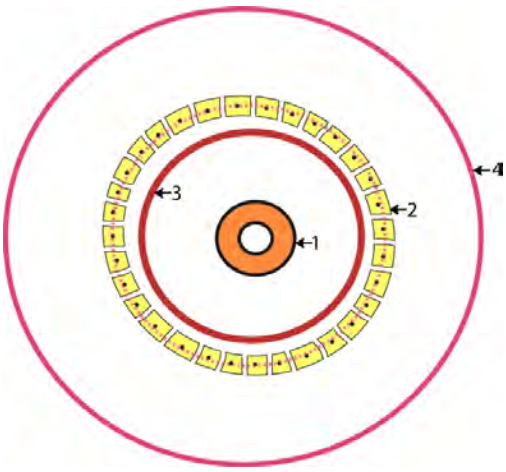
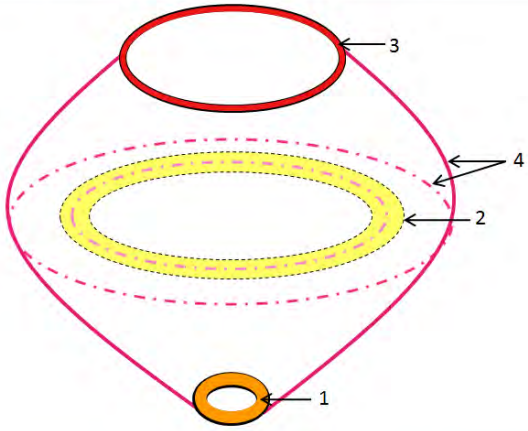
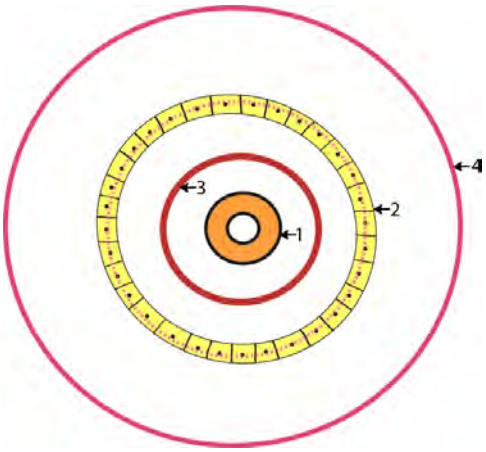
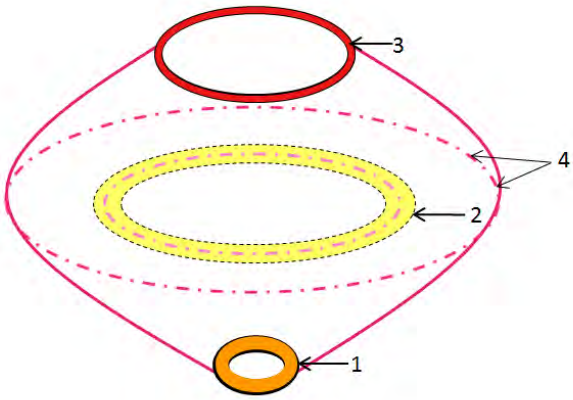
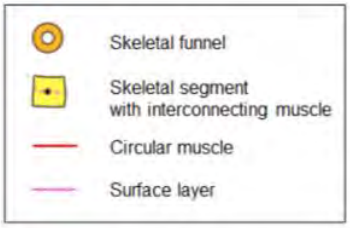
Dorsal view	Lateral view
A Dorsal view of a relaxed sucker	B Lateral view of a relaxed sucker
	
C Dorsal view of a contracted sucker	D Lateral view of a contracted sucker
	
1: The central zone with skeletal funnel at the base of the sucker; 2: Intermediate zone showing skeletal segments and relaxed interconnecting ring muscle; 3: Relaxed ring muscle at the outer periphery of the peripheral zone; 4. Outer ling of sucker.	1: Skeletal funnel at the base of the sucker is pulled back deeper into the body of the haptor creating a vacuum; 2: Skeletal segments draw nearer to one another through the contraction of the interconnecting ring muscle, 3: Ring muscle contracts restricting the size of the opening of the sucker; 4. Outer ling of sucker.
	

Figure 4.5.10: Diagrammatic drawing of dorsal and lateral views of a relaxed and contracted haptoral sucker of *Neopolystoma* sp.

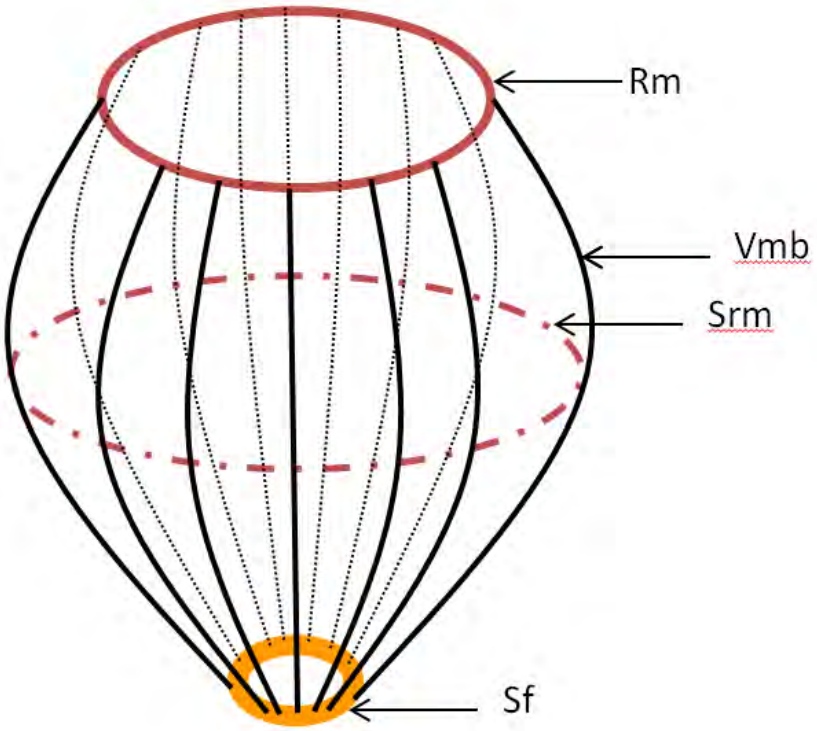


Figure 4.5.11: Drawing of the musculature within the sucker. Ring muscle at the tip of sucker cup contracts and narrows the sucker opening, closing off host tissue to a certain extent. Ring muscle running through the horizontal canals within skeletal ring in the central zone, vertical muscle bands runs through the vertical canals in the skeletal ring. Abbreviations: Rm, ring muscle; Sf, skeletal funnel; Srm, skeletal ring muscle; Vmb, vertical muscle bands.

The bladder lining (Figure 4.5.12 A) is highly flexible. Parasites attach firmly and from here they feed on mucus (Figure 4.5.12 B). The rim of the sucker squeezes firmly on the bud of host tissue drawn into the sucker (Figure 4.5.12 C).

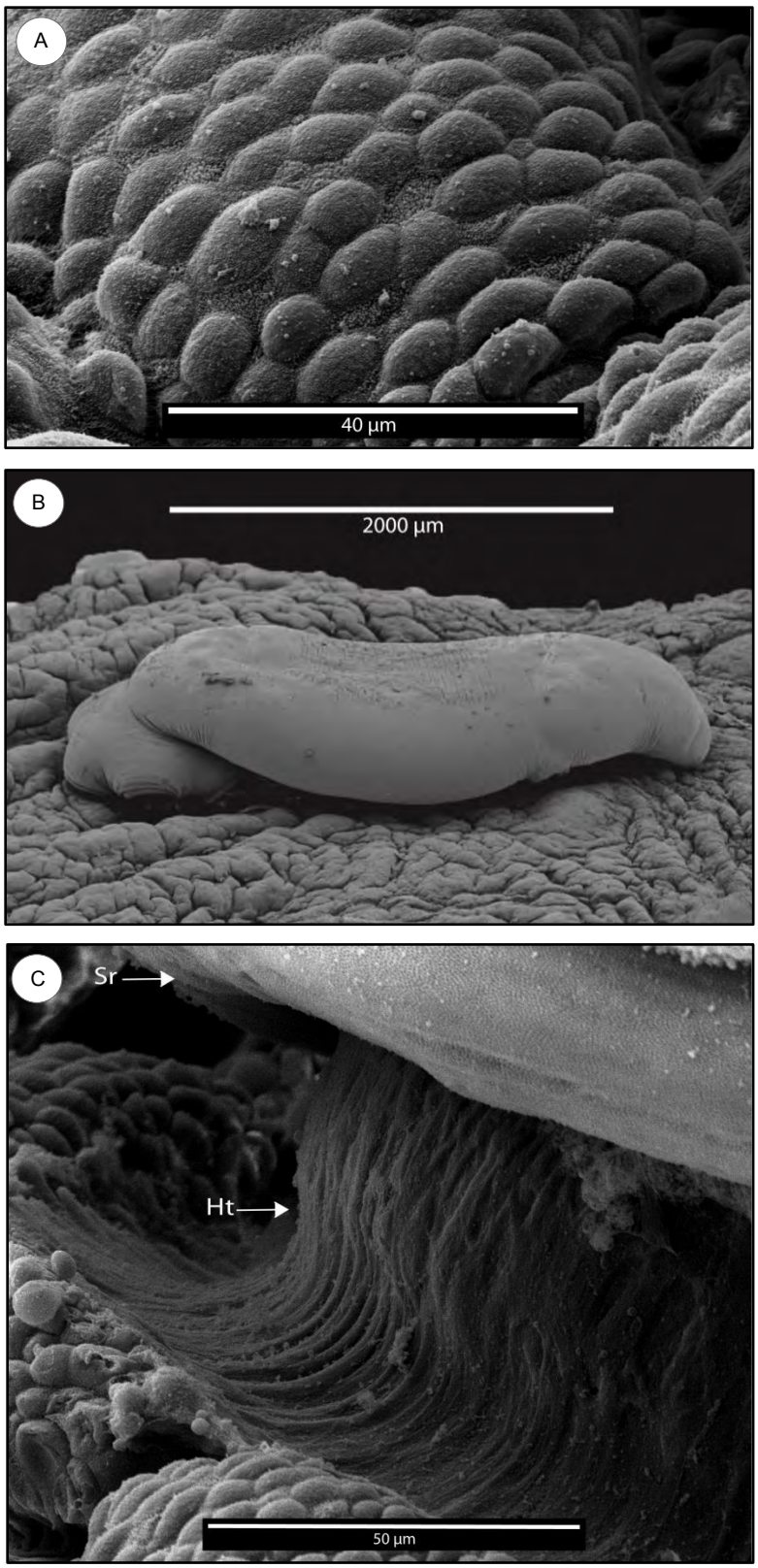


Figure 4.5.12: (A) Scanning electron micrograph showing epithelial tissue of *Trachemys scripta* urinary bladder before any parasite attachment. **(B)** Scanning electron micrograph of *Neopolystoma* attached to *Trachemys scripta* urinary bladder. **(C)** Scanning electron micrograph of a close-up of *Neopolystoma* attached to *Trachemys scripta'* urinary bladder. Abbreviations: Ht, Host tissue; Sr, sucker rim.

Chapter 4.6

Polystomoides

4.6.1 Mature parasites

The general body features for *Polystomoides* (Figures 4.6.1, Figure 4.6.2) are very similar to those described for *Neopolystoma* in 4.5.1, whereas *Neopolystoma* lacked hamuli altogether, *Polystomoide*s has two pairs of hamuli.

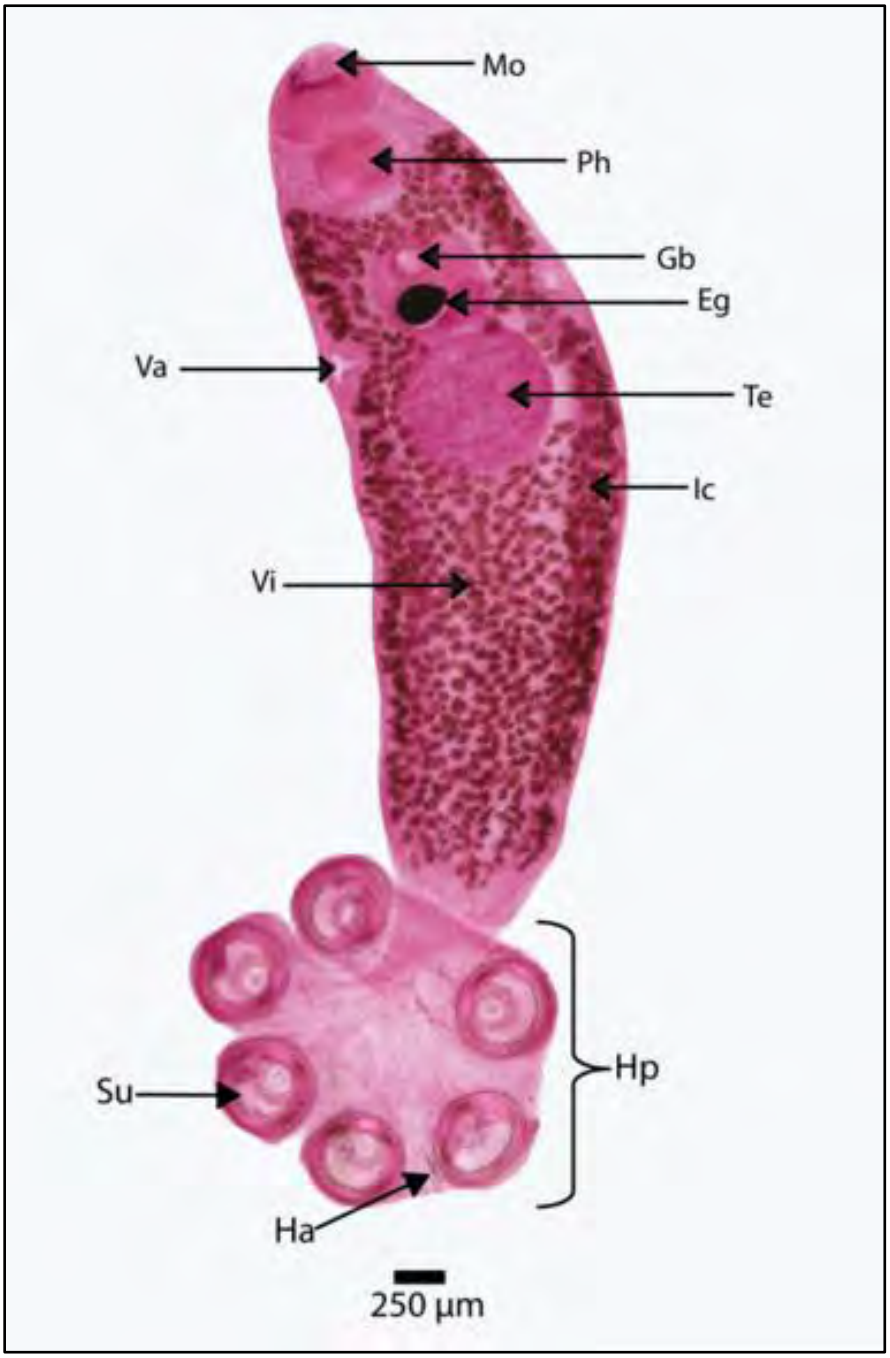


Figure 4.6.1: Light micrograph of a whole mount of *Polystomoides.* Annotations: Eg, egg; Gb, genital bulb; Ha, hamuli; Hp, haptor; Ic, intestinal caecum; Mo, mouth; Ph, pharynx; Su, suckers; Te, testis; Va, vaginae; Vi, vitelline follicles.

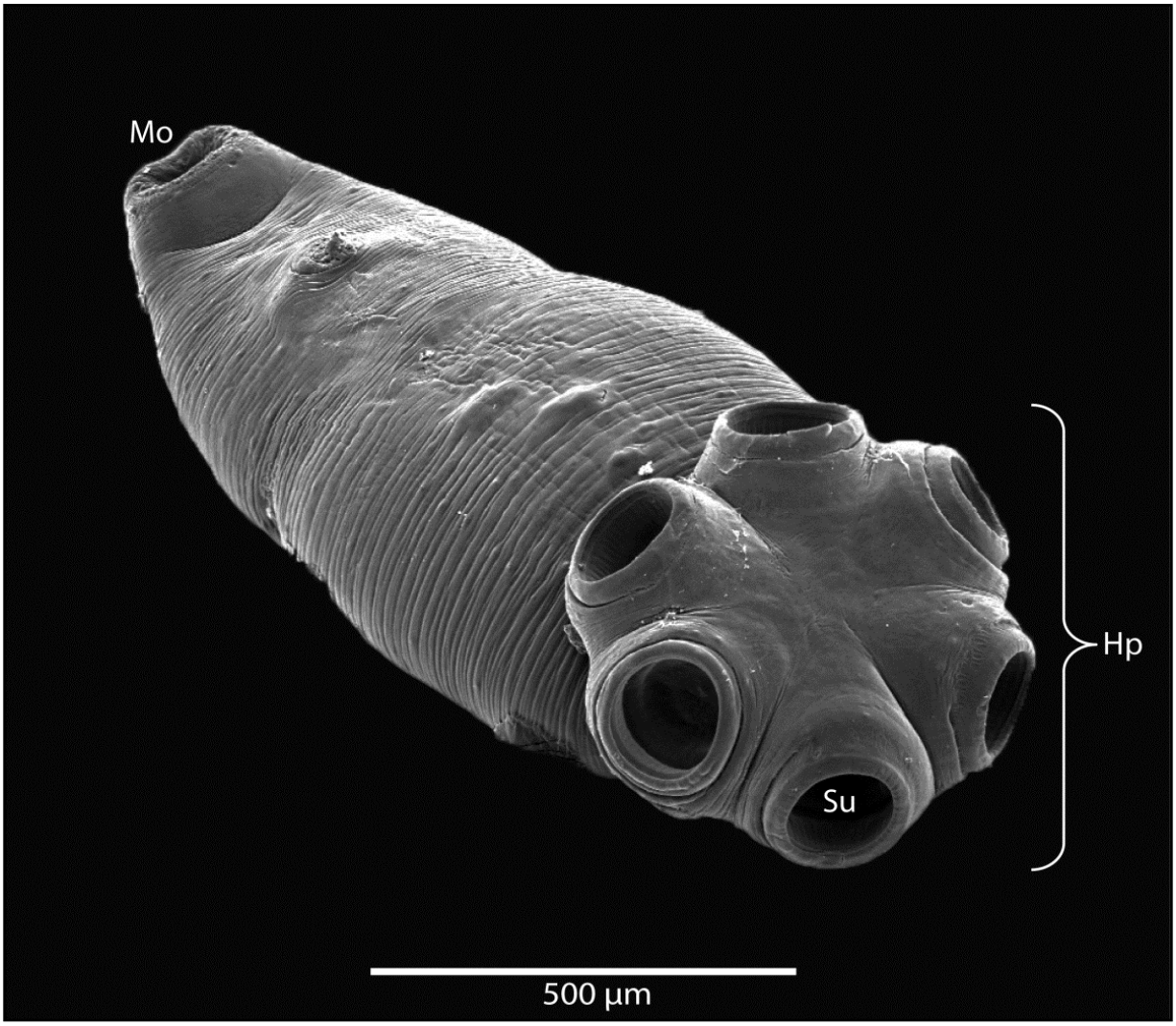


Figure 4.6.2: Scanning electron micrograph of a full length *Polystomoides* specimen, showing the mouth opening (Mo) at the anterior end and the haptor (Hp) armed with six suckers (Su).

4.6.2 Attachment structures

The morphology of the *Polystomoides* oncomiracidium is very similar to that of *Neopolystoma*. Posteriorly in the haptor the oncomiracidium of *Polystomoides* has two pairs of hamulus primordial. Only after the oncomiracidium has attached to a suitable host, hamulus primordial starts to develop (Figure 4.6.3). Only hamuli pair 1 will develop in large falciform hooks. However, both pairs of hamuli are functional and contribute to secure a firm attachment on the host tissue.

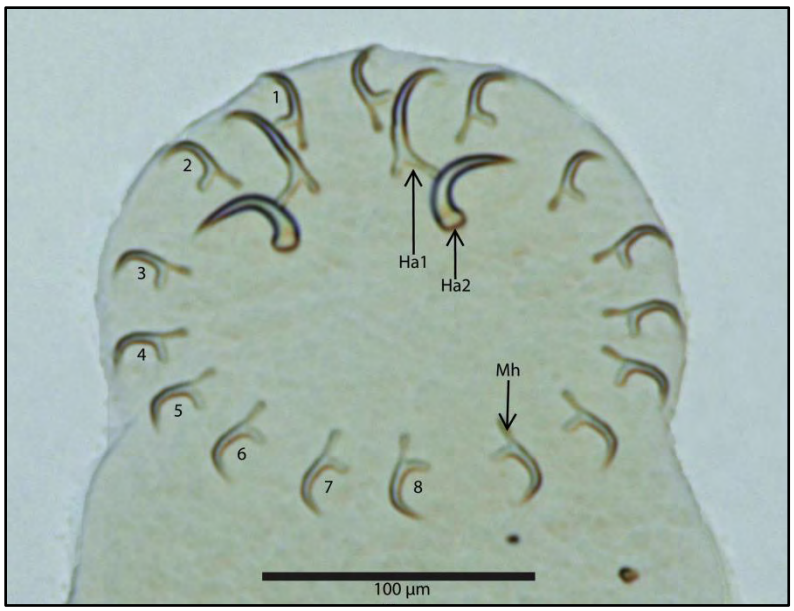


Figure 4.6.3: Light micrograph of the cup-shaped haptor of young parasite showing the 20 sclerites: two pairs of eight marginal hooklets (1 – 8) (← Mh) and two pairs of primordial hamuli (← Ha1 and Ha2).

As parasites mature marginal hooklets are no longer prominently evident, since none of the 16 marginal hooklets undergo further development when the oncomiracidium develops into a mature parasite. Marginal hooklets are, however, still present in the interior of each haptor sucker (Figure 4.6.4).

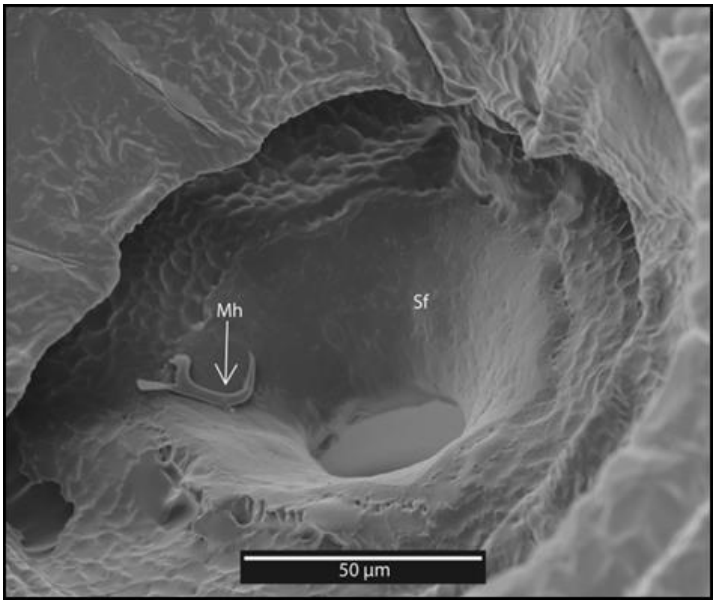


Figure 4.6.4: Scanning electron micrograph of marginal hooklet (Mh), revealed through partial digested with proteinase K enzyme digestion solution, on the edge of the skeletal funnel (Sf), in the centre of a sucker.

Only one pair of hamuli develops into large falciform haptoral hooks, protruding between the posteriormost sucker pair (Figure 4.6.5 B). The hamuli, approximately conical in shape, consist of a broad base with a long, prominently curved, sharp hook. The base with an inconspicuous bipedal root system is referred to as the “handle” for the medial root and the “guard” (Figure 4.6.5 A). Muscle fibres attached to the guard contracts while those attached to the handle relax, causing the hamulus to swing outward and penetrate into the host’s tissue (Figure 4.6.6).

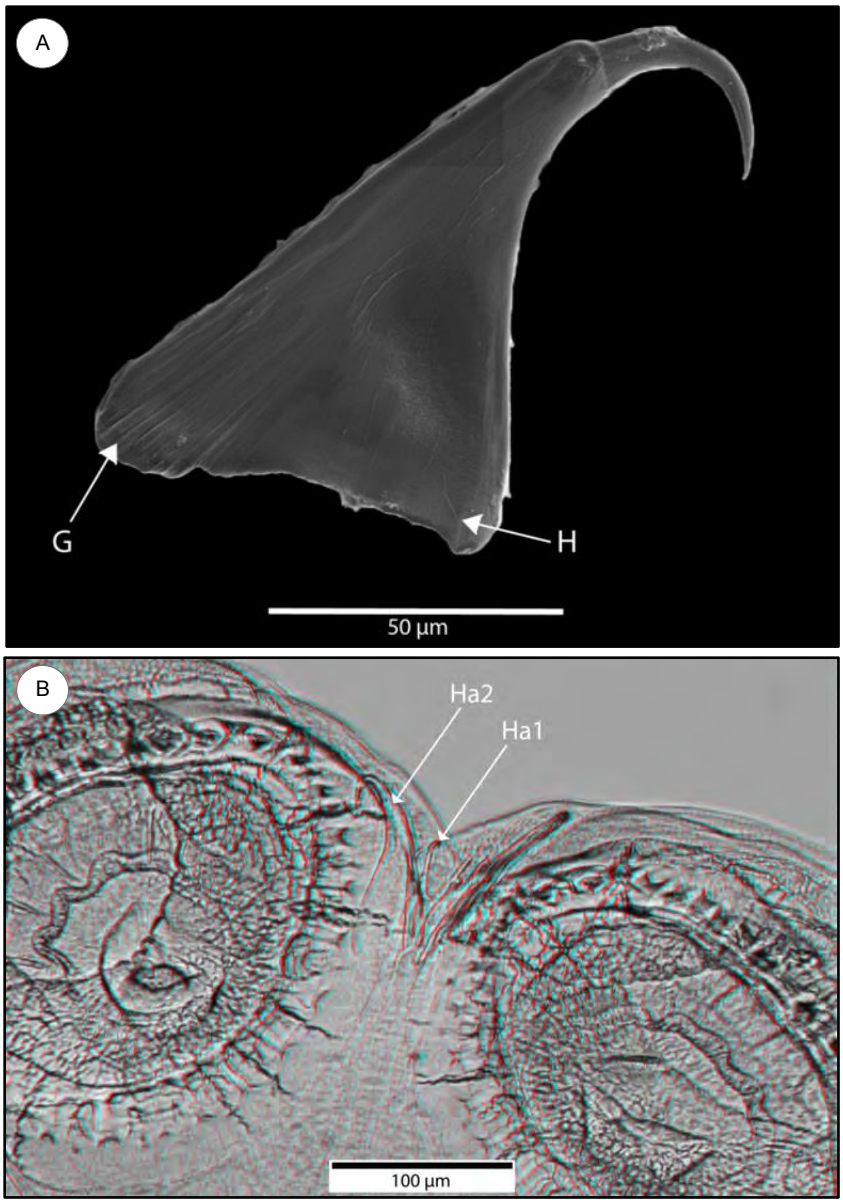


Figure 4.6.5: (A) Scanning electron micrograph of the highly developed hamuli (Ha2), with very subtle spilt between guard (G) and handle (H), revealed through partial digested with proteinase K enzyme digestion solution. **(B)** 3D light micrograph of phalloidin stained *Polystomoides* sp. specimen, showing a close-up the haptor with two pairs of hamuli (Ha1 and Ha2), between the posterior-most sucker pair.

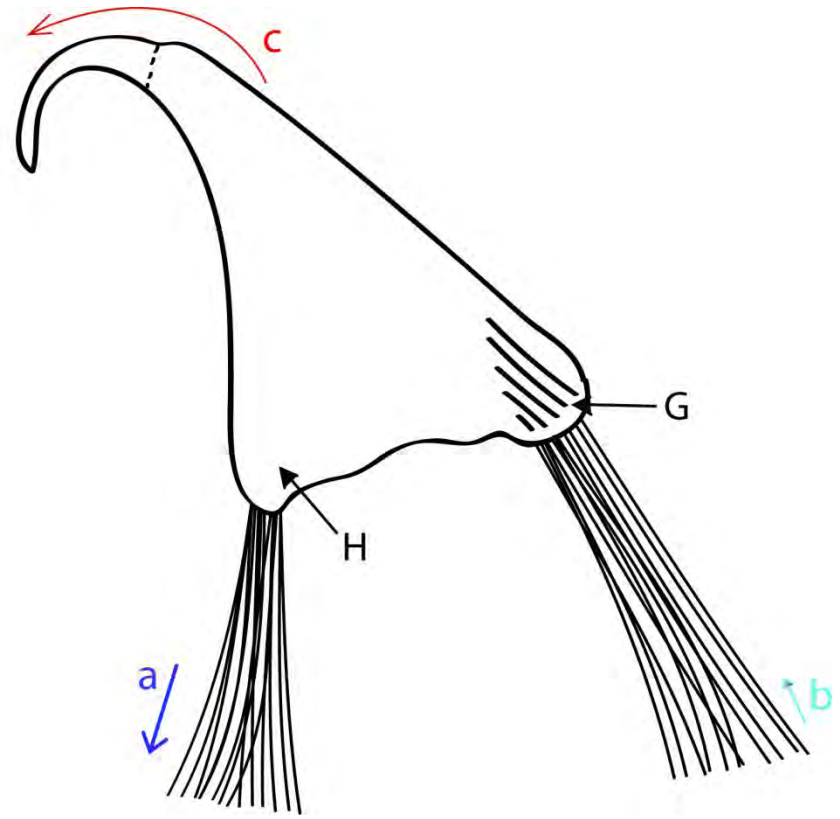


Figure 4.6.6: Drawing to illustrate the mechanism of *Polystomoides* hamuli. Muscle fibres attached to the guard (g) contracts while those attached to the handle (h) relax causing the hamulus to swing outward (c) and penetrate into host tissue.

The morphology of the suckers of *Polystomoides* are very similar as described for *Neopolystoma* (Figures 4.6.7 A–B, Figure 4.6.8 A–F). A funnel shaped structure in the base of the sucker is also present and even more pronounced as in *Polystomoides* (Figure 4.6.4)*;* likewise, with a ring of skeletal blocks in the mid-level of the sucker (Figure 4.6.7 B, Figure 4.6.10, Figure 4.6.11 A–B) with muscle bands running through and in between skeletal elements within the haptor (Figure 4.6.9).

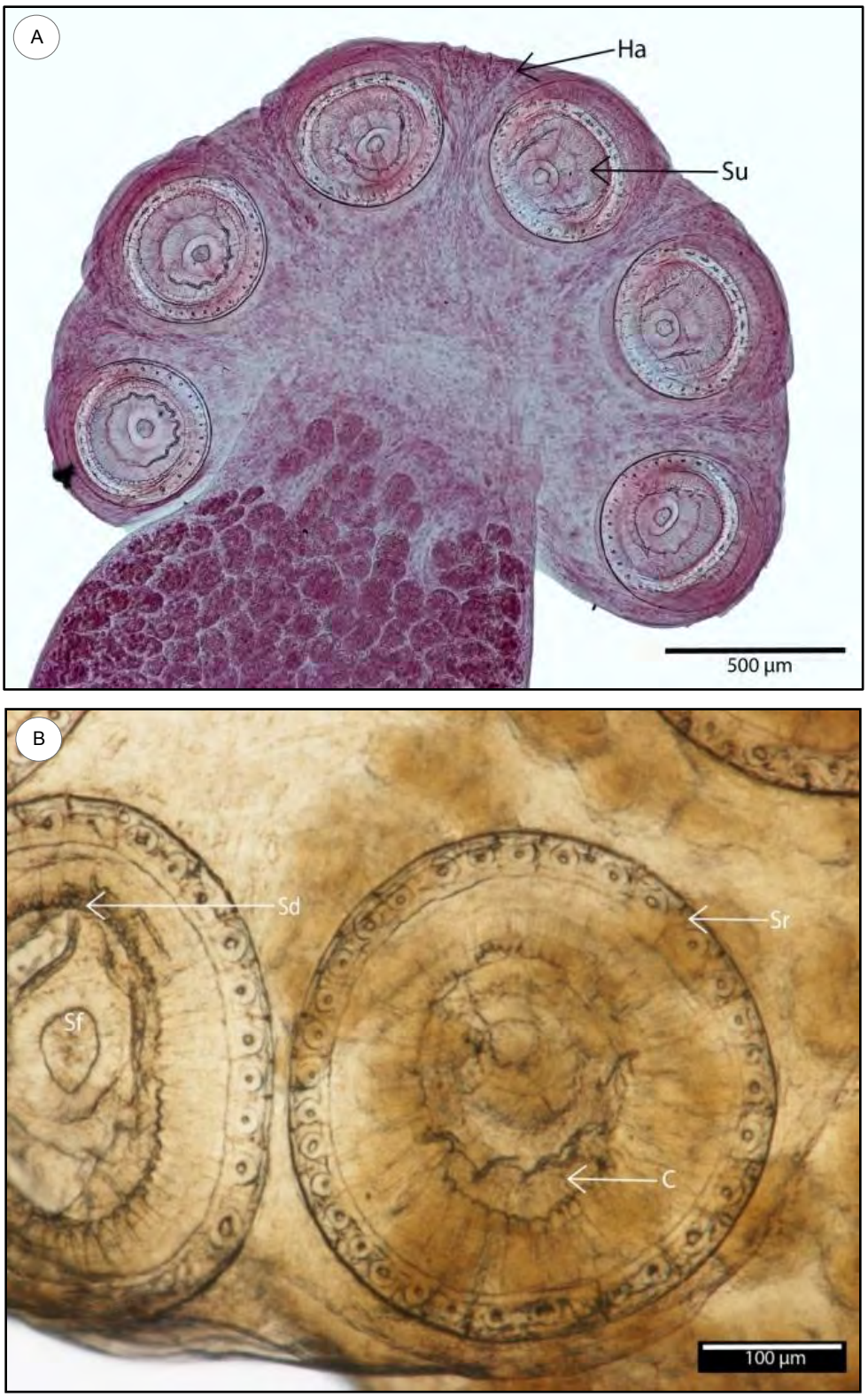


Figure 4.6.7: Light micrographs of *Polystomoides* sp. haptor **(A)**, with six suckers (Su) and two pairs of hamuli (Ha1 and Ha2). **(B)** A close-up of one and a half of *Polystomoides* sp. suckers, showing the skeletal funnel (Sf), costae (C), and skeletal digiti (Sd).

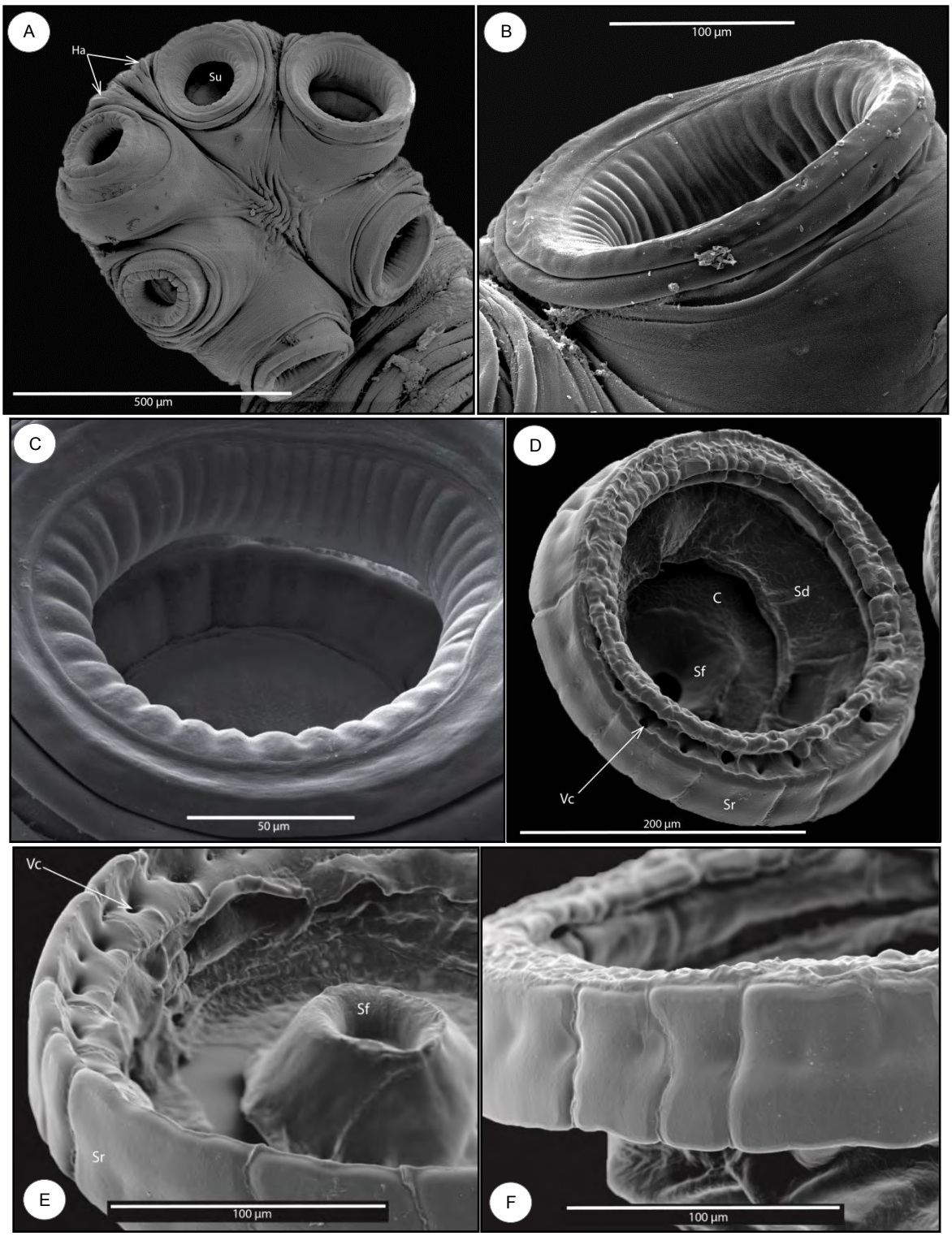


Figure 4.6.8: Scanning electron micrographs of *Polystomoides* haptor. **(A)** Haptor in full, showing the flexible nature of the *Polystomoides*' haptor. **(B - C)** single sucker showing the musculature nature of the sucker. **(D – F)** *Polystomoides* haptor digested with proteinase K enzyme digestion solution. Showing skeletal funnel (Sf), costae (C), skeletal ring (Sr) with perforated skeletal segments and vertical canals (Vc).

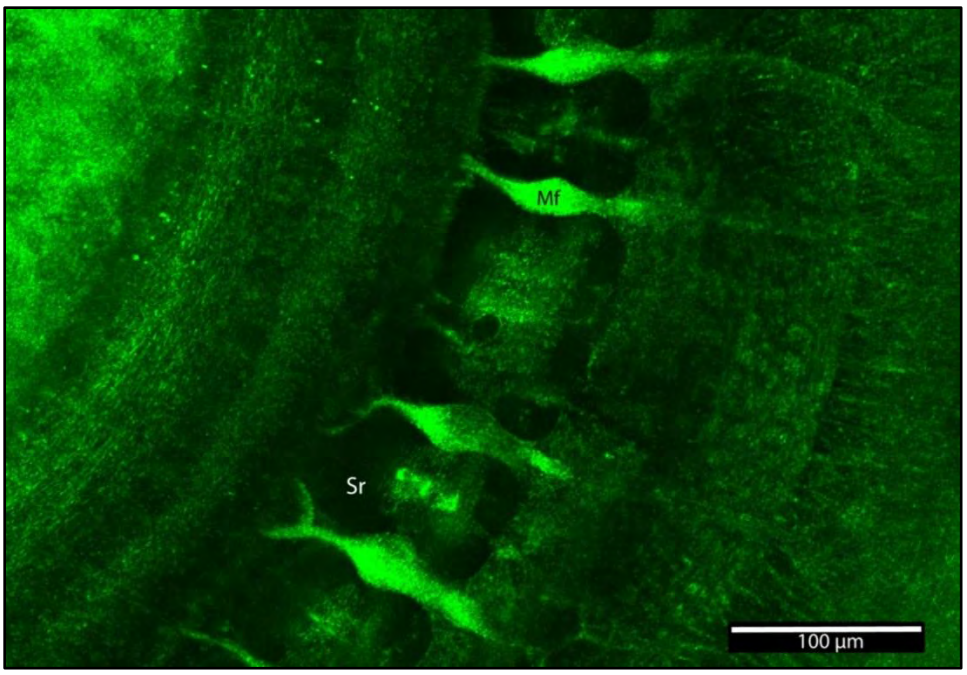


Figure 4.6.9: A confocal scanning laser micrograph of a close-up of a *Polystomoides* sucker showing the internal muscle fibres (Mf) running through the vertical canals in the skeletal ring (Sr) of the sucker. Labelling for f-actin with alexa fluor 488.

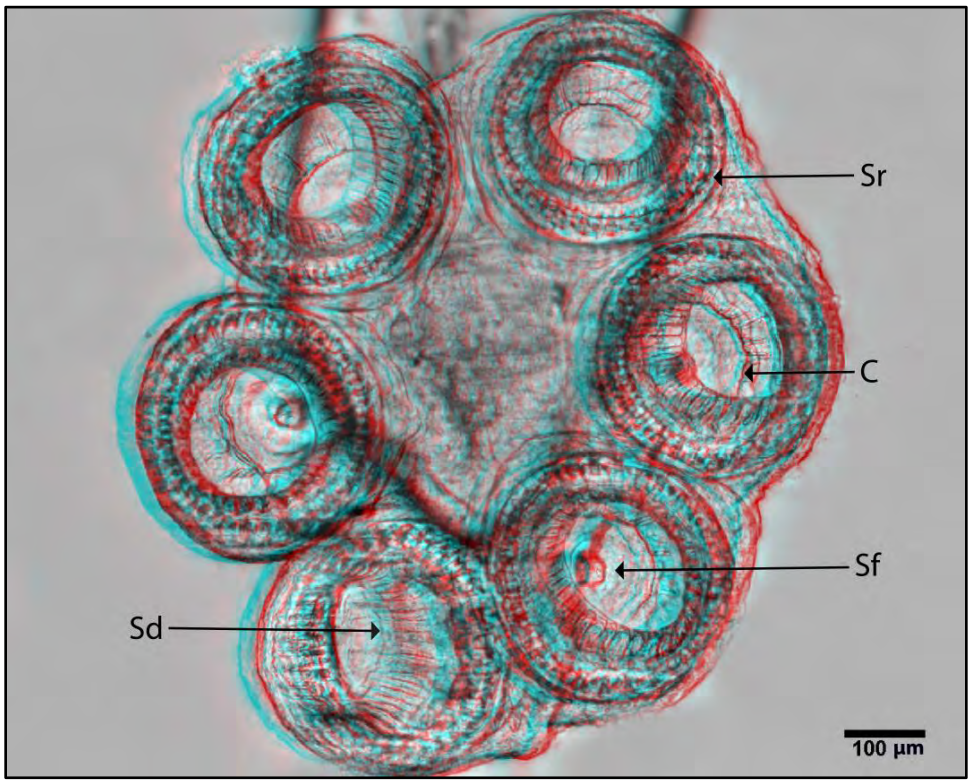


Figure 4.6.10: 3D light micrograph of phalloidin stained *Polystomoides* sp. specimen, showing haptor showing six sucker cups (Su) and hamuli pair (Ha). A skeletal funnel (Sf) is situated at the base of each sucker, which is connected to the skeletal ring (Sr) by a series of costae (C). Skeletal digiti (Sd) extent from skeletal ring to the end of the sucker lining. **Note that this is a 3D image. Please use the anaglyph glasses provided on the inside back cover.**

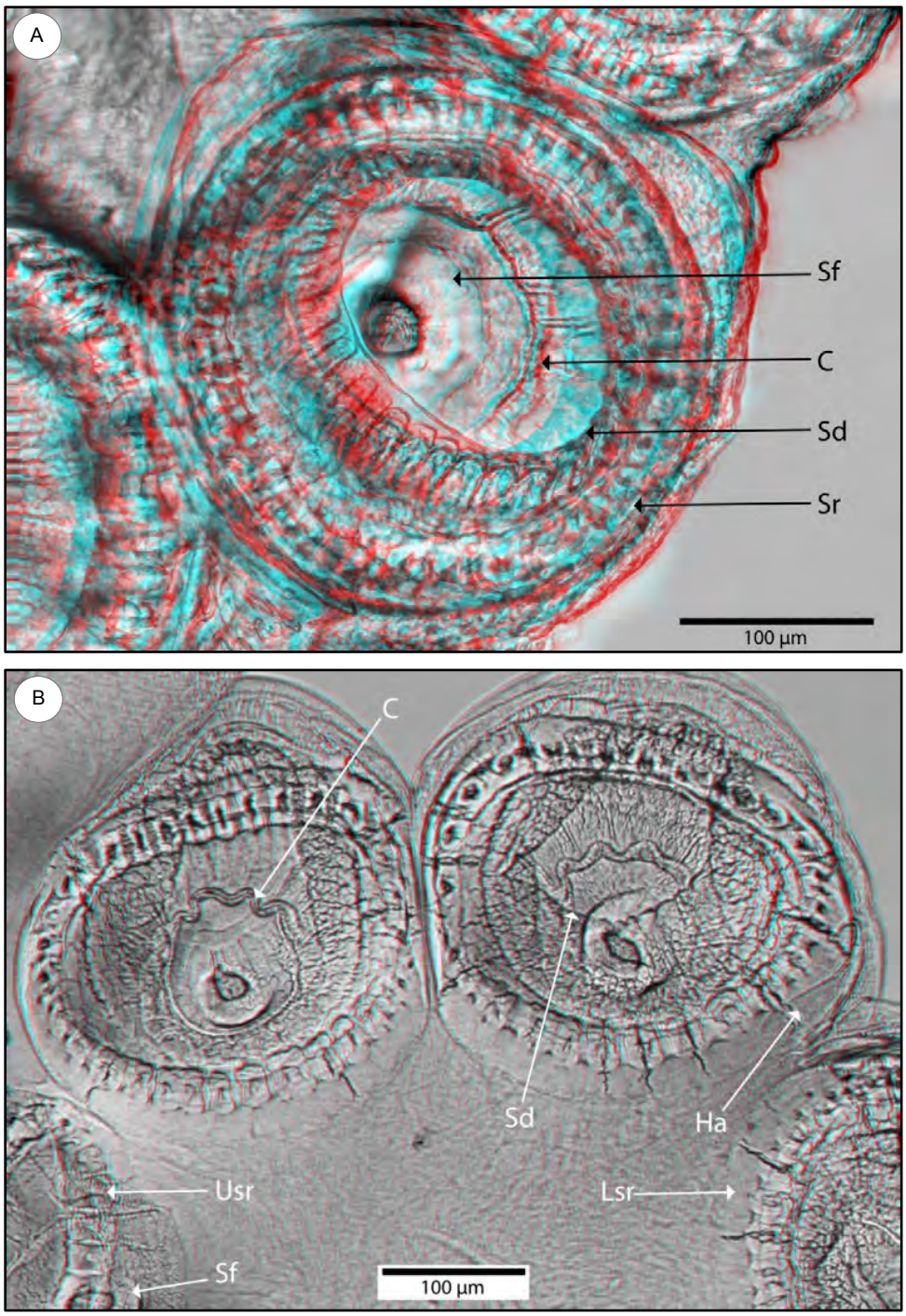


Figure 4.6.11: (A) 3D Light micrograph of a lactophenol cleared *Polystomoides* sp. haptor. At the base of the sucker is a skeletal funnel (Sf) which is connected to the skeletal ring (Sr) by a series of costae (C). Skeletal digiti (Sd) extent from skeletal ring to the end of the sucker lining. **(B)** 3D light micrograph of a phalloidin stained *Polystomoides* sp., showing a close up of *the* haptor. Two hamuli (Ha) pairs at the posterior of the sucker. A skeletal funnel (Sf) is at the base of each sucker (Su), which is connected to the skeletal ring (Sr) by a series of costae (C). Skeletal ring is divided in to an upper (Usr) and lower (Lsr) skeletal ring. Skeletal digiti (Sd) extent from skeletal ring to the end of the sucker lining. **Note that these are 3D images. Please use the anaglyph glasses provided on the inside back cover.**

It is hypothesised that the mechanism for the sucker of this species is similar as that described for *Neopolystoma.* Figure 4.6.12 A is an example of a relaxed sucker, and Figure 4.6.12 B is an example of a contracted sucker.

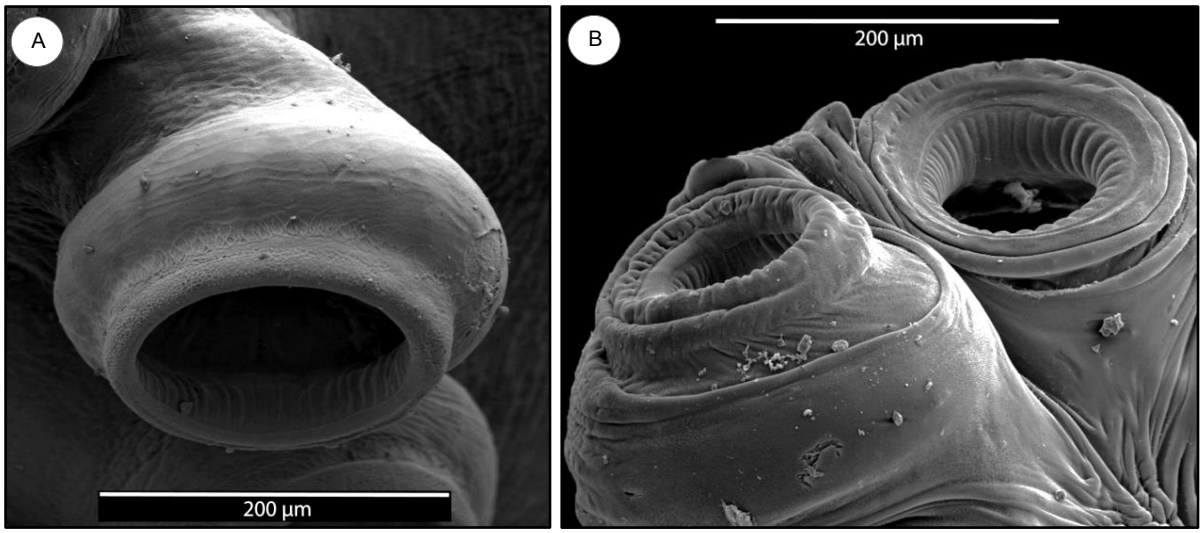


Figure 4.6.12: Scanning electron micrographs of *Polystomoides* sp. Suckers; **(A)** relaxed sucker, **(B)** contracted sucker.

Figure 4.6.13 shows a parasite attached to buccal mucosa, after dissection and the effect it has on host tissue (Figure 4.6.14 A–B). It was noted that parasites are very active and move about readily in a leechlike fashion. After every move, parasites attach firmly to host tissue, spending minimal time unattached.

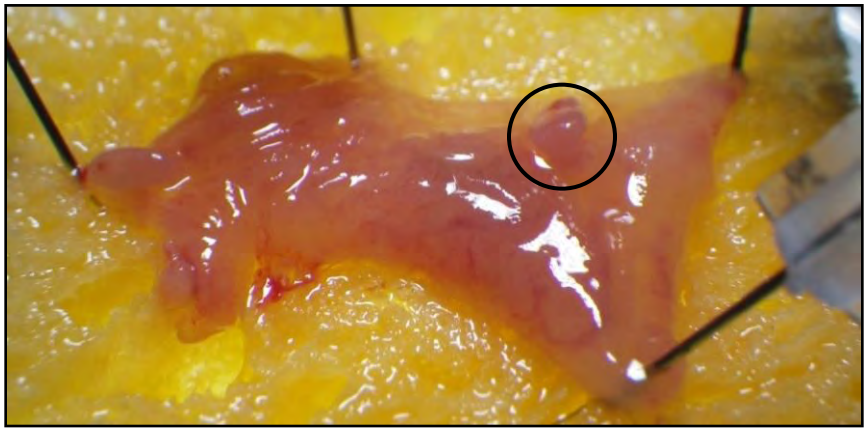


Figure 4.6.13: *Polystomoides* on *Trachemys scripta elegans* buccal mucosa, tissue being stretched and pinned to a small piece of sponge and treated with Todd's fixative.

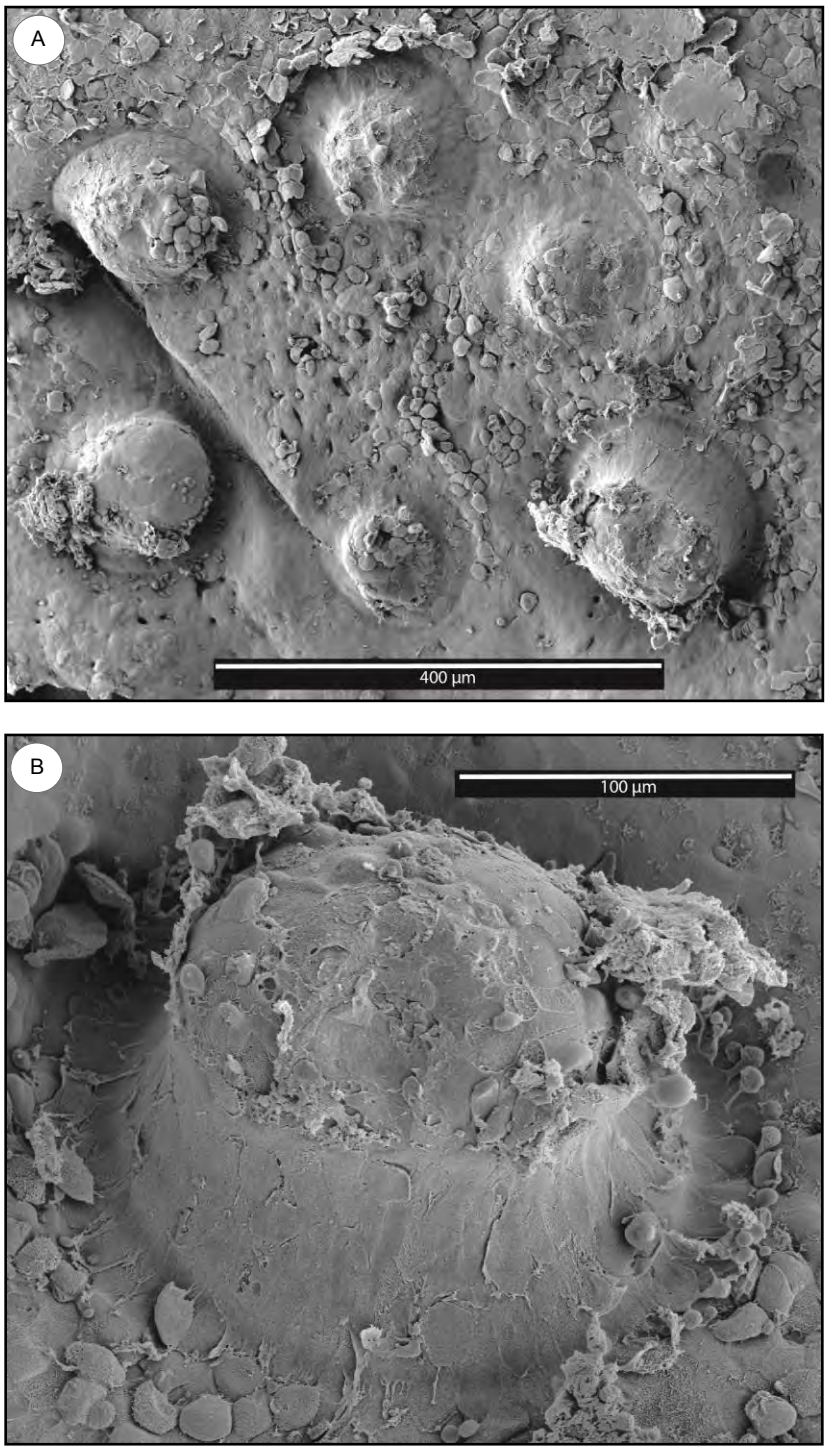


Figure 4.6.14: Scanning electron micrographs of damaged *Trachemys scripta* oral cavity tissue, **(B)** close-up of buds on host tissue, due to firm attachment by *Polystomoides*.

Chapter 4.7

Oculotrema

4.7.1 Mature parasites

The anterior two thirds of *Oculotrema* specimens body appears dark red (Figure 4.7.1), due to the presence of hemoglobin (Thurston, 1970). *Oculotrema* feeds on epithelial cells and mucous and is probably the only known mucous feeding parasite with this feature. *Oculotrema* is extremely flexible; this is attributable to the presence of well-developed circular muscles and the lack of organs within the haptoral and pre-haptoral region of the parasite. The highly flexible and elastic body enables it to stretch and feed across a large area of the eye surface and it has been noted that *Oculotrema* does not detach the opisthaptor while feeding. *Oculotrema* specimens generally occur in clusters on the eye surface (Figure 4.7.1 A). The well-adapted haptor is responsible for attachment; a very firm grip is confirmed by the difficulty removing parasites from host tissue and the observation of damaged eye tissue once the parasite is successfully removed (Figure 4.7.7 A – C, Figure 4.7.8).

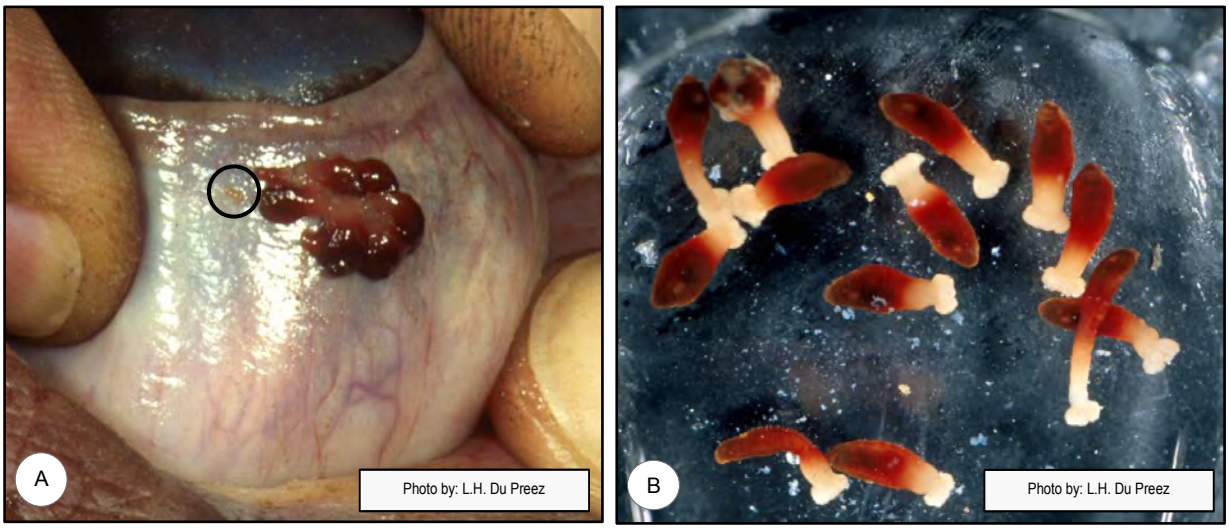


Figure 4.7.1: **(A)** Cluster of live *Oculotrema hippopotami* specimens on the eye of a hippopotamus, immature worm indicated (O). **(B)** Live *Oculotrema hippopotami specimens* in Petri dish.

The body of *Oculotrema* is dorsoventrally flattened and pyriform in shape; the anterior is rounded, narrowing posteriorly (Figure 4.7.2). The mouth is situated at the anterior and opisthaptor at the posterior of the parasite. The false oral sucker and

pharynx are well-developed. The intestinal caeca is without diverticula or anastomoses, only a subtle winding. An ovary with numerous eggs and vagina are present. Testis transversely elongated, situated medially, posterior to the ovary and occupying an enlarged part of anterior body. Vitellaria and intestinal caeca do not extend into the posterior half of the body.

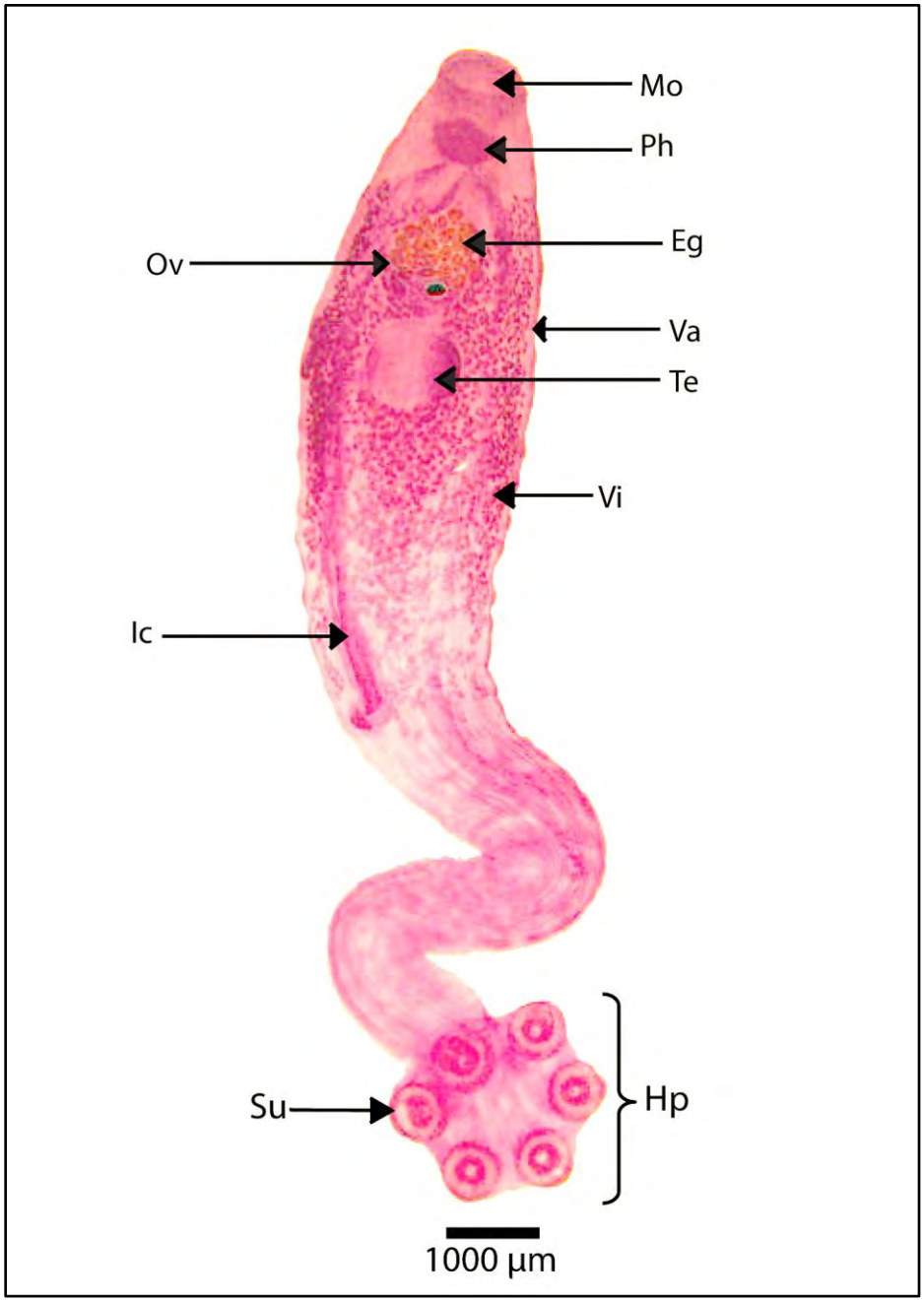


Figure 4.7.2: Light micrograph of a whole mount of *Oculotrema hippopotami*. Annotations: Eg, egg; Gb, genital bulb; Hp, haptor; Ic, intestinal caecum; Mo, mouth; Ov, ovary; Ph, pharynx; Su, suckers; Te, testis; Va, vaginae; Vi, vitelline follicles.

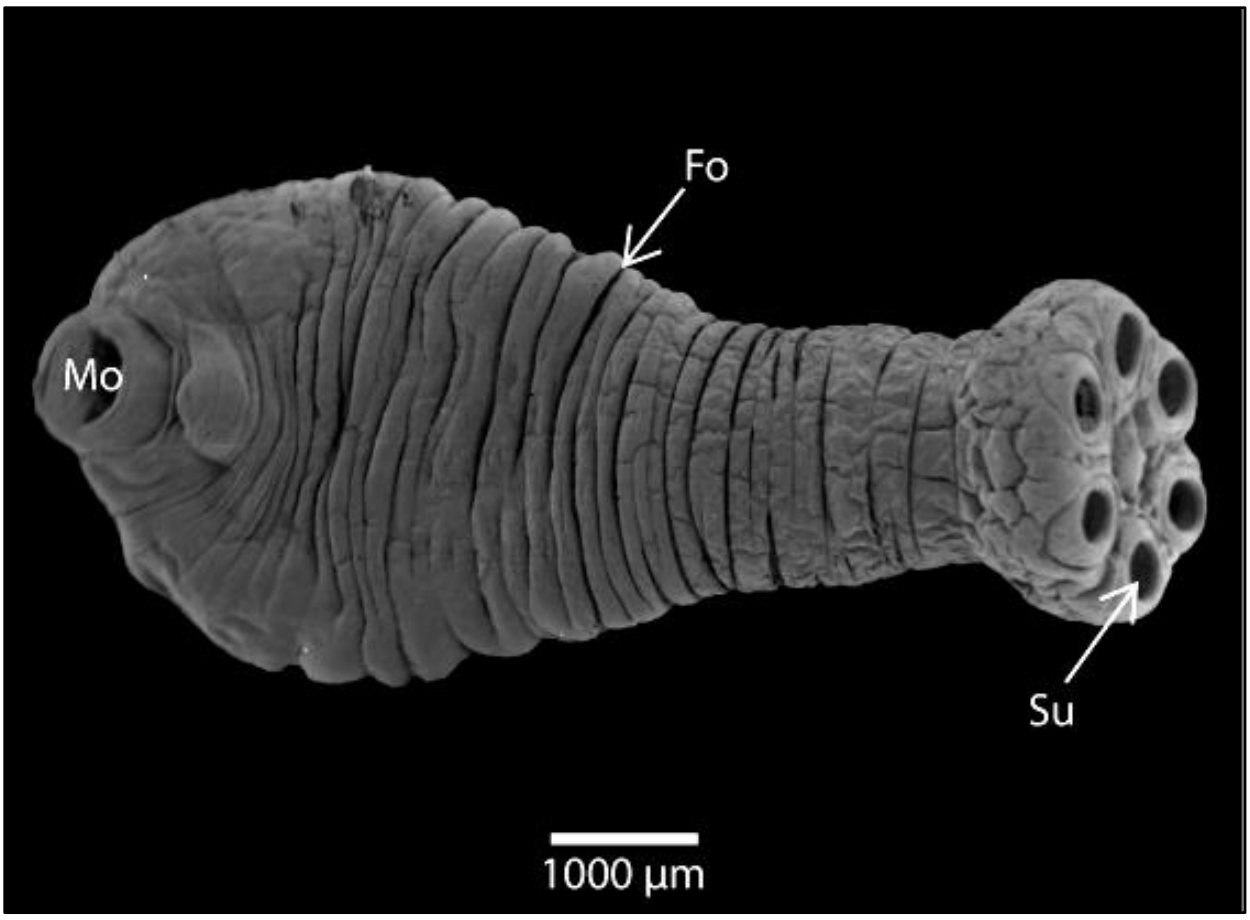


Figure 4.7.3: Scanning electron micrograph of a contracted whole mount of *Oculotrema*, showing the mouth opening (Mo) at the anterior of the parasite, and the haptor (Hp) armed with six suckers (Su) at the posterior. Folding (Fo) of body wall indicates contracted state.

The body of a parasite attached to host is contracted and folded, as in Figure 4.7.3. When live parasites are removed from host and subsequently stored, they are generally relaxed and extended.

4.7.2 Attachment structures

Hamuli are absent, marginal hooklets are not easily noticed and has a general position, similar to those in other polystomatids. Six haptoral suckers are arranged in a circle, opening outwards (Figure 4.7.4 B). Suckers are muscular cups, containing a continuous skeletal lining or ring, dividing muscular cup into halves (Figure 4.7.4 A–D).

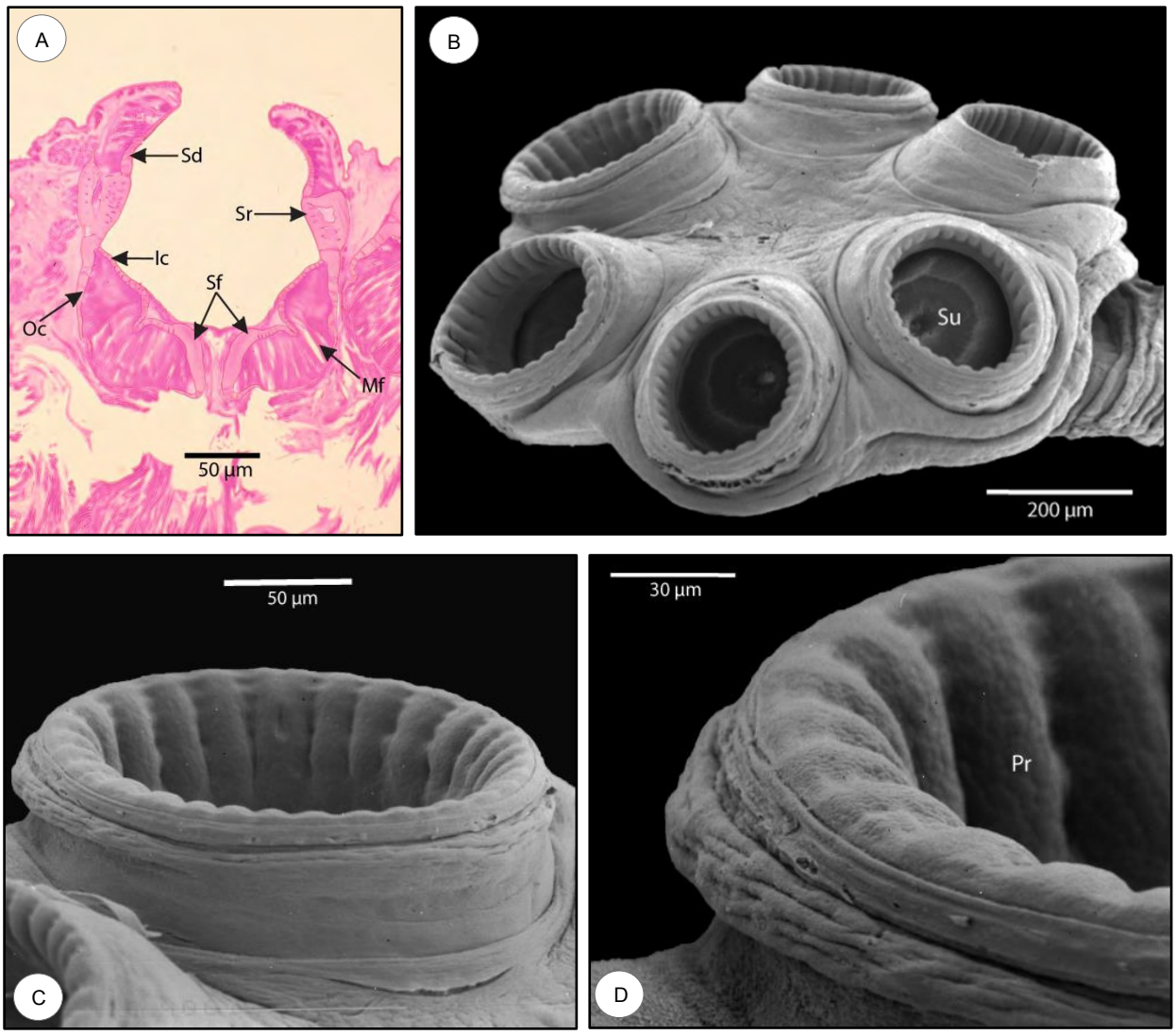


Figure 4.7.4: (A) Light micrograph of a histological section through the middle of a sucker cup of *Oculotrema hippopotami*, showing the skeletal funnel (Sf) at the base of the sucker, which is connected to the skeletal ring (Sr) by a series of inner (Ic) and oueter (Oc) costae. Muscle fibres (Mf) are situated between the inner and outer costae.**(B)** Scanning electron micrograph of the opisthaptor of *Oculotrema hippopotami,* armed with six suckers (Su). **(C)** Scanning electron micrograph of a single sucker. **(D)** Scanning electron micrograph of a close-up of a single sucker, showing the parallel rays (Pr) in the peripheral zone.

The suckers of *Oculotrema* are very similar as described for *Neopolystoma* and *Polystomoides,* comprising of three zones (Figure 4.7.5 A, Figure 4.7.6 A). The intermediate zone is more prominent in *Oculotrema* than in *Neopolystoma* and *Polystomoides* (Figure 4.7.5 B–D, Figure 4.7.6 B).

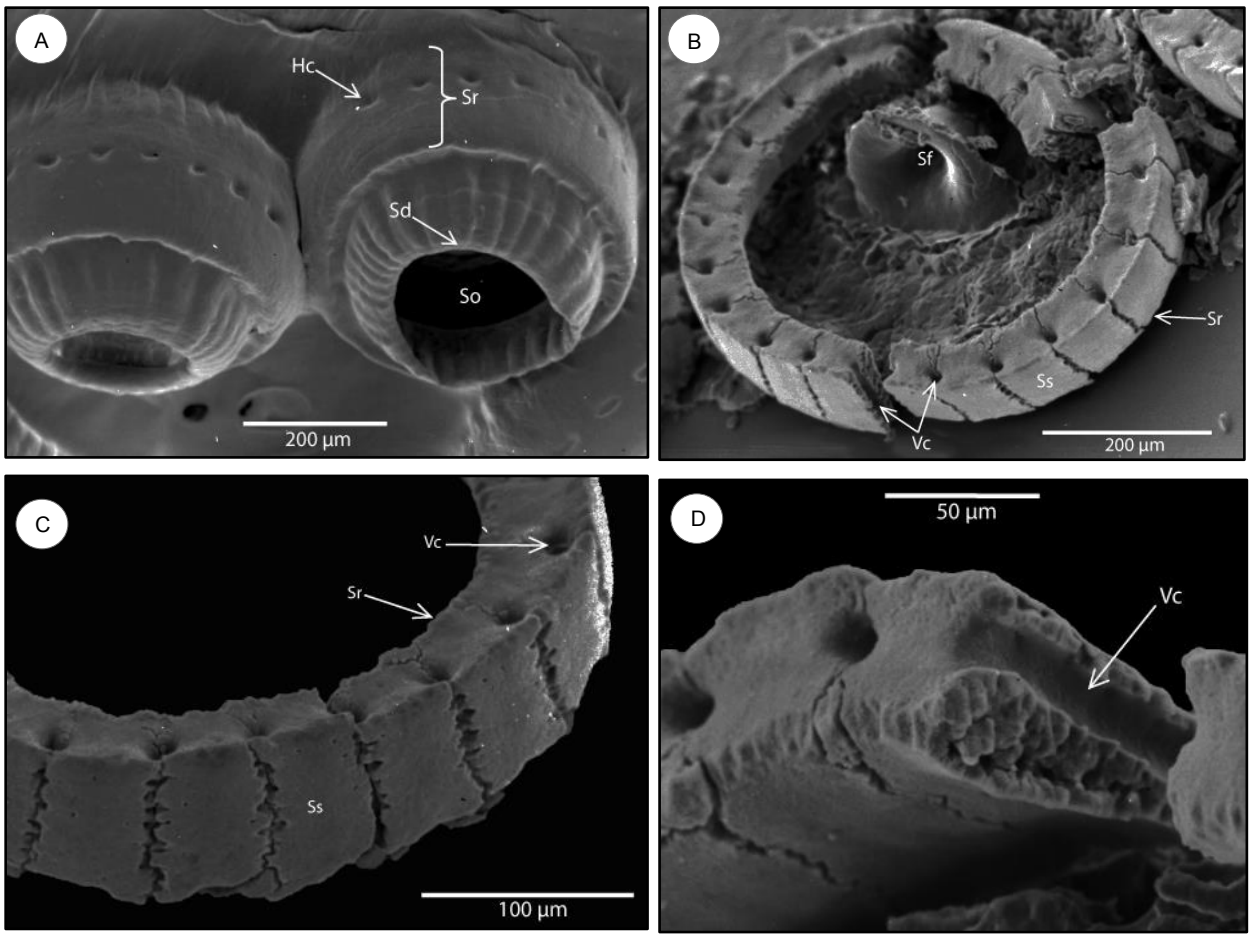


Figure 4.7.5: (A) Scanning electron micrograph showing partially enzyme digested suckers. Peripheral zone, skeletal digiti (Sd) and soft tissue surrounding it, forming the sucker opening (So) is still intact. Horizontal canals (Hc) are present within the skeletal ring (Sr). **(B)** Scanning electron micrograph of a partially digested sucker showing skeletal funnel (Sf) and skeletal ring (Sr); made up of skeletal segments (Ss) with interconnecting vertical canals (Vc). **(C – D)** Scanning electron micrographs showing two partially digested suckers, showing the chitinous skeletal ring (Sr) and the vertical canal running through it.

Suckers are highly manoeuvrable owing to a combination of muscular and collagenous fibres and to the fact that skeletal elements are mostly segmented. Muscle fibres connect digiti and costae to central skeletal ring. Figure 4.7.4 C – D shows digiti covered in muscle fibres appearing as parallel rays within the sucker. The central zone is made up of numerous skeletal segments connecting to inner and outer costi. Radial muscles fibres are found in the central zone between costae, as well as in peripheral zone, between digiti and at the tip of the sucker ring muscle. A ring muscle is also situated in the interior to the skeletal ring, in the central zone. It is hypothesised that the mechanism for the sucker of this species is similar as that described for *Neopolystoma* and *Polystomoides.*

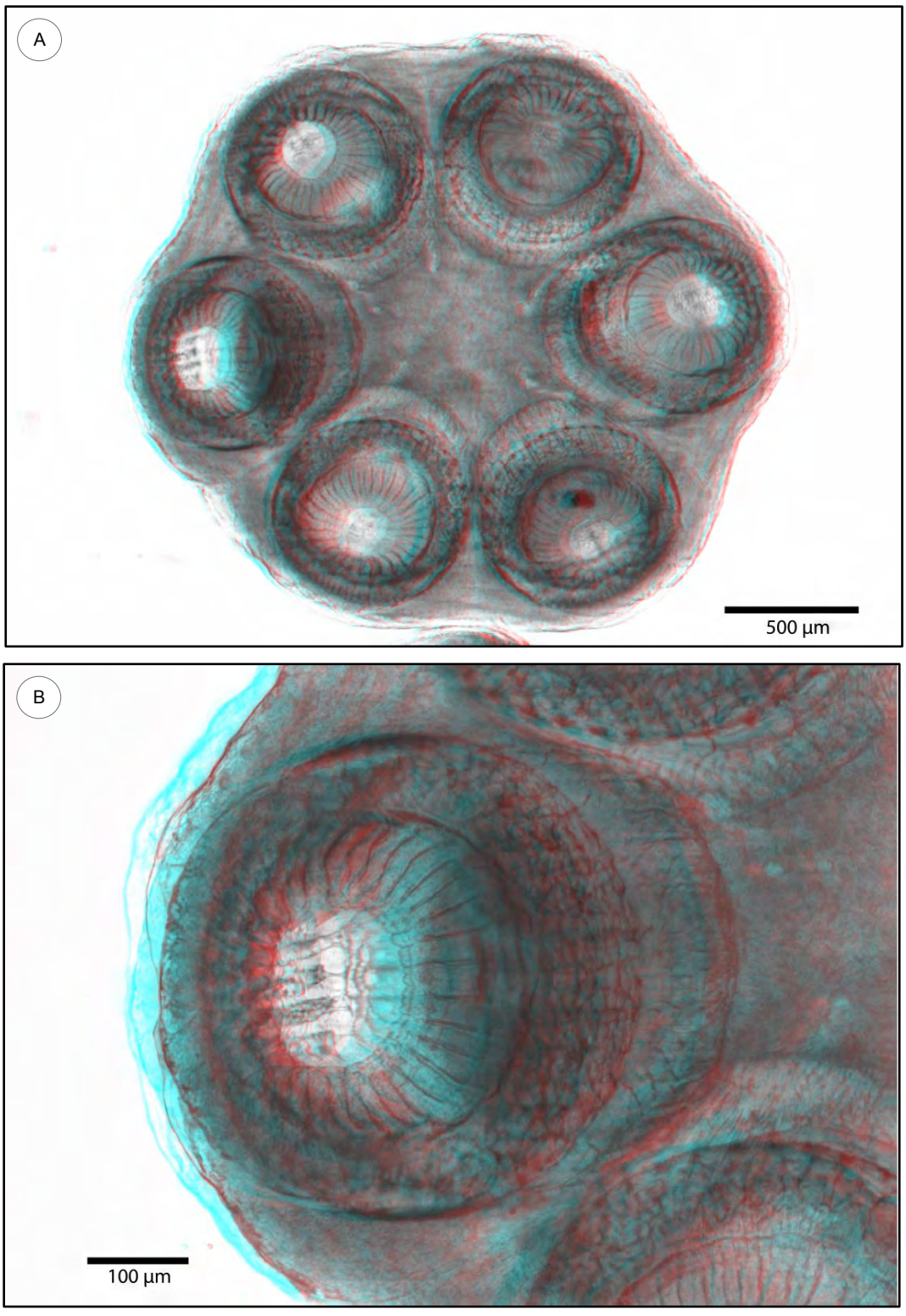


Figure 4.7.6: (A) 3D light micrograph of *Oculotrema* haptor, haptor showing six semi closed sucker cups. **(B)** 3D light micrograph of a close up of a single sucker. **Note that these are 3D images. Please use the anaglyph glasses provided on the inside back cover.**

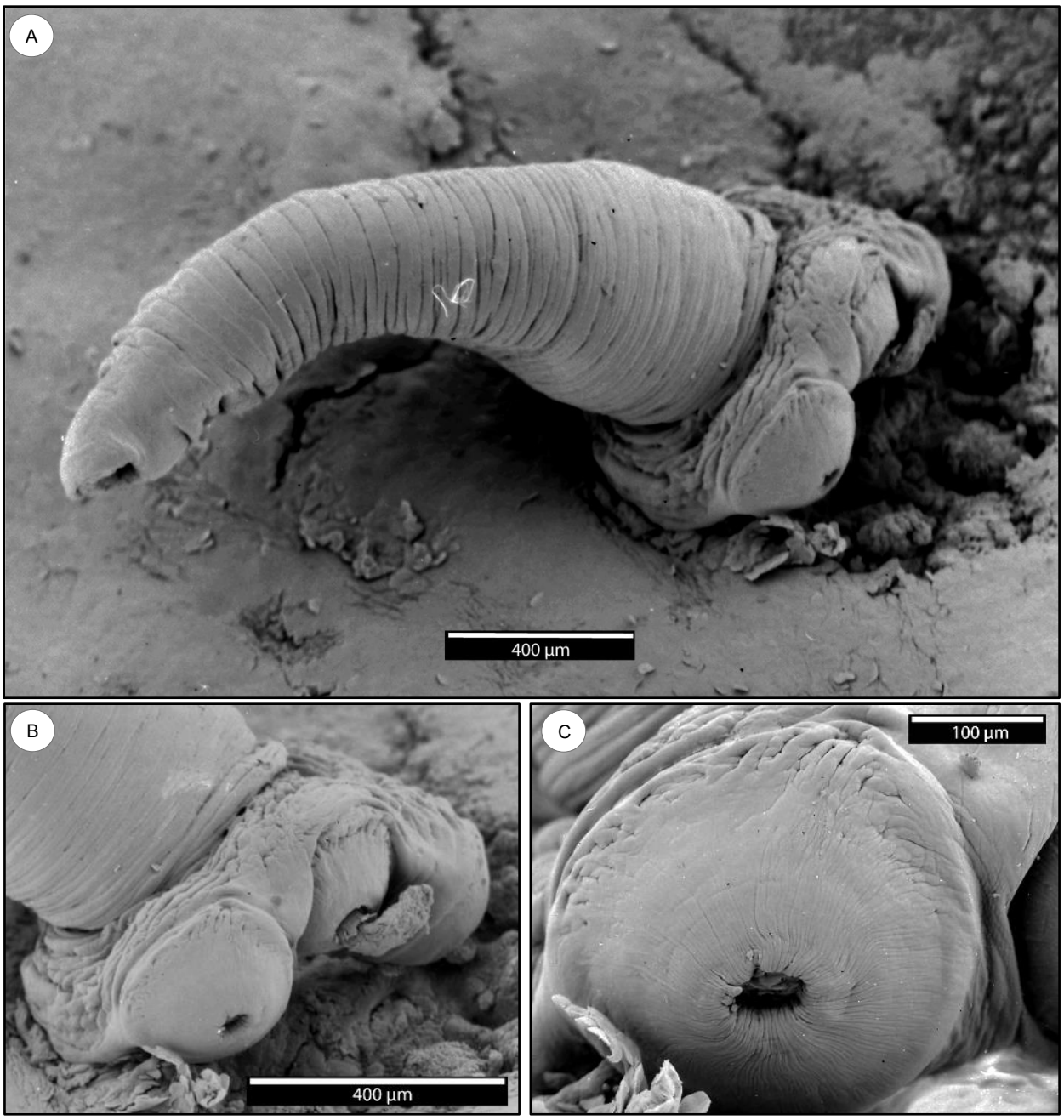


Figure 4.7.7: (A) Scanning electron micrograph showing of *Oculotrema hippopotami* attached to the eye of a hippopotamus. **(B –C)** Scanning electron micrographs showing close-ups of *Oculotrema hippopotami* attached to the eye of a hippopotamus.

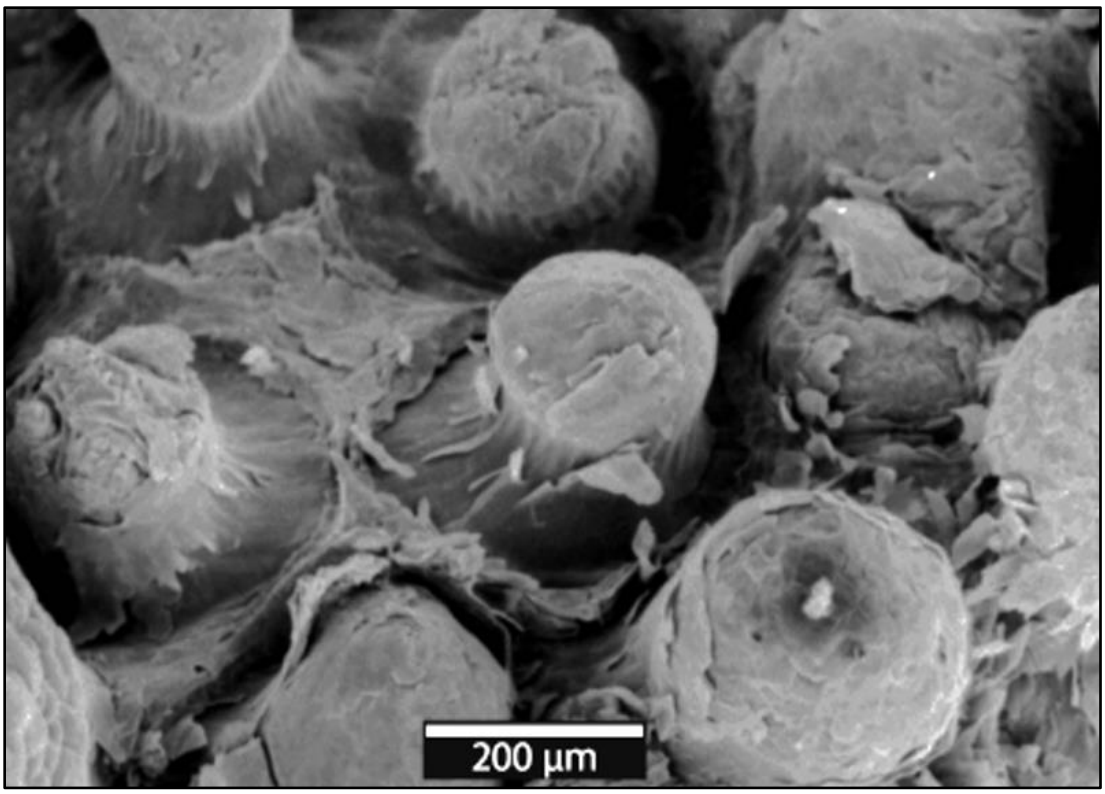


Figure 4.7.8: Scanning electron micrograph of buds made by the parasite's suckers on the host's eye surface.

Chapter 5

Discussion

CONTENTS

5.1 Polystomatid diversity and geographic distribution:

Among the monogeneans, the Polystomatidae is unique in more than one way. In terms of the hosts that the polystomatids parasitize, they comprise of the most diverse monogenean family (Verneau *et al.*, 2009) - emphasising how this group radiated over time. Tinsley (2003) suggested that ancient radiation occurred from a single clade of monogeneans that parasitise fish, the primary hosts of monogeneans.

The Australian lungfish *Neoceratodus forsteri* is the host for *Concinnocotyla*, the only polystomatid known from a fish, and the results from a molecular analysis by Verneau *et al.* (2002) suggest that *Concinnocotyla* was probably the first polystomatid to diverge within the Polystomatidae. *Concinnocotyla australensis* was also placed in a highly supported basal position in another molecular study by Badets and Verneau (2009). *Concinnocotyla* was thus selected as outgroup for the phylogenetic tree obtained in this study, based on morphological characters (Figure 4.1.1). The objective of the tree in the present study was to assist in the selection of species for further morphological study on attachment. Based on the selected morphological characters, monogeneans infecting anuran hosts grouped together to form a monophyletic group, while monogeneans infecting chelonian, caecilian and mammalian hosts form another monophyletic group and the sole fish infecting polystomatid at the basal position of the phylogenetic tree. The following list of three noteworthy groups is also identified in a molecular study that incorporated twelve polystome genera, namely *Diplorchis*, *Eupolystoma*, *Neodiplorchis*, *Neopolystoma*, *Parapolystoma*, *Polystomoides*, *Pseudodiplorchis*, *Polystoma, Concinnocotyla, Protopolystoma, Pseudopolystoma,* and *Sphyranura* (see Badets and Verneau, 2009). The groups include: (1) the clade of chelonian polystomes including *Neopolystoma* and *Polystomoides,* (2) the clade of amphibian polystomes, and the clade of neobatrachian polystomes including *Diplorchis*, *Eupolystoma*, *Parapolystoma* and *Polystoma.* However, host-specificity and identification of polystomes based solely on morphological characteristics is often complicated and in some cases impossible (see Meyer *et al*., 2014). Since amphibian

polystomes are primarily found in the urinary bladder of post-metamorphic frogs, while chelonian polystomes are described from three distinct habitats, namely the urinary bladder, pharyngeal cavity and conjunctival sacs and the possibility of host switching in natural environments cannot be eliminated (Meyer *et al*., 2014). Phylogenetic trees based on molecular tools, where proper dating is possible, are generally more accurate in terms of origin and diversification of species. Nevertheless, the phylogenetic tree obtained in the present study assisted in taxa selection for the present morphological study.

Today polystomes are known from all hospitable continents and some islands. The ability to diversify and establish on other host organisms is probably one of the most important advances of polystomes that contributed to their success and diversity on a global scale. Other hosts include: the Australian lungfish, anurans, caecilians, salamanders, terrapins and a mammal, the hippopotamus.

5.2 Adaptation to habitats:

Alongside their hosts, monogeneans infecting internal sites had to go through a series of adaption events. These include the loss of habitat (gills) due to anuran hosts undergoing metamorphosis, drying of skin as frogs leave the aquatic environment, and interrupted transmission opportunities as frogs would only intermittently frequent water bodies. Van der Linde *et al.* (1984) stated that amphibians have a high tolerance for negative impact and the effect parasites tend to have is seldom noticed. Parasites that have successfully radiated to internal sites of their host are still faced with a number of difficulties. The bladder is not always ideal for parasite infection and presents some unique problems, especially regarding parasite attachment, due to fluctuating levels of salinity and the bladder's flexible nature. When entering aquatic environments, frogs hydrate and as the urinary bladder fills, and tend to urinate more frequently with a reduction in salinity of the urine.

When hibernating, the salinity inside the bladder gradually increases, putting parasites under increased osmotic stress. The thin and contractile membrane of the bladder undergoes sporadic changes in the surface area and thickness causing the haptor of the parasite to readily detach (Tinsley, 1971). However, difficulties infecting internal sites, such as the urinary bladder, are not completely overcome and are indicated by the high mortality rate of established parasites (Tinsley, 1971). The elimination of parasites is most likely due to accidental detachment of parasites within the highly contractile bladder after which they are expelled into the external environment together with the host's urine. The frequency of urination is another factor that can influence the rate of elimination. Parasites infecting X*enopus*, a fully aquatic host, would experience a greater risk of elimination due to an increase in urination frequency in comparison to other, more terrestrial, species. Toads, however, have large urinary bladders in order to store large volumes of water when they hibernate. These urinary bladders are also highly vascularised; allowing sufficient feeding opportunity for a multitude of parasites. Delport (2007) reported that they found up to 132 specimens of *Eupolystoma* in a single *Amtetophrynus garmani* specimen while Tinsley (1973) reported more than 2000 specimens of *Eupolystoma anterorchis* from the urinary bladder of a single *Amietophrynus pantherinus* specimen. This high numbers of polystomes in a single host is not common for chelonian (Rohde, 1965) and other anuran infecting parasites (Gallien, 1935). Attachment is of the utmost importance for polystomes to survive in their respective sites in or on their hosts.

5.3 Attachment of larval polystomes:

Polystomatidae larvae differ morphologically considerably from its adult forms. Locomotion in the free swimming phase of larval development is attained through epidermal cilia while muscle movement and the attachment and detachment of haptoral suckers and the false oral sucker are responsible for movement in the latter phase.

Gliding over the surface and leech-like movement, known as looping, have also been documented after oncomiracidia have made contact with a potential host (Du Preez and Kok, 1997; Du Preez *et al*., 1997). Upon attachment to the host, the developing parasite makes use of the haptor and haptoral sclerits to attach (Llewellyn, 1963). Oncomiracidia of *Protopolystoma* develop within the kidney of the host, a condition only elsewhere recorded in *Eupolystoma* (Tinsley, 1983). In *Pseudodiplorchis* the oncomiracidium enters through the nostrils, migrates to the lungs and from here it goes through the elementary tract to the cloaca and then to the urinary bladder.

The haptor of oncomiracidia differs between various monogenean species in terms of form and type of attachment structures. In some species the haptor faces ventrally, while others face laterally. Marginal hooklets in some species are assisted with an accessory structure known as the domus. In some polystomes, such as *Diplorchis ranae,* marginal hooklets become differentiated from others by becoming larger or take on different shapes (Llewellyn, 1963). In *Polystoma* and *Metapolystoma* it has been documented that marginal hooklet 1 is noticeably bigger, followed by hooklet 8, with hooklets 2-7 smaller and of the same size (Du Preez and Kok, 1992; 1993; 1995; Du Preez *et al.* 2002; 2007). For other polystomes it has been reported that all marginal hooklets are of the same size. This is the situation for *Eupolystoma* (see Du Preez *et al*., 2003), *Madapolystoma* (see Du Preez and Kok, 1992), *Nanopolystoma* (see Du Preez *et al.*, 2008), *Neopolystoma* (see Du Preez and Lim, 2000) and *Oculotrema* (see Du Preez and Moeng, 2004). As can be seen from the results, marginal hooklets lose their function as attachment organs at some stage in the development and are replaced by a different method of attachment, such as suckers and hamuli. Marginal hooklets do not disappear, but are retained. Marginal hooklets 1 and 2 are situated between posterior-most pair of suckers, hooklets 3–5 at the base of the three suckers, and marginal hooklets 6–8 are retained posteriorly between third the anterior-most pair of suckers.

5.4 Attachment of adult polystomes:

In the present study we observed that *Polystoma* and *Eupolystoma* have muscular but soft suckers without any skeletal elements. Histological examination of thin sections through suckers revealed circular and longitudinal muscle fibres. Muscles, responsible for creating the suction, are attached to the back of the sucker cups. While the haptors of both *Polystoma* and *Eupolystoma* are rigid structures, with the suckers directed ventrally, the haptor of *Protopolystoma* is very flexible with suckers directed ventro-laterally. For the specimens of *Polystomoides, Neopolystoma* and *Oculotrema* studied elaborate skeletal elements were observed. These elements were divided in three zones namely the central, intermediate and peripheral zones. The central zone contains the rigid skeletal funnel with costae linking the funnel to elements in the intermediate zone. The primary function of the skeletal funnel is that it provides a firm attachment surface for muscles, which would pull back the funnel to create suction. The intermediate zone is made up of the skeletal ring consisting of brick like structures. Muscle fibres running through canals provide a clamping action which will close the ring around the bud of host tissue sucked into the sucker cup. The outer peripheral zone contains skeletal digital fingers which were observed to be small in both *Neopolystoma* and *Polystomoides* while they were long and very prominent on *Oculotrema.* The skeletal funnel in the central zone is a single rigid structure, while all the skeletal elements in the intermediate and peripheral zones (costae, ring and digitae) are segmented, contributing to the flexible nature of the sucker which is important for its functional clamping mechanism.

Since it has been noticed that parasites found in the urinary bladders of frogs altogether lack skeletal elements within the haptor and relies mostly on muscular fibres within the sucker cups and, in some cases, hamuli to attach to the highly flexible bladder wall. It can be assumed that the means of attachment for anuran infecting polystomes have been successful thus far, even in genera such as *Eupolystoma* that solely rely on muscular cups for attachment. These findings can be interpreted as an

adaptation to the nature of its environment. *Xenopus* has quite a small urinary bladder and based on specimens dissected it was observed that the amount of fluid in the bladder can vary dramatically. It can be hypothesised that the flexibility of the haptor and the orientation of the suckers play an important role in attachment.

On the contrary, parasites found on the eye and in the mouth of hosts have skeletal structures in the haptor. An exception would be polystomes in the urinary bladder of terrapins. However, Williams (1961) mentions the idea that polystomes with an elaborate skeletal structure may reflect a common ancestor, distinct from anuran polystomes. The occurrence of these skeletal elements inside the suckers can probably be associated with the fact that parasites that are found within the mouth, and especially the eyes, of hosts are exposed to greater physical threats of being dislodged. Robust attachment structures are required, since there is a high risk that parasites may be dislodged when hosts open and close their eyes or when a terrapin swallows its food. Nevertheless, urinary bladders contain their own set of challenges, as mentioned before. Chisholm and Whittington (1998) noted that parasites that live in habitats exposed to strong water currents, such as the gills and dorsal skin surface, generally have more complex haptors.

Of the 24 polystome genera 13 have a single pair of large hooks or hamuli, while two have two pairs of hamuli and nine have none. These hooks have two roots referred to as the handle and the guard. The mechanism for these hooks are quite simple as prominent bundles of muscle fibres attached to the guard would, when they contract, swing the hamuli outwards and slam the sharp curved point into the host tissue where they contribute to a firm grip on the host.

Uniform morphology is found in all the members of the subfamily Polystomoidinae Yamaguti, 1968 (Pichelin, 1995), along with the sole polystomatid that infects the mammal, *Oculotrema hippopotami*. Pichelin (1995) stated that no evidence has been found that the circles in the central skeletal ring open to the outside of the cup, however, with the use of enzyme digestive techniques, openings are evident.

Projections from *Oculotrema'* skeletal lining seem to be more elaborate than those of parasites infecting chelonian hosts (Pichelin, 1995). Perforations within the skeletal ring allow spaces for muscle fibres to run though and create a functional unit. The main differentiating factor is the number of hamuli they possess; *Polystomoides* has two pairs of hamuli, *Polystomoidella* one pair, and *Neopolystoma* and *Oculotrema* none. *Nanopolystoma*, a parasite found within the bladder of caecilians, also consist of a "primitive skeletal arrangement" as reported by Du Preez *et al*. (2008) and is basal to all the genera with skeletal elements, see tree in chapter 4.1, Figure 4.1.1.

Based on an extensive study of various polystomes Pichelin (1995) proposed a Polystomatid sucker classification and divided the known genera, at that stage, into groups according to the structure of the haptor, consequently eliminating the need to use the host as primary separating factor. She distinguished three categories, namely Haptoral Sucker Type 1, 2 and 3. **Haptoral Sucker Type 1** comprises of all the genera with suckers made up of simple, muscular cups without accessory sclerits, genera include: *Diplorchis, Eupolystoma, Mesoploystoma, Metapolystoma, Neodiplorchis, Parapolystoma, Parapseudopolystoma, Polystoma, Protopolystoma, Pseudodiplorchis, Pseudopolystoma* and *Riojatrema*. *Kankana*, *Madapolystoma*, *Neoriojatrema, Sundapolystoma* and *Wetapolystoma* can be added to the list. **Haptoral Sucker Type 2** comprises of all the genera with suckers made up of muscular cups with a continuous lining, causing division of the musculature into halves, genera include: *Neopolystoma, Polystomoides, Polystomoidella* and *Oculotrema.* To this list we can add *Nanopolystoma* from caecilian hosts. **Haptoral Sucker Type 3** comprise of only a single genus, *Coninnocotyla*, with suckers that possess an elaborate skeleton of sclerites and reduced musculature.

Chisholm and Whittington (1998) stated that secure attachment to specific sites within hosts is crucial for the survival of many parasites. Although members of the Polystomatidae are essentially characterised by a well-developed haptor with three pairs of suckers, *Sphyranura* is the only genus in the family with merely a single pair of suckers (Sinnappah *et al*., 2001). Whittington *et al*. (2000) stated that it is possible that

anterior attachment by polyopisthocotyleans make more use of suction than adhesion. Adhesive organs such as suckers, in most cases originate from body wall muscles and are often complex in arrangement (Mair *et al*., 1998). Muscle fibres also attach to the root of anchors and sclerits; assisting in the formation of suction at the haptor and facilitate the insertion of anchors or assist in the grasping of mechanisms. Muscle fibres, therefore, assist in the efficient and effective functioning of monogenean attachment organs such as anchors, suckers and clamps.

Several factors need to be taken into account when studying the morphology of monogenean haptors, especially environmental factors relating to host ecology. Such factors play an important role in the adaptation of monogeneans and have possibly led to the change in microhabitats, which in turn explain the variation of haptoral components between parasites. Not all haptoral structures necessarily function in attachment throughout the entire life of the parasite.

FUTURE STUDIES:

- Microscopy underwent a revolutionary development in recent years and various promising techniques are now available to provide further insight into morphology of polystomes.
- Dual stain confocal microscopy techniques and to stain muscle fibres and skeletal elements in haptor and subsequent examination using confocal laser microscopy appears to be very promising. Alexa fluor phalloidin for f-actin and 40 mM chromotrope 2R (C2R) + 3 mM phosphotungstic acid + 0.5% acetic acid to stain the attachment clamps and copulatory spines.
- While life cycles for *Polystoma, Protopolystoma* and *Pseudodiplorchis* are well understood, the life cycles for remainder of the genera are hypothesised and needs to be investigated.
- Morphological changes during the development from egg to oncomiracidium, and immature adult to adult are largely unknown for the Polystomoidinae subfamily. Especially in *Neopolystoma* and *Polystomoides* where skeletal structures are not evident at larval stage using light microscopy.
- Comparative study on rest of differential aspects such as genital spines and larval development.

Chapter 6

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Chapter 7

Appendix A

Table 7.1: Data matrix with the coded characters

			OUTGROUP Concinnod	Diplorchis	Eupolystoma	Kankana	Madapolystoma	Mesopolystoma	Metapolystoma	Nanopolystoma	Neodiplorchis	Neopolystoma	Neoriojatrema	Oculotrema	Parapolystoma	Parapseudopolystoma	Polystoma	Polystomoidella	Polystomoides	Protopolystoma	Pseudodiplorchis	Pseudopolystoma	Riojatrema	Sphyranura	Sundapolystoma	Wetapolystoma
1	Host	0=Dipnoi 1= Urodela 2=Anura 3=Gymnophiona 4=Chelonia 5=Mammalia	0	2	2	2	2	2	2	3	2	4	2	5	2	2	2	4	4	2	2	1	2	1	2	2
2	Found external on host	0=no 1=yes	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
3	Found in bladder	0=no 1=yes	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1
4	Found in mouth	0=no 1=yes	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0
5	Found on eyes	0=no 1=yes	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
6	Eyes in adult	0=no 1=yes	1	1	?	?	0	?	0	0	1	0	?	0	0	0	0	0	0	0	0	0	0	?	0	0
7	Food	0=blood 1=mucus	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0
8	Mouth	0=terminal 1=subterminal	1	0	0	0	0	0	0	1	0	1	0	1	0	0	0	1	1	0	0	0	0	1	0	0
9	Cephalic lobes	0=yes 1=no	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
10	Internal organs	0=through body 1=anterior 2/3	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
11	Gut confluent posteriorly	0=no 1=yes	1	1	1	1	1	1	1	0	1	0	0	0	0	1	1	0	0	0	1	0	1	1	0	1
12	Gut extends into opisthaptor	0=no 1=yes	0	1	1	1	1	1	1	0	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1
13	Gut caecums of equal length	1=no 0=yes	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
14	Medial diverticula absent	0=no 1=yes	1	1	0	0	0	0	0	1	0	1	0	1	1	0	0	1	1	0	0	0	0	1	0	0
15	Medial diverticula small	0=no 1=yes	0	0	1	1	1	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0
16	Medial diverticula extensive	0=no 1=yes	0	0	0	0	0	1	0	0	0	0	1	0	0	1	1	0	0	1	1	1	1	0	0	1
17	Medial diverticula network	0=no 1=yes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
18	Lateral diverticula	0=no 1=yes	0	0	1	0	1	1	1	0	1	0	1	0	0	1	1	0	0	1	1	1	1	0	1	1
19	Anastomosis mostly present	0=no 1=yes	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	1	1	0	1	0	0	1
20	Genitointestinal canal present	0=no 1=yes	1	1	1	?	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	?	1	1

21	Haptor	0=2 lobes 1=1 lobe	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
22	Hamuli present	0=absent 1=1pair 2=2pair	1	1	0	1	0	1	1	1	1	0	0	0	1	0	1	1	2	2	0	0	0	1	1	1	
23	Number of suckers	0=none 1=1 pair 2=3 pars	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	2	2	
24	Hamulis incision	0=absent 1=medium 2=deep 3=no	2	0	3	0	3	2	2	3	1	3	3	3	0	3	1	2	2	2	3	3	3	2	1	0	
25	Suckers with skeleton	0=no 1=bricks 2=fancy	2	0	0	0	0	0	0	1	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0	
26	Sucker symmetry	0=radial 1=bilateral	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
27	Testis position	0=anterior 1=middle 2=posterior	2	0	2	1	2	2	2	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	
28	Testis position 2	0=median 1=lateral	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	
29	Testis compact or diffuse	0=compact 1=diffuse	0	0	1	0	0	1	1	0	0	0	1	0	0	1	1	0	0	1	0	1	1	0	1	1	
30	Number of testis	0=1 1=2 3=many	3	1	0	0	0	0	0	1	1	0	1	0	3	0	0	0	0	0	1	0	0	3	0	0	
31	Copulatory organ	0=none 1=simple 2=complex	2	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	
32	Genital spines	0=no 1=1 set 2=2 sets 3=1or2 sets	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	3	2	1	2	1	1	1	1	
33	Number of genital spines	0=none 1=1-10 2=11-20 3=>20	0	1	1	1	1	1	1	2	1	1	2	0	2	1	1	2	3	2	1	2	1	1	1	1	
34	Ovary position	0=anterior 1=middle 2=posterior	1	0	2	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	
35	Ovary	0=germinal tissue 1=Propar ovary	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
36	Vitellaria	0=diffuse 1=compact	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	
37	Ovary pretesticular	1=No 0=Yes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
38	Vitellaria	0=spread 1=lataral fields 2=lateral	?	1	1	1	?	0	0	1	2	0	0	0	0	1	0	0	0	0	2	0	0	1	0	0	
39	Vaginae present	0=no 1=yes	0	1	0	0	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	0	1	1	1	1	
40	Uterus present	0=absent 1=tubular 2=sacciform	0	1	1	1	1	2	1	0	1	1	1	2	1	1	1	1	1	0	1	0	1	1	1	1	
41	Uterus extends posteriorly	0=absent 1=no 2=yes	2	2	1	2	2	1	1	0	2	1	1	1	2	1	1	1	1	0	2	1	1	0	2	2	
42	Eggs in utero	0=0 1=<10 2=11=20 3=21-50 4=>50	1	4	4	4	3	4	4	1	4	1	3	4	4	1	1	1	1	0	4	1	1	1	4	4	
43	Eggs fusiform	0=no 1=yes	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	
44	Egg shape oval	0=no 1=yes	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	
45	Eggs yello tan	0=yes 1=no	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	
46	Eggs operculated	0=yes 1=no	0	?	1	0	1	0	0	0	?	0	?	0	0	0	0	0	0	0	0	?	?	?	0	1	
47	Neotenic	0=no 1=yes	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	

47	Neotenic	0=no 1=yes	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
48	Internal cycle	0=no 1=yes	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
49	Vivipary	0=no 1=yes	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50	Ovovivipary	0=no 1=yes	0	?	1	1	1	1	1	0	1	0	1	0	1	?	0	0	0	0	1	0	0	0	1	0	
51	Ciliated cells	0=none 1=53 2=55 3=59 4=64	?	3	2	?	?	?	2	?	1	4	?	4	2	?	2	4	4	4	?	?	?	?	3	?	
52	Number of marginal hooklets	0=14 1=16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Chapter 8

Appendix B

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The morphology and attachment of *Protopolystoma xenopodis* (Monogenea: Polystomatidae) infecting the African clawed frog *Xenopus laevis*

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Abstract – The African clawed frog *Xenopus laevis* (Anura: Pipidae) is host to more than 25 parasite genera encompassing most of the parasitic invertebrate groups. *Protopolystoma xenopodis* Price, 1943 (Monogenea: Polystomatidae) is one of two monogeneans infecting *X. laevis*. This study focussed on the external morphology of different developmental stages using scanning electron microscopy, histology and light microscopy. Eggs are released continuously and are washed out when the frog urinates. After successful development, an active swimming oncomiracidium leaves the egg capsule and locates a potential post-metamorphic clawed frog. The oncomiracidium migrates to the kidney where it attaches and starts to feed on blood. The parasite then migrates to the urinary bladder where it reaches maturity. Eggs are fusiform, about 300 µm long, with a smooth surface and are operculated. Oncomiracidia are elongated and cylindrical in shape, with an oval posterior cup-shaped haptor that bears a total of 20 sclerites; 16 marginal hooklets used for attachment to the kidney of the host and two pairs of hamulus primordia. Cilia from the 64 ciliated cells enable the oncomiracidium to swim for up to 24 h when the cilia subsequently curl up, become non-functional and are shed from the body. The tegument between the ciliated cells bears a series of sensory papillae. The body of the mature parasite is elongated and pyriform and possesses an opisthaptor armed with three pairs of suckers and two pairs of falciform hooks to ensure a firm grip on the flexible internal surface of the urinary bladder.

Key words: Monogenea, Polystomatidae, *Protopolystoma xenopodis*, *Xenopus laevis*, Morphology.

Résumé – Morphologie et attachement de *Protopolystoma xenopodis* (Monogenea : Polystomatidae) infectant le xénope du Cap *Xenopus laevis*. Le xénope du Cap *Xenopus laevis* (Anura : Pipidae) est l'hôte de plus de 25 genres de parasites représentant la plupart des groupes d'invertébrés parasites. *Protopolystoma xenopodis* Price, 1943 (Monogenea : Polystomatidae) est l'un des deux monogènes infectant *X. laevis*. Cette étude a porté sur la morphologie externe des différents stades de développement du cycle de vie de *P. xenopodis* en utilisant la microscopie électronique à balayage, l'histologie et la microscopie photonique. Les œufs sont libérés de façon continue et sont dispersés quand le xénope urine. Après un développement réussi, un oncomiracidium actif et nageant librement quitte la capsule de l'œuf et localise un potentiel xénope post-métamorphique. L'oncomiracidium migre vers le rein où il s'attache et commence à se nourrir de sang. Le parasite migre ensuite vers la vessie où il atteint sa maturité. Les oeufs sont fusiformes, longs de 300 µm, avec une surface lisse et sont operculés. Les oncomiracidia sont allongés et cylindriques, avec un hapteur postérieur ovale en forme de coupe qui porte un total de 20 sclérites ; 16 petits crochets marginaux utilisés pour se fixer sur le rein de l'hôte et 2 paires de primordia d'hamuli. Des cils portés par 64 cellules ciliées permettent à l'oncomiracidium de nager pendant 24 heures, après quoi les cils se recroquevillent, deviennent non fonctionnels et sont éliminées de la surface du corps. Le tégument entre les cellules ciliées porte une série de papilles sensorielles. Le corps du parasite mûr est allongé et piriforme et porte un opisthohapteur armé de trois paires de ventouses et deux paires de crochets falciformes, assurant une prise ferme sur la surface flexible interne de la vessie urinaire.

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Introduction

The African clawed frog *Xenopus laevis* (Daudin, 1802) [2] (Anura: Pipidae) is primarily aquatic, which allows it to serve as a host or intermediate host for several parasites. *Xenopus laevis* is host to a rich assemblage of more than 25 parasite genera from seven invertebrate groups [33]. These parasites are relatively distinct from those in other anurans, shown by their isolated taxonomic position [33]. Parasites infecting *Xenopus* also benefit from the fact that large numbers of frogs are often confined to relatively small areas of suitable habitat, predominantly when water levels drop during dry seasons [27].

Protopolystoma xenopodis (Price, 1943) [22] (Monogenea: Polystomatidae) is one of two monogeneans to infect this anuran host, the other being *Gyrdicotylus gallieni* Vercammen-Grandjean, 1960 [42], that is found in the buccal region of the host. Monogeneans are mainly ectoparasitic on fish, but the family Polystomatidae radiated onto the tetrapods and are found on the skin and gills of the Australian lungfish *Neoceratodus forsteri* (Krefft, 1870) [16], urinary bladder of frogs, gills and skin of salamanders, cloaca and phallodeum of caecileans, on the eye, nostrils, mouth, cloaca or urinary bladder of freshwater turtles and on the eye of the hippopotamus *Hippopotamus amphibius* Linnaeus, 1758 [1, 19, 38]. These host organisms are ecologically related through their association with freshwater habitats that favours parasite transmission. Polystome reproduction is generally limited to the periods when hosts are in water. Strict host specificity as observed in anuran polystomes minimises the chance of host-switching to other amphibian species [12, 38]. Amongst anuran polystomes *P. xenopodis* is probably one of the best studied species with respect to general biology and environmental factors affecting reproduction, transmission and survival [9–11, 35].

Adult *P. xenopodis* are found in the urinary bladder where they continuously produce eggs (mean 9 eggs/worm/day, at 20 °C) [11]. Egg production and output is highly sensitive to temperature fluctuation [10, 11, 39]. Reproduction is continuous, throughout the life of the mature worm, but does fluctuate according to the water temperature [10]. Eggs are expelled into the external environment and infective oncomiracidia hatch in about 22 days. Oncomiracidia swim and actively search for a potential host. Once contact has been established with a potential host the oncomiracidium enters the cloaca and migrates to the kidneys where it develops for approximately 2–3 months. It then migrates back to the urinary bladder where egg production begins 3–4 months postinfection at 22 °C [34, 37, 38]. *Protopolystoma xenopodis* has no uterus and eggs are expelled directly into the host's urinary bladder [32]. Although cross-fertilisation seems to be the preferred choice amongst polystomes, self-insemination probably occurs via the ovo-vitelline duct in solitary individuals [44]. In comparison with most parasitic platyhelminths, *P. xenopodis* is characterised by low prevalence and intensity in natural populations and low rates of egg production (all reducing output of infective stages into the environment), a relatively long developmental period to egg hatching (delaying transmission) and slow development postinfection to maturity (extending generation time) [38].

This study focussed on the external morphology of *P. xenopodis* using scanning electron microscopy (SEM), histology and clear mount. Egg, larval, subadult and adult stages of *P. xenopodis* will be examined.

Material and methods

Wild *X. laevis* were caught in July 2013, using baited 20-L bucket traps fitted with an inward directed funnel. Traps were set at various sites in and around the city of Potchefstroom, North-West Province, South-Africa. Permit number 028-NW-11; North-West University ethical clearance: NWU-00005-14-S3. Traps were baited using chicken and/or beef liver, left overnight and retrieved the following morning. To prevent frogs from swallowing the bait, the liver was placed inside a gauze bag which was placed inside the trap. Captured *X. laevis* were individually screened for the release of parasite eggs. Frogs were individually placed in 500 mL plastic tubs containing approximately 250 mL water and maintained at room temperature. After 24 h, the frogs were transferred into clean water and the residual suspended debris was allowed to settle. The supernatant was progressively decanted and the remaining volume was screened using a stereo microscope. A gentle rotating action was used through centripetal force to concentrate sediment particles and eggs in the centre of the tub. The presence of characteristic golden, shiny, fusiform eggs would indicate a positive infection. Tubs with infected hosts were marked. Eggs, larval and adult stages of the life cycle were collected and subsequently prepared for microscopy. Eggs earmarked for incubation were transferred to 6 cm diameter Petri dishes containing dechlorinated water and incubated at 24 °C. The incubation period for *P. xenopodis* was approximately between 22 and 25 days. Development of eggs was monitored using a dissecting microscope, and when fully formed oncomiracidia were observed moving within the eggs, the Petri dishes containing these eggs were placed in direct sunlight for approximately 30 s, resulting in rapid hatching. Hatched oncomiracidia were studied live before they were fixed. Fully embryonated eggs at the point of hatching together with some empty egg shells were collected and fixed in 70% ethanol.

Hosts were euthanised by placing them in a 3% ethyl-4-aminobenzoate (MS 222) solution (Sandoz) for approximately 15 min before they were dissected. The urinary bladder and kidneys were inspected for the presence of parasites. The dark colour as a result of the blood pigment haematin in the gut channel makes it easy to spot *P. xenopodis* within the transparent bladder, but it is less easily spotted within the kidney. The kidneys were flattened between two glass slides and parasites identified by their dark intestines and movement in the kidney tissue. Four positively infected kidneys were fixed in Bouin's fixative for histological sectioning. Parasites were removed through microdissection; however, it was difficult to remove specimens without damaging them. The bladder was carefully removed and placed in a Petri dish containing a 0.03% saline solution after which it was cut open and parasites were removed and fixed under coverslip pressure either in 10% neutral buffered formalin for light microscopy or in Todd's fixative for SEM [40]. To fix parasites still attached to the bladder wall, a piece of thin cotton thread was used to tie off the bladder and Todd's fixative was then carefully injected into the bladder using a 1-mL syringe.

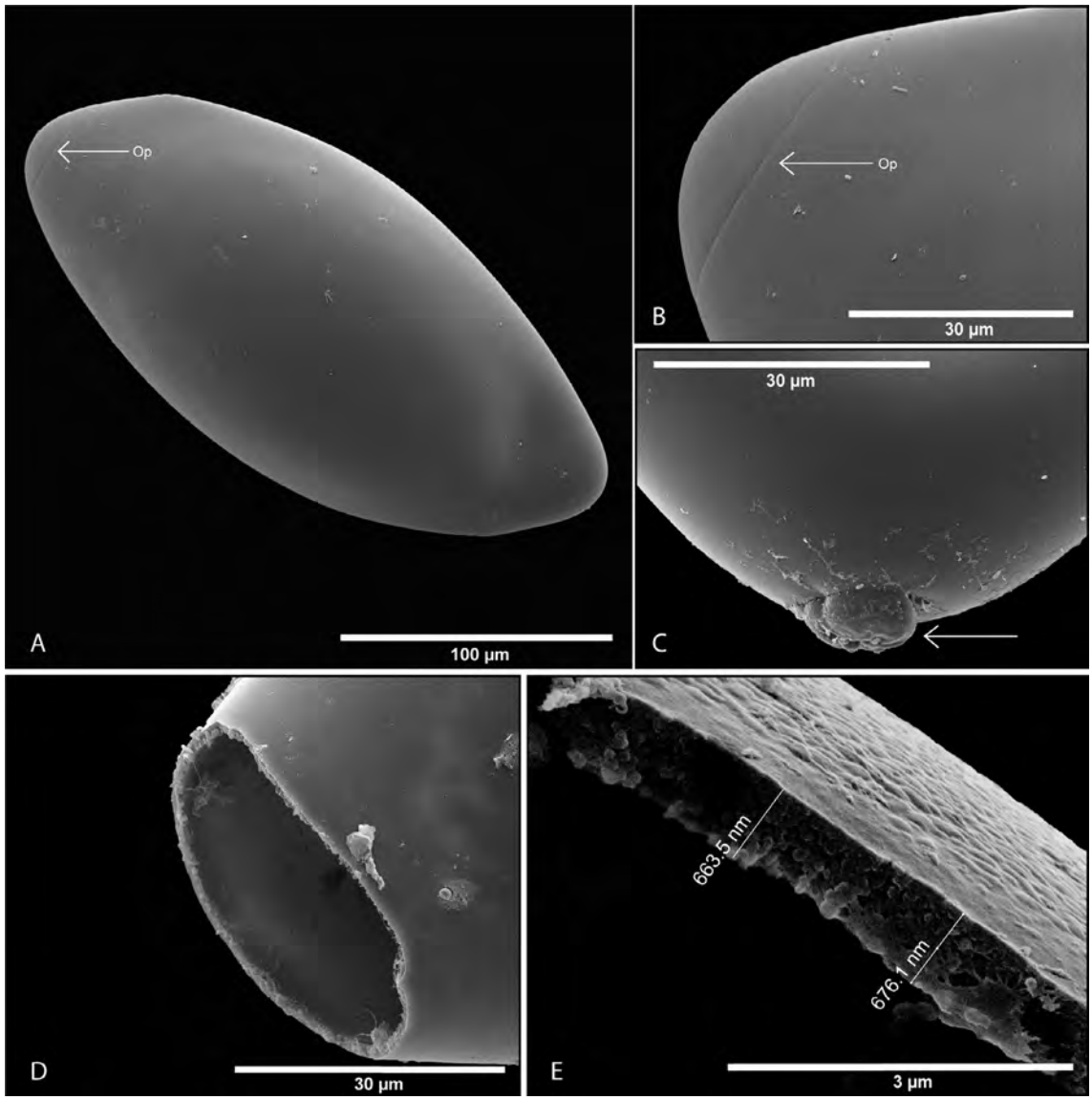

Figure 1. Scanning electron micrographs of *Protopolystoma xenopodis* egg features. (A) Fully embryonated egg, operculum visible (← Op). (B) Operculum becoming visible as the egg develops (← Op). (C) A residual structure on the non-opercular side of the egg. (D) An empty egg shell after the oncomiracidium has left. (E) Egg shell indicating the thickness of an individual parasite egg shell at the opercular opening.

In order to study sclerites of oncomiracidia, 10 specimens were mounted temporarily in lactophenol to clear the specimens. Coverslips were secured using clear nail varnish. Marginal hooklets were studied using a Nikon E800 compound microscope and measurements taken using Nikon NIS Elements software.

For histological sectioning, material was fixed in Bouin's fixative and subsequently transferred through a graded series of ethanol in 10–15-min steps starting at 30%, then 50% and stored in 70% ethanol. For sectioning, material was further dehydrated in an ethanol series of 70%, 80%, 90% and twice in 100% for 10–15 min each. Dehydrated material was cleared in xylene-ethanol mixture for 10 min and finally in two changes of pure xylene for 20 min each. Material was impregnated with paraffin wax at 60 °C for 24 h; impregnated material was embedded in paraffin wax with a melting point of 65 °C in a Histocene embedding machine. Material was sectioned at 5 µm on a Reichert Yung motorised microtome. Wax sections were placed on a glass slide covered with an albumin adhesive solution, stretched on a stretching plate and dried overnight at 40 °C in an oven. Sections were stained in routine Harris' haematoxylin and eosin and mounted using Entellan.

For scanning electron microscopy, specimens fixed in Todd's were washed three times in 0.05 M cacodylate buffer for 15 min each and then washed three times in distilled water for 15 min each. Samples were then dehydrated in an ethanol series; 70%, 90%, 100% and 100% for 15 min each. The samples were critical-point-dried (CPD), mounted on aluminium

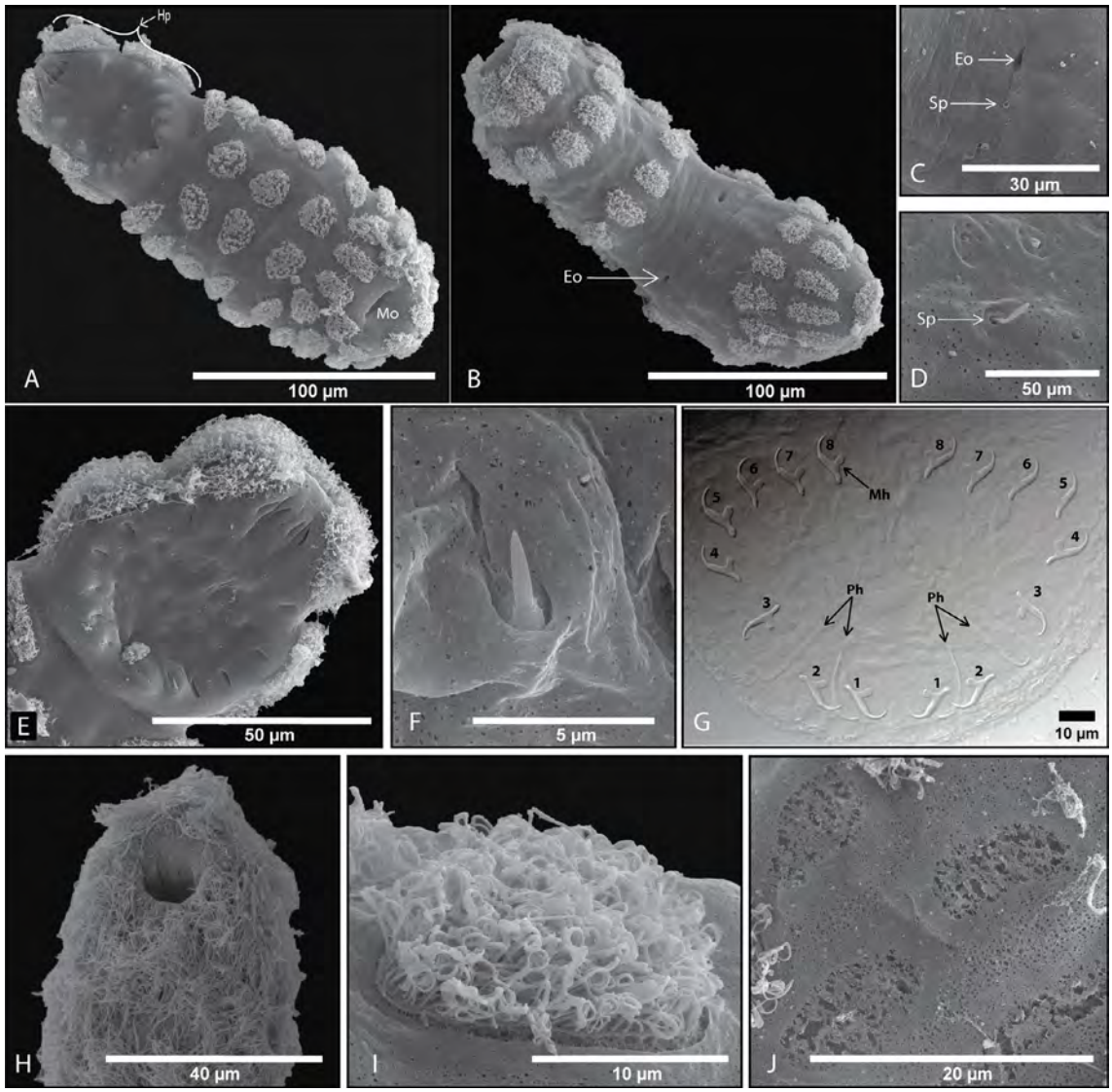

Figure 2. Scanning electron micrographs and one light micrograph (G) of the *Protopolystoma xenopodis* oncomiracidium. (A) Ventral view with posterior haptor (Hp) and anteriorly placed mouth opening (Mo). (B) Dorsal view with two excretory openings in the centre (← Eo). (C) Dorsal view with sensory papillae (← Sp) next to excretory opening. (D) Sensory papillae (← Sp) situated all over the body surface of the parasite. (E) Haptor with 16 retracted marginal hooklets and two pairs of large primordial hamuli. (F) Emerging marginal hooklet. (G) Light micrograph of a lactophenol cleared haptor showing the 20 sclerites; two pairs of eight (1–8) marginal hooklets (Mh) and two pairs of primordial hamuli (Ph). (H) Anterior side of the oncomiracidium covered with cilia. (I) A single ciliated cell with cilia coiling up before cells are shed. (J) Scars after intact cells were shed at the end of the swimming phase.

stubs with the use of double-sided carbon tape, sputter-coated with gold palladium and examined with a FBI ESEM Quanta 200 scanning electron microscope.

Results

Egg morphology (Fig. 1)

Eggs of *P. xenopodis* are fusiform and operculated (Fig. 1A). The surface is smooth with no visible appendages or filaments attached. In unembryonated eggs, the operculum line is not visible but in incubated eggs, a clear groove where the egg capsule will break open can be observed (Fig. 1B). At the apex of the non-opercular end of the egg, a small residual structure is sometimes noticeable (Fig. 1C). This residual structure was never observed at the operculated side of the egg. Examination of empty egg shells (Fig. 1D) revealed that the operculum breaks off completely with no indication of a hinge structure to keep the operculum attached. Five eggs measured using a scanning electron microscope were 240.9

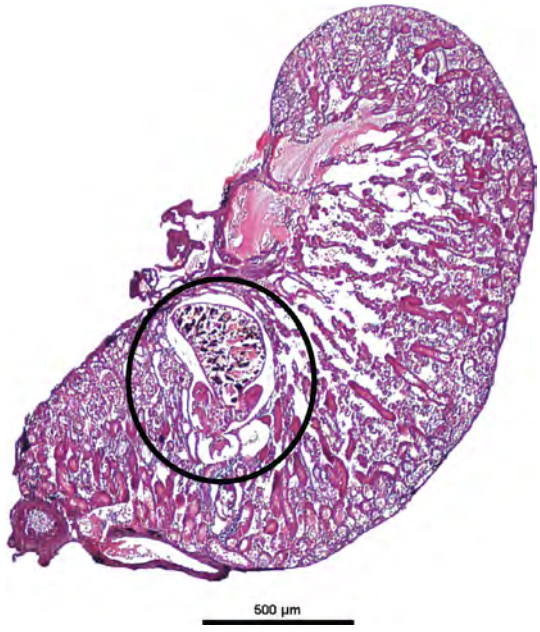

Figure 3. Histological section through a *Protopolystoma xenopodis* within the kidney of a *Xenopus laevis* specimen. The position of the parasite is indicated in the figure.

(230.9–252.1) µm in length and 103.3 (86.8–113.6) µm in width. The egg wall of a single egg measured was 670 nm thick (Fig. 1E).

Oncomiracidium (Fig. 2)

The body of the oncomiracidium is elongated and cylindrical (Figs. 2A and 2B), 199.3 (193.8–202.6) µm in length, measured from four specimens, using a scanning electron microscope. The mouth is ventral and subterminal. The haptor is directed ventrally, concave and elongated longitudinally (Fig. 2E). Its length is nearly one third of the total body length (Fig. 2A). The haptor bears a total of 20 sclerites. There are 16 marginal hooklets (Fig. 2G), each approximately 13–14 µm, arranged in a circle alongside the primordial of the four hamuli (Fig. 2G). Primordial hamuli protrude posteriorly from the centre of the haptor with a total length of approximately 28–30 µm. The second smaller pair of hamuli lies between the posterior and posterior lateral hooklets (hooklets 1 and 2); these small hamuli are thin, gracile structures, approximately 19–20 µm long. The sclerites are mostly withdrawn (Fig. 2E) when the oncomiracidium is not attached to its host; however, they may sometimes protrude, as shown in Figure 2F. The oncomiracidium is covered in 64 isolated ciliated cells arranged in a symmetrical pattern. Oncomiracidia will actively swim for up to 24 h. In actively swimming oncomiracidia, the cilia are long and appear as a continuous carpet of cilia over the oncomiracidium (Fig. 2H), making it difficult to identify individual ciliated cells. Oncomiracidia that do not find a host in time will lose their ability to swim and become moribund. SEM examination of these moribund oncomiracidia revealed that the cilia curl up and became dysfunctional (Figs. 2A, 2B and 2I). Entire cells are shed, leaving markings on the body (Fig. 2J). The tegument between the ciliated cells bears a series of sensillae (Figs. 2C and 2D); these inhabit relatively constant positions with regard to the ciliated cells.

Subadult – kidney stage (Fig. 3)

The oncomiracidia locate a potential frog host and enter the body through the cloaca. Oncomiracidia migrate through the urinary duct to the kidney. The immature parasite attaches inside the kidney (Fig. 3) using its 16 marginal hooklets and most likely the two pairs of developing hamuli. They start feeding on blood and develop in the kidneys. As the parasite matures, it develops six suckers which replace the marginal hooklets as primary attachment organs. The development of suckers was not documented in the present study. The black pigment in the parasite's gut is through the accumulation of haematin, indicating a sanguinivorous diet [28]. Parasites were observed migrating down the urinary duct towards the bladder where they continue to develop and reach maturity.

Adult parasite – bladder stage (Figs. 4 and 5)

The mouth is subterminal and ventral, with an upper lip protruding over the mouth (Fig. 4B). The body is pyriform, narrowing posteriorly anterior to the haptor. Marginal hooklets are no longer functional but are retained in the body, and attachment to the bladder wall is achieved through six muscular and very flexible suckers (Fig. 4C). Unlike most other polystomes where suckers face ventrally, suckers of *P. xenopodis* face ventro-laterally. Compared with other polystomes the haptor is very flexible, to the extent that whenever a parasite is removed from the bladder and placed in a Petri dish, the haptor folds over and suckers attach readily to the body proper. A wedge-shaped infolding in the anterior margin of the haptor between sucker pair 3 was observed (Figs. 4A and 4C). When attached to the urinary bladder the soft transitional epithelial is drawn into the sucker and when a parasite is removed from the bladder the sucker imprint is clearly visible (Figs. 5A and 5B). Suckers are adapted for attachment to highly contractile substrata such as the urinary bladder.

Attachment is further secured through two pairs of hamuli (Fig. 4E). Only one pair of hamuli develops into large falciform haptoral hamuli, approximately 208 (180–243) µm in length, whilst the second pair of hamuli develops into smaller hamuli, approximately 28 (27–30) µm in length. Suckers are supple and assist in successful attachment to urinary tissue of its host (Figs. 4D and 4F). The body surface is free from ciliated cells or scar tissue as the parasite matures, but still possesses multiple sensillae. Adult parasites in the bladder start producing eggs.

Discussion

Relative to their body size, the eggs of most monogeneans are quite large [32]. Egg shape varies and, according to Schmidt et al. [24], the shape is determined by the oötype walls. Surface structures and appendages such as filaments are common for monogenean eggs and, according to Van der Linde [41], these structures can be functionally explained in that filaments attach to objects. Egg filaments are absent in polystomes.

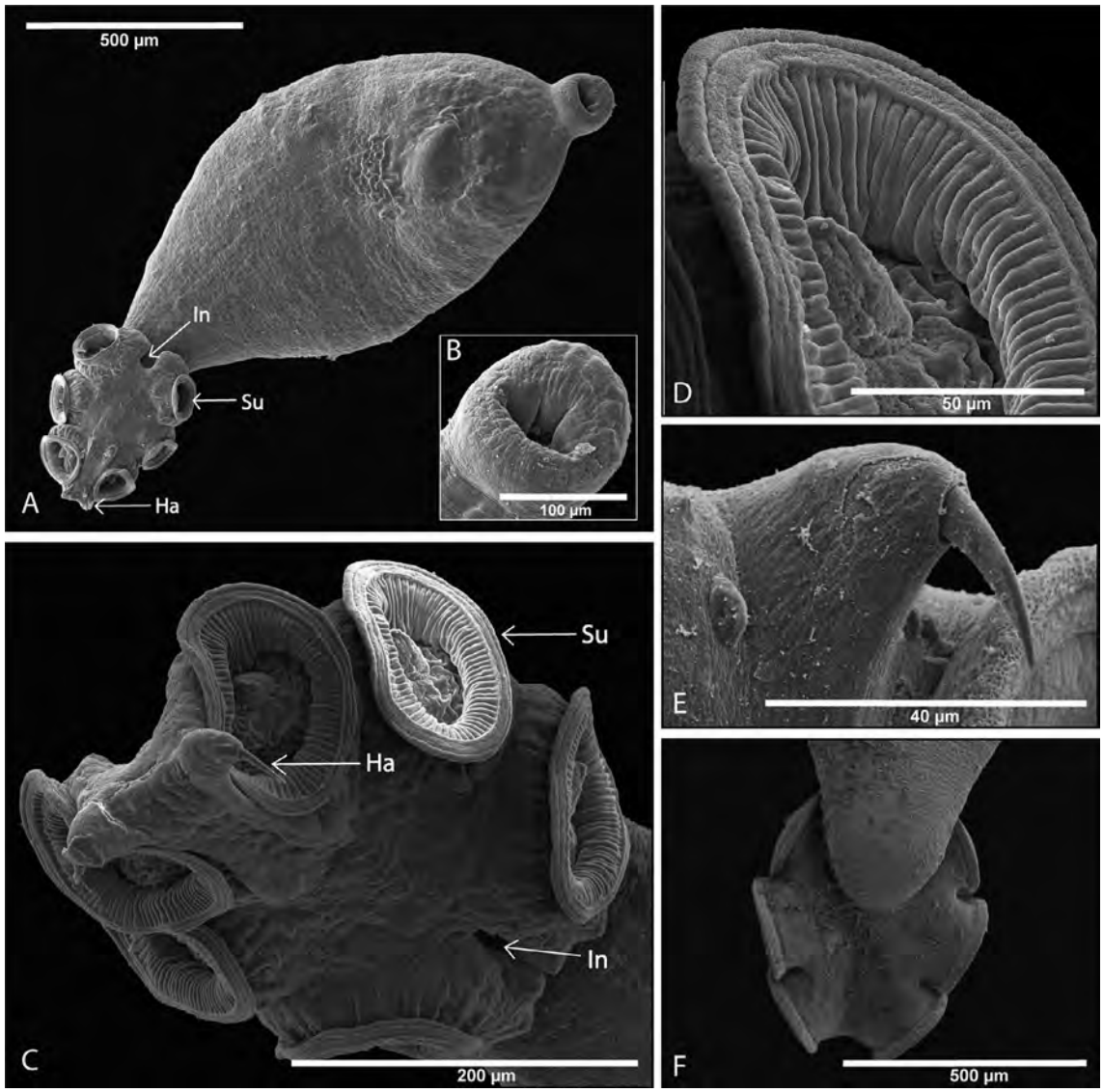

Figure 4. Scanning electron micrographs of mature *Protopolystoma xenopodis* specimens. (A) Ventral view of the parasite showing the mouth opening in the anterior of the parasite and the haptor armed with suckers (← Su), hamuli (← Ha) and a wedge-shaped infolding (← In) between the third sucker pair at the anterior margin of the haptor. (B) Ventral mouth opening. (C) Haptor with six ventral suckers (← Su), hamuli (← Ha) and a wedge-shaped infolding (← In) between the third sucker pair. (D) Single sucker showing the musculature of the sucker. (E) Hamulus point. (F) Dorsal view of the haptor.

Within the Polystomatidae, the shape of the egg varies from oval-round [18, 26], pear-shaped [43], elliptical [5, 21, 43], spindle-shaped [7] to fusiform or diamond-shaped [35] with a smooth surface and no functional appendages. The thickness of the egg shell varies considerably between species. Du Preez et al. (2010) [8] described the egg shell of *Madapolystoma* Du Preez, 2010 [8] as a thin membrane. *Eupolystoma* Kaw, 1950 [13] likewise has a thin transparent membrane as an egg shell with no operculum [30]. The membrane simply ruptures when the oncomiracidium hatches, leaving a collapsed structure with no discernible shape behind. On the other extreme is the thick egg wall of *Oculotrema* with a reported thickness of 3.3 µm [6]. Between the two extremes, a variety of egg shell thicknesses exists and as a rule, polystome eggs are operculated. The eggs of *P. xenopodis* are operculated and have a wall that is 670 nm thick. The opercula on the eggs of *Polystoma australis* Kok et Wyk, 1986 [15], by comparison, are hinged and remain attached once the oncomiracidia have exited [3]. In the present study, no eggs were found with the operculum still attached to the egg casing after the oncomiracidium hatched [41], however, noted that the operculum sometimes does stay attached but did not report a hinge as in the case of *Polystoma* [3]. The colour of polystome eggs varies from whitish in the case of the thin-shelled types of eggs produced by the species of *Eupolystoma* and *Madapolystoma* to the dark, shiny, golden colour of those produced by *Oculotrema* Stunkard, 1924 [25]. The significance of egg shape and shell thickness needs to be studied from an evolutionary perspective. Where eggs develop and hatch inside the host as in the case of *Eupolystoma*, the egg shell is a thin membrane. Eggs that are

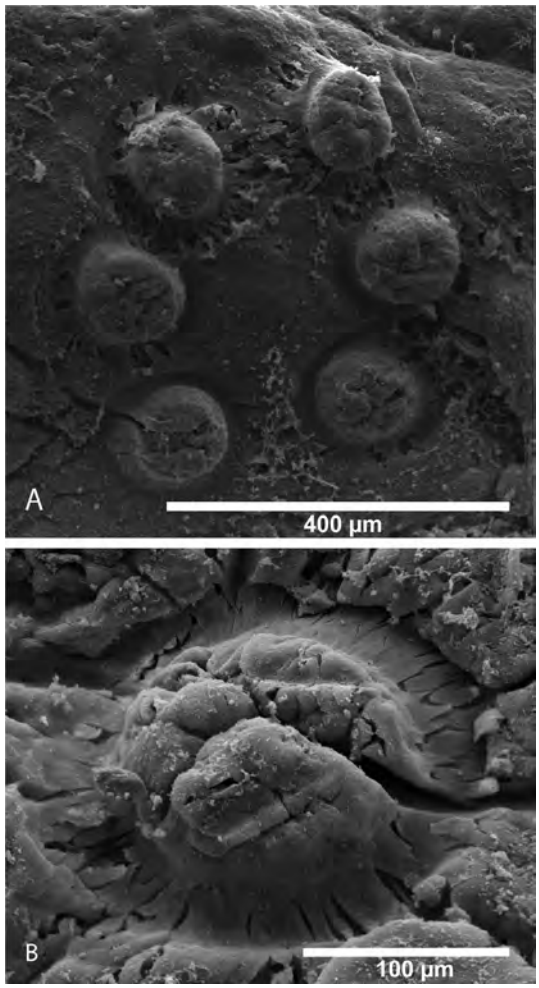

Figure 5. Scanning electron micrographs of buds made by the parasite's suckers on the host's bladder surface (A) and high magnification of a single bud (B).

expelled and develop outside the protection of the host's body as in the case of *Polystoma* and *Protopolystoma* have thicker egg shells. Polystomes that live under the eyelids of their hosts as in the case of *Neopolystoma* in turtles and *Oculotrema* in the hippopotamus, have eggs with a very thick egg shell [6] that provides protection.

The number and placement of ciliated cells that were observed in this study were similar to those detailed by Tinsley and Owen [38]. Five groups were documented: (1) apical group – 2 cells anterior; (2) cephalic group – 2 × 14 cells, dorsal and ventral; (3) medio-anterior group – 2 × 3 cells, ventral; (4) medio-posterior group – 2 × 6 cells, dorsal and ventral; and (5) haptoral group – 2 × 8 cells, dorsal and lateral. The shapes of the ciliated cells vary in different regions: medially situated cells are often broadly rounded whilst lateral cells are elongated and ellipsoidal [38]. The locomotory cells of monogeneans may be isolated or contiguous [17]. Tinsley [29, 31] briefly described the ultrastructure of polystomatid ciliated cells. The coiling of cilia of moribund oncomiracidia as observed for *P. xenopodis* in the present study (Fig. 2J) has not been reported for polystome larvae. In the present study we observed that when cilia coil up, oncomiracidia lose their ability to swim, and one can assume that oncomiracidia that have not located a host by the time cilia start to lose their functionality will be doomed. Du Preez and Kok's [4], a study on *Polystoma australis* Kok and Van Wyk, 1986 [15] found that cilia are shed, leaving naked cells. It would thus appear that different mechanisms may be at work. The oncomiracidia of some monogenean species shed their ciliary cells immediately after making contact with the host [4, 14, 15, 20], whilst others do not [36]. There seems to be agreement that entire ciliated cells are shed after larvae are established on their hosts. According to Tinsley [31] "the intact cells are thrown off the body surface" of polystomatid oncomiracidia following successful infection.

The mature *P. xenopodis* has a reported lifespan of about 2.5 years [32]. The body surface is smooth, and contains multiple sensillae. Sensory papillae are spread across the surface area of the oncomiracidium, enabling it to navigate and identify potential hosts. Two types of sensillae may be distinguished: small circular "buttons" with a relatively thin encircling wall which are distributed over the surface of the body in no discernible symmetrical configuration, and larger, ellipsoidal, thick-walled sensillae which occur in four regions in *P. xenopodis* (normally three pairs lateral to the mouth, three pairs between and behind the eyes dorsally, two pairs at the junction of the body with the haptor ventrally and several pairs on the posterior lip of the haptor) [38]. Compared with other polystomes, the haptor is exceptionally flexible and manoeuvrable with suckers orientated ventro-laterally. The wedge-shaped infolding in the anterior margin of the haptor between sucker pair 3 has not been reported for any other polystomes. This infolding contributes to the flexibility of the haptor, expanding the area the suckers can reach. As the parasite matures the functional role of attachment of the 16 marginal hooklets are replaced by the six suckers. The suckers are supple and assist in the successful attachment to urinary tissue of its host. The flexible haptor and suckers resemble those of the neotenic form of *Polystoma* which attach to the branchial filaments of tadpoles. Williams [44] stated that the morphology of *P. xenopodis* was in general essentially similar to the neotenic adult of *Polystoma integerrimum* (Frölich) Rudolphi, 1808 [23].

Since the host is permanently aquatic it undergoes continuous osmotic influx, which results in frequent and regular urination (approximately every 2–3 h) [10]. This implies a frequent change in the volume and thus tension on the bladder wall. The thin highly contractile membrane of the bladder thus undergoes intermittent sudden changes in surface area and thickness, potentially causing the haptor to readily detach if rapid stretching or contracting of the bladder tissue is stimulated [27]. In this sheltered site of infection, successful suctorial attachment mechanisms to a highly contractile substrate are of great adaptive advantage to *P. xenopodis*. The flexibility of the parasite's haptor and suckers along with the two pairs of hamuli probably play a crucial role in attachment within a frequently changing environment.

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