

TAXONOMIC REVIEW AND GENERAL BIOLOGY OF THE GENUS
***Eupolystoma* (MONOGENEA: POLYSTOMATIDAE)**

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Summary

Polystomes are parasitic flatworms infecting mainly anuran hosts. Of the 21 known polystome genera only *Eupolystoma*, *Metapolystoma*, *Polystoma* and *Protopolystoma* are known from Africa. Although the genus *Polystoma* has a world wide distribution, *Metapolystoma* and *Protopolystoma* are limited to Africa while *Eupolystoma* is limited to Africa and India. The genus *Eupolystoma* currently consists of five species. These species include *Eupolystoma anterorchis* parasitic in *Amietophrynus pantherinus*, *E. vanasi* from *Schismaderma carens*, *E. alluaudi* from *A. regularis*, *A. gutturalis*, *Nectophrynoides malcomi* and *Pyxicephalus adspersus* from Africa. Two species, *E. chauhani* from an unidentified *Bufo* sp. and *E. rajai* from an unidentified *Rana* sp., are both known from India.

Eupolystoma produce both ciliated and unciliated oncomiracidia. Ciliated oncomiracidia leave the host and infect post-metamorphic naive hosts. The oncomiracidium enters the cloaca; migrate to the kidney, then to the Mulerian ducts and to the bladder where the parasites then reach maturity. Because the hosts are usually explosive breeders, parasite transmission may be risky. *Eupolystoma* also produce non-ciliated oncomiracidia. They do not leave the parent host but attach alongside the parent to develop into mature parasites.

Due to large intraspecies variation and limited interspecies variation, huge emphasis is placed on morphometric measurements of sclerotised parts and in particular the marginal hooklets. A set of 13 measurements were taken of the sclerotised marginal hooklets of the *Eupolystoma* species. Through mathematical modelling the combination of measurements with the highest classification potential was determined for the genus *Eupolystoma*. The best combination of measurements did not produce a significantly better result as the protocol for the genus *Polystoma*. We thus suggest that the *Polystoma* protocol be used for the genus *Eupolystoma*.

Opsomming

Polystoma is parasitiese platwurms wat hoofsaaklik amfibiërgashere infekteer. Van dié 21 bekende genera wat amfibiërs infekteer, word *Eupolystoma*, *Metapolystoma*, *Polystoma* en *Protopolystoma* in Afrika aangetref. Terwyl die genus *Polystoma* wêreldwyd voorkom, is die verspreiding van *Metapolystoma* en *Protopolystoma* beperk tot Afrika en *Eupolystoma* is beperk tot Afrika en Indië. Die genus *Eupolystoma* bestaan tans uit vyf spesies. Hierdie spesies sluit *Eupolystoma anterorchis* uit *Amietophrynus pantherinus*, *E. vanasi* uit *Schismaderma carens* en *E. alluaudi* uit *A. regularis*, *A. gutturalis*, *Nectophrynoides malcomi* en *Pyxicephalus adspersus* vanuit Afrika in. Die twee spesies, *E. chauhani* van 'n ongeïdentifiseerde *Bufo* sp. en *E. rajai*, wat van 'n ongeïdentifiseerde *Rana* sp. beskryf is, is beide afkomstig van Indië.

Eupolystoma produseer gesillieerde en ongesillieerde onkomirasidia. Die gesillieerde onkomirasidia verlaat die gasheer en kan ander gashere infekteer deur die kloak binne te dring en na die niere te migreer. Daarna keer dit terug deur die Müllerbuis en dan die blaas waar die parasiet volwassenheid bereik. Omrede die gasheer 'n eksplosiewe broeier is en die parasiet transmissie onveilig kan wees produseer *Eupolystoma* ook ongesillieerde onkomirasidia. Hierdie onkomirasidia verlaat nie die gasheer nie en heg langs die volwasse parasiete in die blaas en ontwikkel tot volwassenheid.

Aangesien die intraspesievariasie taamlik groot is en daar beperkte interspesievariasie voorkom, word groot klem op die morfometriese afmetings van skleriede en veral die marginale hakies geplaas. 'n Stel van 13 afmetings is van die marginale hakies van die *Eupolystoma* spesies geneem. Deur wiskundige modellering is die kombinasie van afmetings met die die hoogste klassifikasiepotensiaal vir die genus *Eupolystoma* vasgestel. Aangesien die beste moontlike kombinasie van afmetings nie betekenisvol beter vaar as die protokol vir die genus *Polystoma* nie, word voorgestel dat die *Polystoma* protokol ter wille van stabiliteit ook vir die genus *Eupolystoma* gebruik word.

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Chapter 1: Introduction

1.1 General Introduction

“Parasites account for only a fraction of the biomass in the overall community, and to most ecologists throughout this century they have been not only invisible but unimportant. This view has changed slowly over the past 15-20 years, and now a bridge exists between parasitology and community ecology.”

Poulin, 1998.

Amphibians are parasitised by a wide range of protozoans, mites, leeches, nematodes, acanthocephalans, flukes and tapeworms. The study of amphibian flatworms is not new to science. As far back as 1737 Swammerdam reported a worm in the lung of a frog. The first monogenean was observed by Roesel von Rosenhof when he reported a “leech” in the urinary bladder of a frog. The parasite turned out to be *Polystoma integerrimum* (see Prudhoe & Bray, 1982). Rudolphi (1809-1810) listed all known parasites of amphibians and came up with five species of trematodes, one monogenean and one cestode. Since those early days, the field of research received considerable attention and several scientists made significant contributions.

The phylum Platyhelminthes is subdivided into four classes: Turbellaria (non-parasitic free living flatworms), Monogenea (monogenetic flukes), Trematoda (digenetic flukes) and Cestoda (tapeworms). This study will focus only on monogeneans. The far majority of monogeneans are parasitic in poikilothermic vertebrates with the majority being small fish parasites. Monogenean evolution has been studied by several parasitologists. Parasitic flatworms most likely evolved from rhabdocoelid-like urbellarians having

facultative commensal associations with several aquatic invertebrates (Prudhoe & Bray, 1982). These associations probably later became obligatory. One can visualise that fish preying on these infected invertebrates could contract new parasitic associations through host switching. Amphibians evolved from primitive sarcopterygiins and one can speculate that the parasites speciated with their hosts gradually becoming more host specific. Although ancestral Monogenea were presumably external parasites of early aquatic vertebrates, parasitising on epidermal cells or flakes of skin, mucus and other secretions (Erasmus, 1972), modern monogeneans radiated and today consist of a vast diversity of highly specialised and successful parasites (Tinsley, 1976; Whittington *et al.* 2000).

Poulin (2001) stated that monogeneans are diverse both in terms of morphology and numbers. They are generally host specific and have a direct life cycle with no intermediate hosts involved. To adapt to their different hosts and environments, monogeneans have specialised in attachment organs (Chisholm & Whittington, 1998) and attachment areas (Llewellyn, 1964). This adaptation relates predominantly to morphological specialisation for attachment by the haptor of the parasites (Cribb *et al.* 2002). Monogenean parasites can also be found internally in the uteri and the bladders of fish and externally they can be found on the eyes of turtles, in the mouth and gills of lungfish, on the gills of salamanders and a single specimen, *Oculotrema hippopotami* (Stunkard, 1924) is found under the eyelids of the hippopotamus (Moeng & Du Preez, 1997). Tinsley (1976) stated that the colonisation of parasites on mammals are ancient and may possibly be due to the divergent morphologies of the adult parasites. Other forms of Monogenea parasitise on Amphibia where the adult parasite are found in the urinary bladder, stomach, intestine, skin and gills of frogs and tadpoles (Williams, 1994).

For a century platyhelminth parasites referred to as monogenetic trematodes have been classified almost exclusively according to their adult parasite characteristics. Bychowsky (1962) and Llewellyn (1957) exploited the

use of larval characters as taxonomic characteristics documenting intraspecies variation in order to obtain a more detailed classification. Today, the larvae of about 75% of the families of monogeneans are known, and those not yet known will most likely not differ much in terms of adult characteristics (Llewellyn, 1963).

Llewellyn (1963) stated that the three main facets of post-oncomiracidial development of the Monogenea include growth, development of the genitalia and the development of definitive adhesive apparatus. The internal arrangements of most monogenean forms have been well studied. Internally all Monogenea have a general pattern of a mouth, pharynx, oesophagus and either a basic or complexed blind ending intestine of gut caecum. Anterior, the mouth is either terminal or sub-terminal. Normally the anterior mouth can be plain or extended into a labia that is usually associated with the false oral sucker. The buccal cavity leads into the muscular pharynx that consists of three types of muscle fibres (Chappel, 1980).

Halton *et al.* (1974) stated that the spermatogonial cells of the reproductive system of a Polyopisthocotylean (*Diclidophora merlangi*) (Kuhn, 1832), are undifferentiated and that it displays numerous free ribosomes and relatively little cytoplasm. Spermatocytes are produced by successive mitotic divisions that are characterised by the expansion of the endoplasmic reticulum and the development of the Golgi complexes. Incomplete cytokinesis follows the nuclear division so that the spermatocytes and subsequent stages join and develop syncytially. During spermateleosis, the nucleus of the spermatid condenses and migrates into a conical shaped projection of cytoplasm. Before the spermatozoa of the Polyopisthocotylean are released from the residual cytoplasm, a centriole type structure and basal bodies that are anchored by a pair of rootlets produce axial filaments that grow out from the spermatid and fuse with the nuclear projection (Halton *et al.* 1975).

Vitelline cell development has been studied in three polyopisthocotyleans and one monopisthocotylean (Halton *et al.* 1974). In all the cases, the vitelline cell development has proved to be very similar. The immature vitelline cells are typically embryonic and undifferentiated. For differentiation into maturing vitelline cells, the development of extensive GER, Golgi complexes and the production of dense droplets of shell-protein are needed. When maturity is breached, the protein synthesis stops (the GER disintegrates) and the cell develop food-reserves in the form of yolk bodies (glycogen and lipid). This is then released into the vitelline ductlet. The processes occur in all the follicles simultaneously and all stages of development can be found in any one of the follicles.

The digestion system also been observed with *Dicliophora merlangi* (Polyopisthocotylea). Halton (1974) described that the blood proteins of the host are isolated by endocytosis and broken down in an interconnecting network of channels that form an essential but changing part of the cell. From the granular endoplasmic reticulum the involved digestive enzymes originate where they are packaged in the Golgi apparatus and transferred to the channels in the Golgi vesicles. The haemoglobin absorption and the activity of the Golgi are stimulated by the introduction of the host protein into the gut lumen. The residues of the haematin after digestion are extruded into the lumen via exocytosis involving membrane fusion.

The two orders of the Monogenea (Monopisthocotylea and Polyopisthocotylea) differ specifically in their diet. Polyopisthocotylea appears to be exclusively sanguiniforic however some species also prove to feed on mucus (turtle, hippopotamus and salamander parasites) (Halton & Jennings, 1965) where Monopisthocotylea primarily feed on epidermal tissue and mucoid secretions of the host. Histologically the intestinal epithelium reflects the main differences of the two orders. A continuous gastrodermis is present in Monopisthocotylea whereas Polyopisthocotylea are generally characterised by a discontinuous gastrodermis; the entire epithelium of the latter is replaced with

every meal after ingestion. A further difference between the two orders is the digestive processes. Monopisthocotyleans digest their food material extracellular, within the lumen of the intestine. A folding of external tegument lines the mouth cavity and is connected by cytoplasmic processes to subjacent perikarya (Halton & Stranock, 1976). Digestion in Polyopisthocotylea can occur intracellular and extracellular. With the blood feeders (Polyopisthocotylea), the haemoglobin is absorbed by endocytosis across the microvilli of haematin cells where vesicles are formed. A direct numerical relationship between these vesicles and the host's blood in the adjacent lumen of the caeca exists. Once the haemoglobin is in the haematin cell, it is digested by enzymes that originate from the granular endoplasmic reticulum. Waste haematin residue is removed from the cells by continuous exocytosis. Morphological studies indicate that the foregut of the Polyopisthocotylea is very complex but the physiological significance of why this is so, remains to be elucidated (Chappel, 1980). Because Polyopisthocotyleans are mainly sanguinivoric it might be argued that they are more advanced because they parasitise primarily amphibians and chelonians as well as one mammal, the hippopotamus, while the Monopisthocotylea prefer fish as their hosts (Williams, 1994).

Polyopisthocotyleans probably evolved after archaic monogeneans invaded the branchial chambers of fish. During the phylogeny of Polyopisthocotylea, the posterior suckers at first developed around the marginal hooklets, replacing them later on as functional anchoring structures. As essential larval features, hooklets are characteristic in most of the modern Polyopisthocotylea. The initiation of the posterior haptor was evidently correlated with the occupation of the new host site where the parasite more or less permanently attaches to gill lamellae or filaments, or, if possible, in a chamber where respiratory currents are present (Bentz *et al.* 2003). Therefore, suckers are tentative adaptations for grasping gill lamellae or filaments and may have arisen because the host is being parasitised on in an enclosed environment of

the branchial chamber. By living in the chamber, protection was provided as well as the opportunity for a sanguinic lifestyle on the vascular gills of the host. Digestion of the sanguinivoric Polystomatidae occurs through the caecal epithelium that contains haematin containing digestive cells for the digestion of the haemoglobin. After the digestion, intracellular accumulation of haematin occurs. Haematin is insoluble and the parasite compensates by eventually discharging the product in membrane bound vacuoles (simple extrusion) (Tinsley, 1974).

More contemporary polystomes possess a bucco-intestinal canal from where the intestinal contents are discharged. It has also been recorded that modern polystomes have an intestinal epithelium that consists of haematin-laden cells (digests haemoglobin) and supportive tissue (Williams, 1994). Whittington *et al.* (2000) also suggested that evolution of the attachment organs may have occurred so that the parasite can adapt more successfully to the vascular tissue of their host.

Attachment of the parasite to the host is of the utmost importance for survival. In a study conducted on three different Polyopisthocotyleans, it was concluded that each one of the species had developed different attachment aids. All of the Polyopisthocotylea, have hold fast units ranging from springlike clamps to muscular clamps to suckers with sclerotised rims (Wright & Dechtiar, 1974). In the case of the Polyopisthocotylea, a basic haptorial structure is modified, as marginal hooklets are present in series of two, six, and eight, as well as in combination with suckers. Some of the marginal hooks in most polyopisthocotyleans became differentiated from each other by adapting in shape and size. With the Polystomatids, the domus accompanies all 16 marginal hooklets. Some of the Monogeneans' marginal hooklets become modified at the end of the oncomiracidial development. According to Llewellyn (1963), the number of marginal hooklets is of utmost importance for taxonomic criteria, making this study inevitable. One of the main characteristics of the

polystomatids is that the oncomiracidium has 16 marginal hooklets in addition to the primordia of the hamuli (Bychowsky, 1962). As the parasites grow and the hamuli and suckers develop, the marginal hooklets become non-functional. They are, however, retained with hooklet pairs I and II between the most posterior suckers, hooklets III, IV and V are in the base of the sucker pairs 1, 2 and 3 and hooklet pairs Vi, VII and VIII are retained between sucker pair 3. (Llewellyn, 1963; 1968).

The hamuli of adults develop in the intervals between hooks II and III on each side of the haptor of most polystomatids and monocotylids. This must take place so that there are two pairs of marginal hooklets (I and II) on the posterior margin of the haptor in the middle of the median hooks (Llewellyn, 1963).

In general the Polystomatidae display a high level of host specificity (Tinsley, 1982; Du Preez & Kok, 1997; Tinsley & Jackson, 1998). In the light of specificity, this group of parasites illustrate themselves as an ideal model for studying evolutionary development which can lead to more knowledge being acquired about host / parasite relationships. This culminates into learning new historical events and the true time factors of the events that took place (e.g. drifting of Gondwanaland) (Dr L Du Preez, Personal Communication¹).

Together Polystomatidae evolved with their hosts (Amphibia) as the *terrestrial habitat proved competent with other ancestral amphibians*. Evolution of polystomatids trace the continuous and progressive development of host-parasite relationship as the exploitation of the new habitat took place. With the terrestrial adaptation of the amphibians, ancestral polystomes survived by colonising the vascularised bladder to avoid dessication and starvation. Although

¹ Dr L.H du Preez, School of Environmental Sciences and Development, North-West University, Potchefstroom Campus, South Africa

water was a prime requirement for parasites to reproduce, access was only granted during the reproduction period of the amphibians (Williams, 1994).

Previously, taxonomists used a basic philosophy to classify by indicating how morphologically related one species was to another. Contemporary taxonomy now appears to rather emphasize differences between two species, thus obtaining systematic relationships. There are two distinct approaches to the classification of the Polystomatidae; firstly, that of the comparative morphologist and the experimentalist or ethologist that yields morphological species, and secondly an apparent “biological species”.

There are, however, limitations with these two approaches. Morphological species are simply factual and rely entirely on distinct characters to indicate variation between species to allow species differentiation, but because of the lack of variation and the variation between species, morphological characters that identify species within Polystomatidae have caused confusion. Biological species arise from the assumption of a high degree of host-specificity and states that the behaviour of parasites under laboratory conditions is natural and invariable. It is generally known that host-specificity is an accepted theory within the Polystomatidae, especially the genus *Polystoma* but it has, however, not yet been experimented on other genera of the Polystomatidae (e.g. *Eupolystoma*), and therefore remains controversial (Van Niekerk, 1992).

1.2 Taxonomic Procedures

Technological improvement outdated new parasitic species descriptions in the early 1900's. In order to assess its classification performance, various statistical classification techniques have been developed and evaluated (McLachlan, 1992). Taxonomic classification is often hampered by problems, because of the need to over-simplify and the use of morphometric descriptors. To complicate matters, species boundaries are often poorly defined because of: (1) limited distinguishable morphometric features, (2) the simplicity of the forms under study, (3) overlaps among the species in morphometric space and (4) large intraspecies variation (Anton & Duthie, 1981). Flatworms have flexible, soft bodies. The degree to which a specimen is flattened during the fixation process, affects the body measurements limiting the value of body measurements as informative morphological characteristics. Flatworms do, however, possess sclerites consisting of sclero-proteins (Hyman, 1951), and these structures are frequently used as taxonomic characters.

Malmberg (1970) only measured the marginal hooklet of the *Eupolystoma* in length which already indicated a great interspecies difference. Also, according to Malmberg (1970), hooklet "one" (that was actually hooklet two and three) was measured. It was indicated that hooklet "one" was indeed shorter than hooklet eight (hooklet seven and six). This might be true, but no indication was given as to where the true hooklet one might be. He also stated that the blade of the marginal hooklet displays minimal variation between species.

Llewellyn (1963) first noticed that the hooklets vary between species and remain remarkably constant throughout monogeneans. He also mentioned that the most posterior hooklets of one Polystome (*Polystoma integerrimum*) were larger in size than the rest of the marginal hooklets of this parasite.

Murith (1979) recognised the potential of using the stability of the marginal hooklets as a specific taxonomic character. She studied the most posterior pair of marginal hooklets (C1) in a study of polystome systematics. She proposed a taxonomic protocol by measuring the hooklets of infesting larvae of the Polystomatidae (Figure 1.1). Based on marginal hooklet measurements, Murith (1979) was able to point out that various polystome species infect the same host species *Dicloglossus occipitalis*. This was surprising, as polystome parasites are known to be host specific (Bourgat & Salami-Cadoux, 1976; Combes, 1966; Combes & Channing, 1979; Du Preez & Kok, 1997; Euzet *et al.* 1974; Kok & Du Preez, 1987; Kok & Van Wyk, 1986; Maeder, 1973; Maeder *et al.* 1970; Murith, 1982; Tinsley, 1974). Other French workers adopted this protocol.

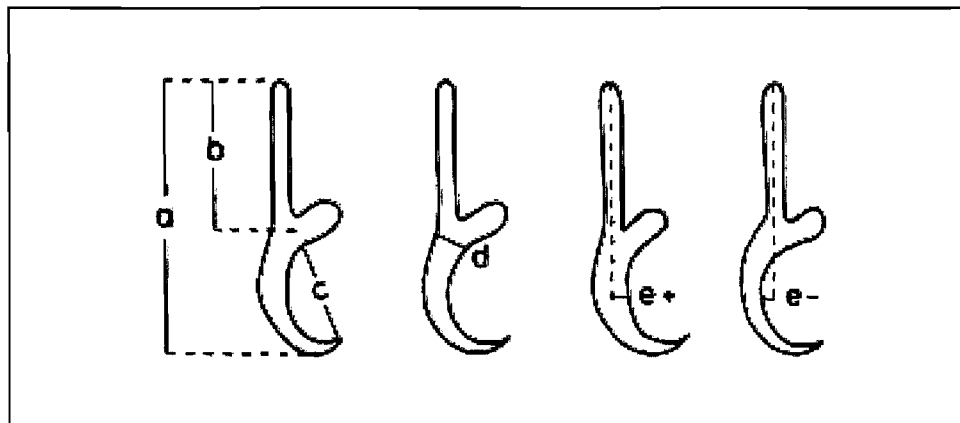


Figure 1.1: Diagram from Murith (1979) illustrating the parameters used in her suggested protocol.

On the opisthaptor of the parasite, the most posterior marginal hooklets were named C1. The total length and handle length of the marginal hooklet C1 was measured for a number of species by Bourgat & Murith (1980). Murith (1982) continued on this by calculating the ratio of the total length against handle length for marginal hooklet C1 (a/b) ratio. The proposed set of measurements (Figure 1.1) indicated how marginal hooklet morphometrics can be useful to identify or separate different species of a genus.

A few problems arose with the proposed measurement system by Murith (1982). Measurement b (Figure 1.1) has poor repeatability and is not objectively measurable because the measurement is from a fixed point to an estimated position at the middle of the base of the guard. Measurement “e+” and “e-” can also fall in this category, although measurement “e-” is also negative.

The most contemporary protocol was established by Du Preez & Maritz (2005) using the computer software Matlab®. The ultimate goal of Du Preez & Maritz (2005) was to use generally available software like MSeExcel® for accurate classification of Monogenean parasites. Thirteen measurements were taken of the posteriormost pair of marginal hooklets (C1) of at least 10 specimens per species (Figure 1.2). The aim was to determine the measurement or combination of measurements mathematically with the highest classification potential that would separate species the best. For the genus *Polystoma*, the best product of parameters has been calculated at (P1 x P9) and (P1 x P2). For each species measured, the products of parameters are plotted resulting in species clusters being formed (Du Preez & Maritz, 2005).

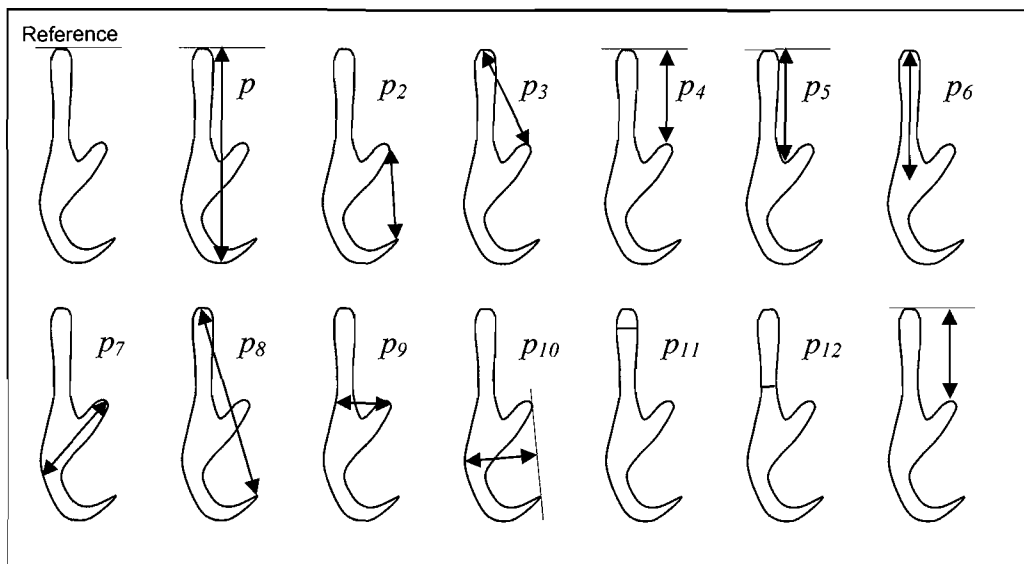


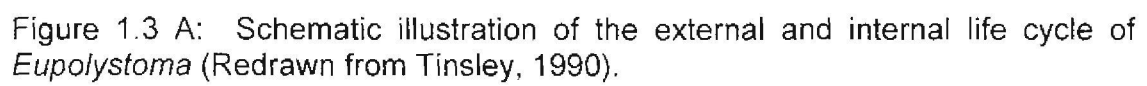
Figure 1.2: Illustration of the 13 measurements measured on marginal hooklets C1.

1.3 The polystome genus *Eupolystoma* (Kaw, 1950)

Previously little information was available on the genus *Eupolystoma* (Kaw, 1950). Some taxonomic contributions have been made by De Beauchamp (1913), Kaw (1950), Pandey (1969), Tinsley (1978), Beverly-Burton (1962), Yamaguti (1963), Euzet and Combes (1967), and Du Preez *et al.* (2002) that included descriptions of the anatomy and biology of five individual species of the genus, but no taxonomic protocol has been described for *Eupolystoma*.

Eupolystoma's taxonomic classification has been controversial in the last century. The first member of the genus *Eupolystoma* was originally named *Polystomum alluaudi* (De Beauchamp, 1913), but after taxonomic assessment, both the parasite and its hosts have been revised. According to Tinsley (1978), *P. alluaudi* was placed in the genus *Parapolystoma*. Yamaguti (1963) revised the genus again, erecting the genus *Beauchampia*. The genus was then been changed for a fourth time to *Eupolystoma*. Tinsley (1978) is the most productive single contributor to the knowledge of polystomatids in Africa. He published on the various aspects of the taxonomy and biology of *Eupolystoma* (Tinsley, 1978).

When the eggs of *Eupolystoma* are deposited from the uterus, the oncomiracidia hatch rapidly in both the host urine and water. If the eggs are discharged from the bladder during urination, the ciliated larvae are released so that they can infect the same or other host individuals (external life cycle) through the cloaca (Figure 1.3 A & B). With the internal cycle, the eggs are deposited in the bladder of the host between urinations; the unciliated oncomiracidia can emerge and attach themselves directly to the bladder wall of the same host (autoinfection) (Tinsley, 1977; 1990).



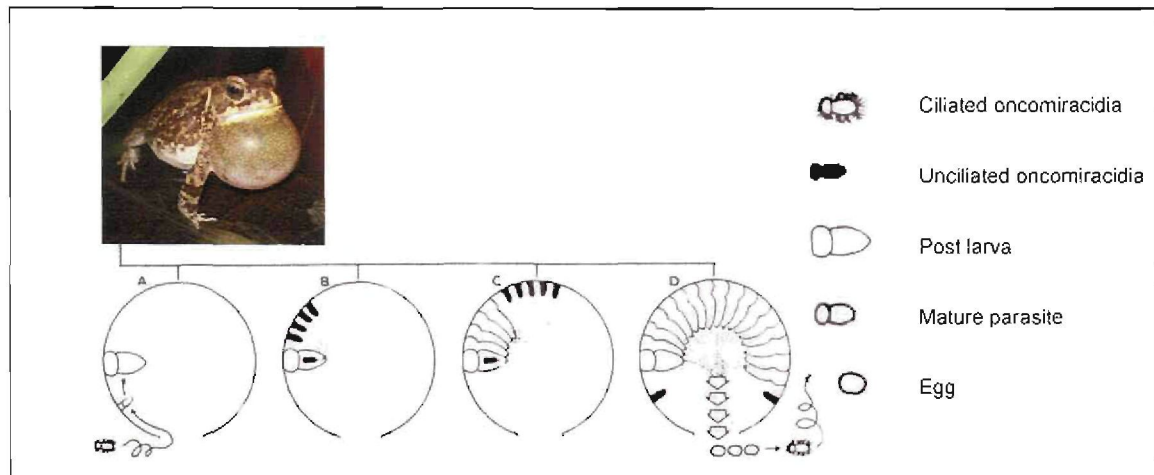


Figure 1.3 B: Schematic illustration of oncomiracidium production (Redrawn from Tinsley, 1990).

1.4 *Eupolystoma* species

1.4.1 *Eupolystoma anterorchis* (Tinsley, 1978)

The species description of *Eupolystoma anterorchis* was based on parasite specimens retrieved from the host, *Amietophrynus pantherinus* (Western leopard toad) from Noordhoek (34°06'S, 18°21'E) and Clovelly (34°07'S, 18°26'E), Cape Peninsula, South Africa during 1971-1976. Tinsley routinely ordered *Xenopus laevis* specimens from a supplier in the Cape for student training in the United Kingdom. In one of these shipments a number of Western Leopard Toads were included. He dissected them and discovered that they were infected with what appeared to be a new species of *Eupolystoma* (Tinsley, personal communication²). Heavy infestations of hundreds of parasites per frog were observed (Tinsley, 1978).

Tinsley (1978) also observed all stages of the parasite, from the freshly attached oncomiracidia to reproducing adults side by side in the urinary bladder of the host. The kidneys and Mulerian ducts were only occasionally parasitised

² Dr R.C. Tinsley, Dept Biology, University of Bristol, Bristol, United Kingdom

on by more recently infecting larvae. The oncomiracidia which had not yet had a blood meal were located in the urinary bladder next to the established adults and small adult parasites. *Amietophrynus pantherinus* is restricted to South Africa and limited to coastal regions from in Noordhoek and Clovelly in the Cape peninsula (Minter *et al.* 2004). The host mainly prefers a habitat that is grassy or open bushveld areas and has a breeding habitat that is usually in deep permanent waterholes and dams (Carruthers, 2001). *Amietophrynus pantherinus* is an explosive breeder and breeding activity will usually peak over a very short period of only two days late in August. The reproductive cycle of the parasite is synchronised with that of the host, as parasite transmission can only take place during this brief reproduction event.

Eupolystoma anterorchis differ morphologically from other species, because the position of the testis that is distributed from the posterior caecal union medially leading towards the ovary and laterally along the lower and ventral-lateral surfaces of the intestinal caeca. The shape of the intestine are without lobes or branches in some cases where others indicate prominent diverticula of variable lengths; usually they are short, but in exceptional cases, it will form transverse intercaecal anastomosis in the post-ovarian region. The follicular vitellaria forms two masses that are lateral and concentrated more posterior, however, it can extend along the intestine to the pharyngeal level. The intestinal pattern is an established characteristic of the genus *Eupolystoma* due to the fact that it is more or less ring-shaped with an unbranched caeca (Tinsley, 1978).

Up to about 300 eggs are retained in the long tubiform uterus. These eggs develop *in situ* and are ready to hatch the moment that the environmental signal is received. Tinsley (1977) observed that the stimuli for the deposition of the eggs are osmotic (when the worms are transferred from the host's bladder into fresh water they hatch immediately).

According to Price (1973) the overall reaction of egg production for parasites is adapted to respond to the reproduction cycle of the host. *Eupolystoma anterorchis* has adapted its egg production to its host's sporadic breeding period.

1.4.2 *Eupolystoma vanasi* (Du Preez, Tinsley & De Sa, 2003)

Eupolystoma vanasi is parasitic in the Red toad *Schismaderma carens* (Smith, 1848). The toad is a monotypical primitive genus in relation to other *Amietophrynus* spp. in the Bufonidae. They breed in deep pools or dams in the early Spring and mid-Summer. These frogs are diversely dispersed in the Savannah biome (Du Preez *et al.* 2003).

Eupolystoma vanasi is the largest of the known species and differs from other members of the genus by a combination of characters. The intestine of the parasite is bifurcate and has small lateral diverticula. No median branches have been observed and the caeca can be observed confluent posteriorly, in a small area anterior to haptoral anastomosis. The testes are diffused, consisting of several follicles dispersed ventrally, medially and posteriorly. The follicles are also limited to a small area just anterior to the haptorial anastomosis. Lateral to the intestine in the posterior half of the body, two lateral follicles are formed by the vitellaria (Du Preez *et al.* 2003).

1.4.3 *Eupolystoma chauhani* (Pandey, 1968)

The description of *Eupolystoma chauhani* is based on two specimens collected from the urinary bladder of an unidentified *Bufo* sp. in Gazipur, India. Morphologically, the intestinal caeca lacks a diverticulum and an anastomosis is present at the anterior border of the opisthaptor. A branch in the middle of the posterior anastomosis extends into the opisthaptor posteriorly to the last pair of suckers (Pandey, 1969). The follicular testes are posterior to the ovary and the follicular vitellaria elongates laterally along the intestinal caeca from the pharyngeal level to the posterior end. A median vitelline duct is also formed by the union of two transverse vitelline ducts that open at the ootype (Pandey, 1969).

The larva has only six larval hooks that are T-shaped with a long blade that is present at the hind border of the opisthaptor between the last pair of suckers (Pandey, 1969). This can be disregarded simply because the parasite could have been transferred incorrectly with the fixation process, leaving the haptor to be upside down for the morphological description. Tinsley (1978) remarked that *E. chauhani* has 18 marginal hooklets, suggesting that the extra pair of marginal hooklets between the posterior suckers might be hamuli. Hamuli have not been mentioned, indicating that this may not be a *Eupolystoma* sp. It is clear that not only from the lack of literature, but the missing holotype as well, that this parasite may have been incorrectly classified.

1.4.4 *Eupolystoma rajai* (Kaw, 1950)

Specimens of an unknown polystome were found in an unidentified *Rana* sp. in Kashmir, India. The intestinal caeca elongates laterally throughout the body and merge in front of the posterior haptorial suckers. No prehaptorial anastomosis was noticed from the caeca. The testes are median and post-ovarian in the case of *E. rajai*. The genital bulbus has eight genital spines. Morphologically, the vitelline follicles are characteristically scattered throughout the body of the parasite, from the intestinal branching up to the posterior haptor (Kaw, 1950).

Tinsley (1978) concluded that both the Indian parasitic species (*E. chauhani* and *E. rajai*) are problematic. These parasites are distinguished by the characteristic posterior ovary and the elongated uterus but lack some of the features that are traditionally used in the polystomatid taxonomy. Some of these features include the intestinal branches and haptorial hamuli. Similar morphological patterns have also been noted in both the African and the Indian regions that can possibly be explained by continental drift (Tinsley, 1978).

1.4.5 *Eupolystoma alluaudi* (De Beauchamp, 1913)

Data that includes the presence of the bucco-intestinal canal, the genito-intestinal canal, the positions of the vitelline glands and the testes reflected in the species *Eupolystoma alluaudi* that is parasitic in the bladder of the prominent host *Amietophrynus regularis*. It has also been observed that the invading larvae of this Togolaise species firstly migrate to the kidneys of their host (in this case *A. regularis*) were they go through a developmental period of three weeks before returning through the Mulerian ducts to the bladder. Salami-Cardoux (1975) stated that this is rare behaviour for *Eupolystoma*. It has, however, also been observed in other species of *Eupolystoma*. In 1913, De Beauchamp described *Polystomum alluaudi* in an expedition to Alluaud and Jeannel in Oriental Africa. Only one specimen was found in a temporary pond with the toads *Amietophrynus regularis* and *Amietophrynus taitanus*.

Euzet & Combes (1967) mentioned that if one should observe the geographical distribution of *E. alluaudi*, a problem would emerge in collaboration with the biological cycle of the parasite in relation to the ethology of the parasite host and the biotope in which this specific parasite evolves. Further, if the presence of a fully formed larva in the eggs of all the *Eupolystoma*, it can contribute to the classification of the genus. This may be the only indication of a different cycle to that of the other Polystomatidae (Euzet & Combes, 1967).

According to De Beauchamp (1913), none of the other authors that were studying the species observed testes or vaginae, nor the genital hooks. The presence of the buccal-intestinal and genital intestinal canals were also not observed by these authors (De Beauchamp, 1913). This might indicate that the species is still misclassified or that a complex of species can still emerge within the *E. alluaudi* itself.

In 1935 Ozaki proposed to place *E. alluaudi* in the genus *Parapolystoma*, but none of the characteristics fit and the proposal was not valid. After this,

Beverley Burton (1962) found species of De Beauchamp and restored the name *Parapolystoma alluaudi* (Euzet & Combes, 1967) and claimed that the Rhodesian material was similar in general morphology and pattern of the intestinal caeca to *P. alluaudi* and was thus tentatively assigned to the species (Beverley-Burton, 1962).

Yamaguti (1963) noticed incompatibilities and created *Beauchampia* with *Beauchampia alluaudi* as the type of species. The genus was characterised by:

- An ovary situated behind the vaginas (anterior),
- a post-ovarian uterus occupying the intercaecal space,
- embryonic eggs,
- a digestive tube with haptorial anastomose,
- a haptor without hamuli.

De Beauchamp's material (1913) did not substantiate this. According to De Beauchamp (1913), one can think that an ovary existed in the anterior quarter. In detail, the figure of De Beauchamp showed only one crushed and deformed egg. The real ovary was only discovered later and known to be situated in the posterior part of the body, just in front of the haptorial anastomose. Now, the parasite became the same as *Eupolystoma rajai* and it is not known if the species are the same. It is only confirmed that the two species are in the same genus, *Eupolystoma*.

During an epidemiological expedition to Northern Chad (1960), J-A Rioux collected Polystomatidae in a unidentified amphibian (probably *Bufo*) which had all the characters of *E. alluaudi*. This was the third discovery of the parasite.

Eupolystoma alluaudi has four known hosts that indicate a complex of species. Bourgat & Salami-Cadoux (1974) found the parasite in Togo and Cameroon parasitic in *Amietophrynus regularis*. It has also been found in Ethiopia in the host *Nectophrynoides malcolmi*, only in the urinary bladder (with no mentioning of the urinary ducts nor the kidneys). Other hosts include

Pyxicephalus adspersus present in Zimbabwe and *A. gutturalis* widely distributed in South Zimbabwe (Van Niekerk, 1992). Normally, members of the order Monogenea exhibit a strong degree of host-specificity (Hargis, 1957). It has been observed in *Polystoma* that the parasites are host specific, opening a major research field in the *Eupolystoma* genera. The geographical distribution of the four hosts stresses the fact that a complex of species exist within *E. alluaudi*.

There are two separate types or aspects of host-specificity among monogeneans. Infra-specificity is where the parasitic species is only present in a single taxon, the term genus specificity may also be applied where the parasite can parasitise on several congeneric hosts (Hargis, 1957). This may not be the case with *E. alluaudi*, where the 4 hosts are not even in the same family; for this, the term supra-specificity would be better suited. Supra-specificity can be defined as the limitation of a natural group of parasites to a natural group of host species; this includes the occurrence of the worm members of any supra-specific taxon, subgenus, genus, subfamily etc. on members of any supra-specific category of hosts (Hargis, 1957).

1.5 Study objectives

The objectives of this study were to:

- Study the South African species of *Eupolystoma* by re-describing known and describing new species.
- Review the *Eupolystoma* genus and resolve the *Eupolystoma alluaudi* species complex.
- Describe new species where necessary.
- Determine the set of marginal hooklet measurements that will produce the highest classification potential and to develop a protocol for the genus *Eupolystoma*
- Determine the level of host-specificity in *Eupolystoma*.
- Study the reproductive cycle of *Eupolystoma* to determine the role of the kidney and Mulerian duct phases.

Based on the literature and personal observations a few hypotheses were formulated:

- Preliminary results indicated that there are two new South African species.
- *Eupolystoma alluaudi* consist of a complex of species.
- The optimal set or combination of marginal hooklet measurements will separate valid species
- *Eupolystoma* is host specific but to a lesser extent than the polystome genus *Polystoma*.
- The Mulerian ducts as well as the kidneys of the host will be affected by the parasite infestation and that this specific infestation may be a trait of the genus, making it an additional character for identification purposes.

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Chapter 2: General Study area, Material and Methods

2.1 Study areas

2.1.1 *Eupolystoma* sp 1 from *Amietophrynus garmani*

In order to obtain parasite specimens, various fieldtrips to the Malelane area in the Mpumalanga Province of South Africa were undertaken where this parasite is known to occur. *Amietophrynus garmani* were collected at Berg and Dal restcamp in the Kruger National Park, (25°29'03,6" S, 31°29'40,8" E), Malelane Village (25°29'39,5" S, 31°30'56,3" E) and Fish Eagle bend (25°29'57,7"S, 31°30'00,9"E). All three locations were within a 10 km range (Figure 2.1).

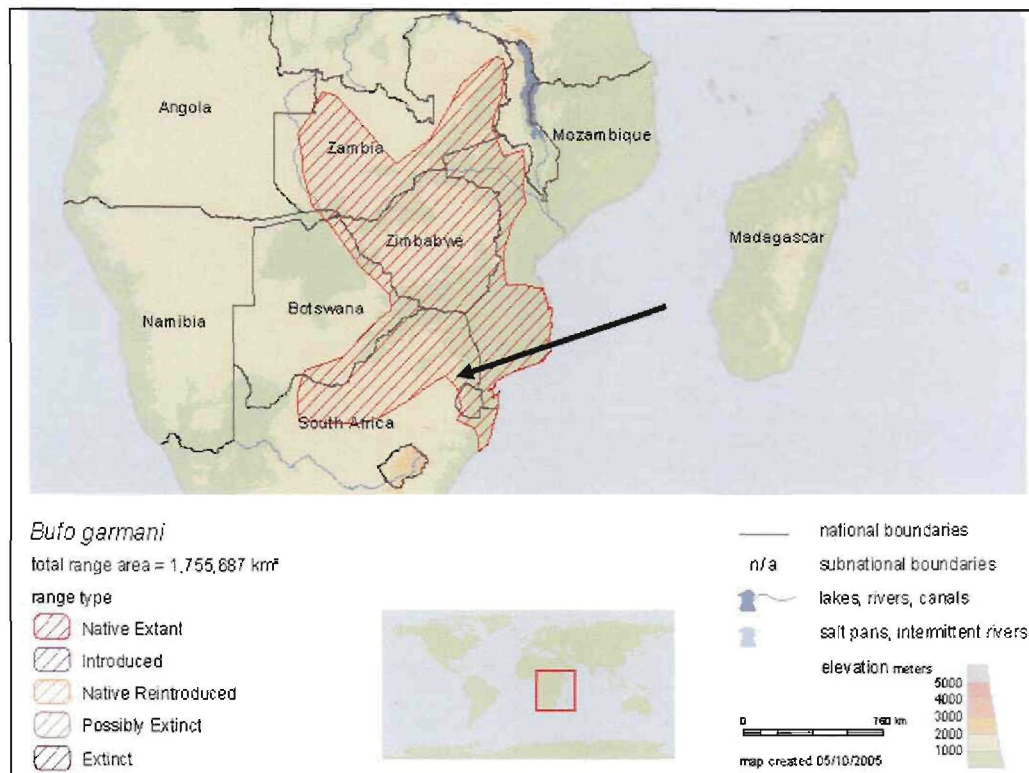


Figure 2.1: Geographical distribution of *Amietophrynus garmani* with collecting sites indicated (Source of map: IUCN; Global Amphibian Assessment, 2005).

2.1.2 *Eupolystoma anterorchis* from *Amietophrynus pantherinus*

In an attempt to obtain specimens of *Eupolystoma anterorchis* from its host *Amietophrynus pantherinus*, two fieldtrips were undertaken to the Noordhoek and Clovelly areas of the Cape Peninsula (Figure 2.2).

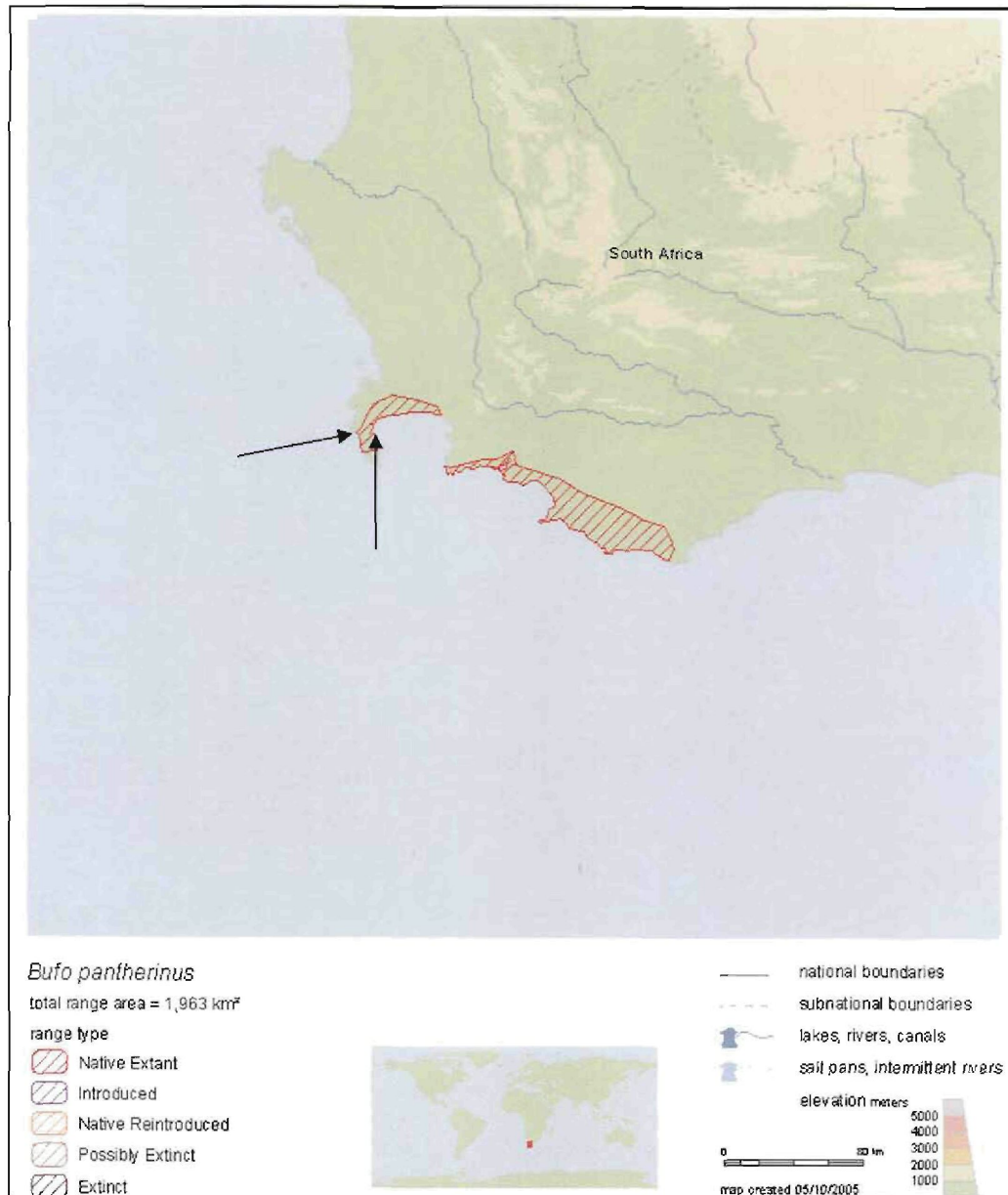


Figure 2.2: Geographical distribution for *Amietophrynus pantherinus* in the Cape Peninsula with collecting sites indicated (Source of map: IUCN; Global Amphibian Assessment, 2005).

2.2 Collection of parasites

Parasites needed to be retrieved from the urinary system of the frogs for further processing. Prior to dissection, the anurans were sedated with an overdose of MS 222 (Sandoz) and pinned in a wax dissection tray (Figure 2.3). The urinary bladder, Wolfian ducts and kidneys were removed and placed in amphibian saline in glass Petri dishes for inspection.

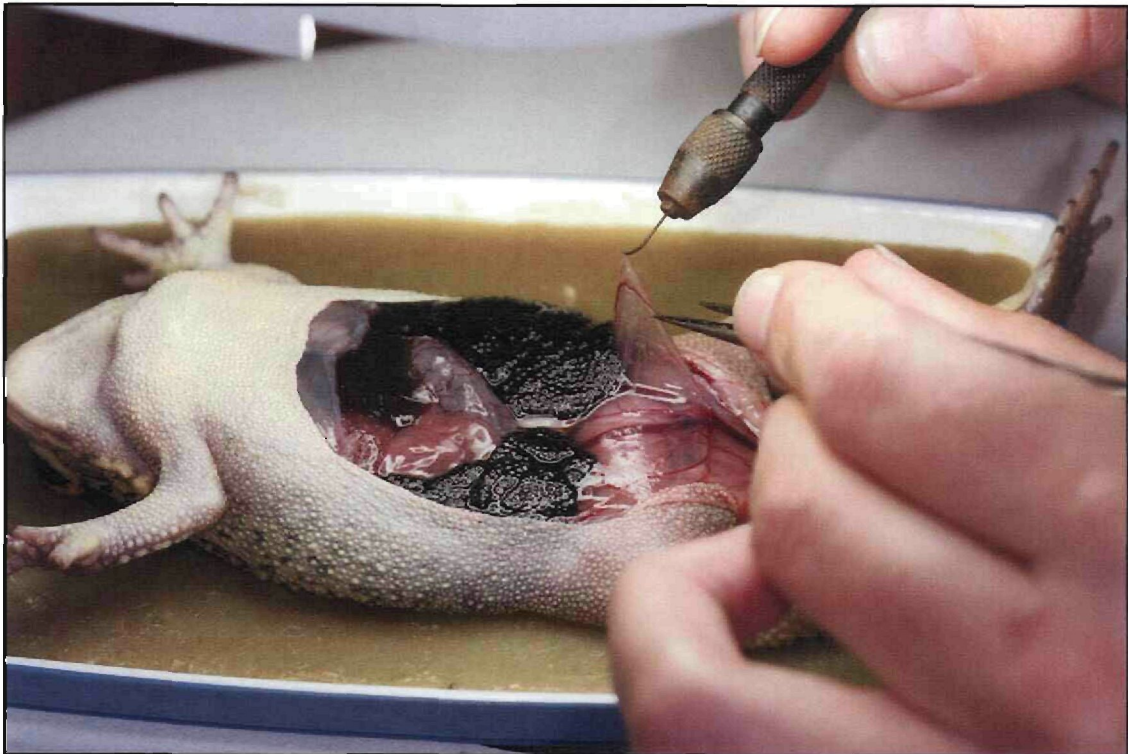


Figure 2.3: Microscopical examination of an adult *Amietophrynus garmani*.

2.2.1 Collecting of *Eupolystoma* sp 1 from *Amietophrynus garmani*

The first specimens of this polystome were discovered by L.H. du Preez during a parasitology symposium in a single infected specimen of *Amietophrynus garmani* found at the swimming pool in the Berg & Dal Restcamp in the Kruger National Park. A fieldtrip was conducted on 6 December 2005 after a downpour of rain had initiated breeding activities. Twenty-eight *A. garmani* specimens were collected by hand during the early morning hours (5:00-7:00), in and around the swimming pool at the Berg-and-Dal rest camp in the Kruger Park (Figure 2.4).

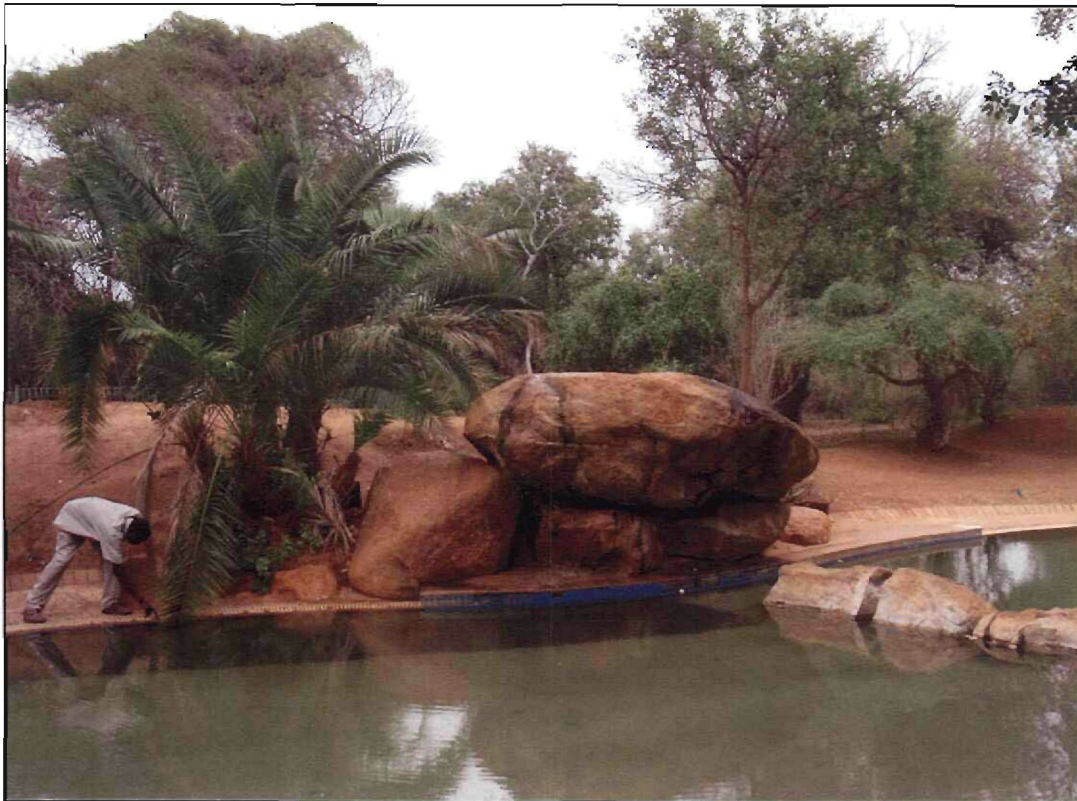


Figure 2.4: Photograph indicating the environment where *Amietophrynus garmani* were found at Berg-and-Dal rest camp in the Kruger National Park, South Africa.

The frogs were kept and transported to the African Amphibian Conservation Research Group (AACRG) facility in Potchefstroom in a cooler box

with moist soil instead of water to prevent spawning and parasite transmission in transit.

During the second field expedition on 3 October 2006 fifty-six *A. garmani* were collected. A third fieldtrip on 18 September 2007 a drastic decline in the population size of *A. garmani* was found due to the removal of vegetation that surrounded Berg-and-Dal rest camp's swimming pool consequently only two males were collected.

2.2.2 Collecting of *Eupolystoma anterorchis* from *Amietophrynus pantherinus*

Du Preez conducted a fieldtrip to the Cape Peninsula during middle August 2007. This was before the onset of the peak breeding event and only six road-killed specimens were examined. A second trip was undertaken on 1 – 6 September 2007 during which 74 live *A. pantherinus* specimens were collected at night at Noordhoek on the Silvermine Road. Because of their conservation status vulnerable (Minter *et al.* 2004), only one of the frogs were dissected

Seven reproductively adult frogs were screened by leaving the frogs individually in plastic containers with 10mm deep water. The water was then poured through two plankton sieves with mesh sizes of 500 µm and 100 µm respectively. The first net collected all the larger faeces and debris whilst the smaller net collected the eggs of the parasites and all the smaller debris.

In addition to this, as part of a South Africa National Biodiversity Institute (SANBI) molecular project, 113 road kills of *A. pantherinus* were collected between 12 August and 5 September 2007, labelled and frozen. These specimens were thawed and dissected to inspect for the presence of polystomes. Eight kidneys and mulerian ducts of four adult frogs were removed and fixed in Bouin's fixative for histology.

2.2.3 Other *Eupolystoma* spp.

All other *Eupolystoma* specimens in the AACRG collection as well as a number of specimens on loan were examined. These specimens included *Eupolystoma vanasi* collected in the Northern Province and KwaZulu-Natal, South Africa (63 specimens). *E. anterorchis* collected in Noordhoek and Clovelly in the Cape peninsula (6 specimens). *Eupolystoma alluaudi* from *Amietophrynus regularis* collected in Togo, Dapango during 1977 (47 specimens) from the British

Museum and Genève, 75 *E. alluaudi* specimens collected from *A. maculatus* in Nigeria. Twelve specimens of *E. alluaudi* parasitising *Nectophrynoides malcomi* that were collected in Ethiopia, were also received from the British Museum. Despite endless attempts, we were unable to locate specimens from the two Indian species *E. chauhani* from Kashmir and *E. rajai* from Lucknow.

2.3 Fixation and preparation

For histological examination the Mulerian ducts and kidneys were fixed in Bouin's fixative for 24 hours and then stored in 70% EtOH. Oncomiracidia and mature parasites were fixed under coverslip pressure in 70% EtOH or 10% neutral buffered formalin. Some specimens were fixed in 96% EtOH for DNA sequencing. During the fixation process a single parasite was positioned in a drop or two of saline on the microscope slide. A clean cover slip was lowered on the parasite to flatten it slightly. While administering the fixative on the one side of the cover slip, the slide was tilted on a paper towel to draw the saline from underneath the cover slip, replacing the saline with fixative. Parasites were fixed overnight and then removed and stored in either 70% EtOH or 10% NBF (Figure 2.5).

Semi-permanent lactophenol or ammonium picrate preparations were made from oncomiracidia and some of the smaller parasites. The coverslip was secured using clear nail varnish. Because lactophenol and ammonium picrate both make the parasites transparent, their positions on the preparations were marked on the coverslip with a permanent marker.

In order to prepare permanent mounts of parasites, they were stained in acetocarmine or alum carmine and mounted in Canada balsam (Appendix A).

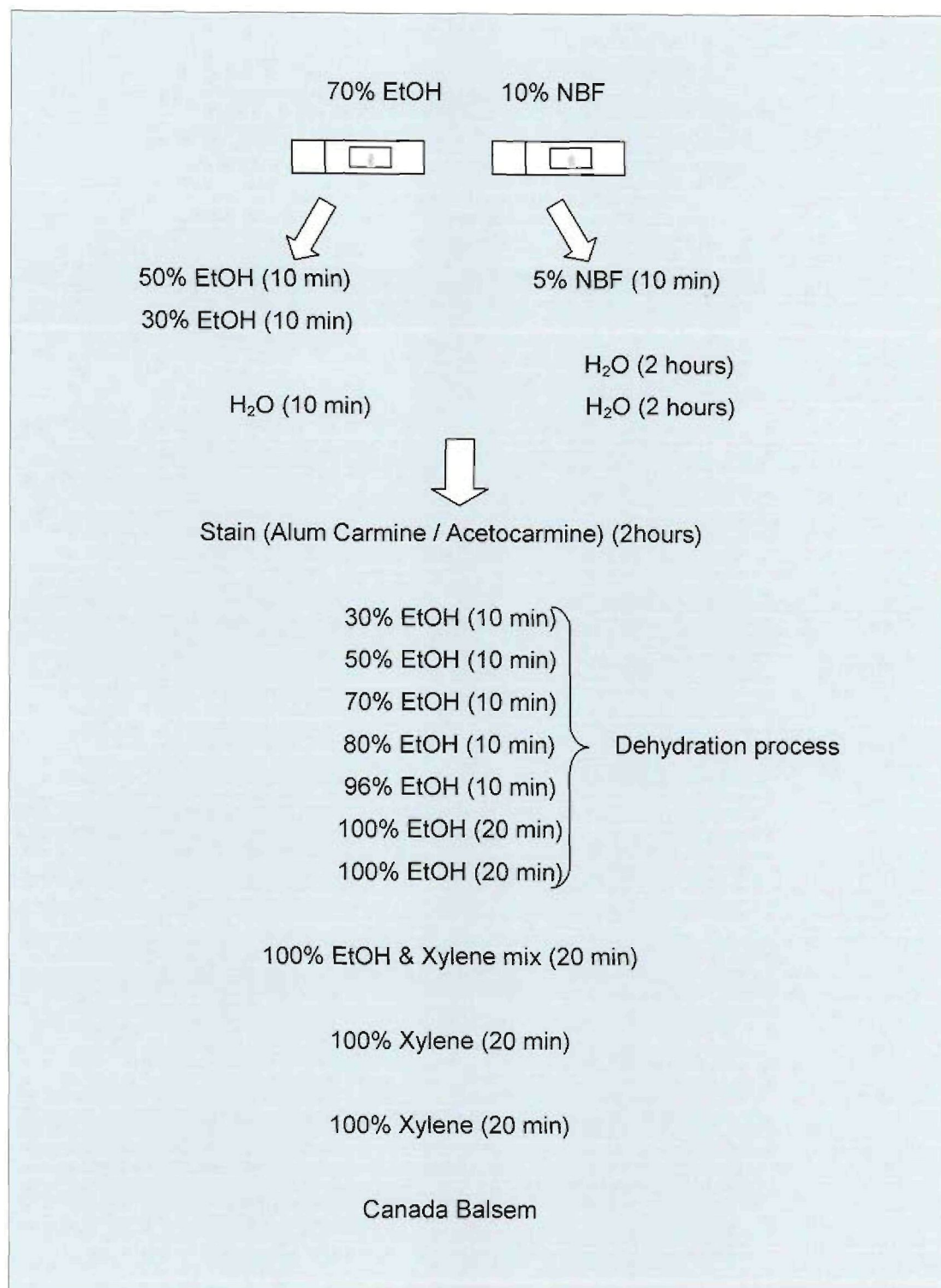


Figure 2.5: Schematic diagram outlining the staining procedure. Parasites were stained using Alum Carmine or Acetocarmine (Appendix A)

2.4 Histological Evaluation

Preserved kidney and Mulerian duct tissue were dehydrated in graded alcohol, cleared in xylene, impregnated in paraffin wax and embedded in wax using a Slee embedding centre (Figure 2.6).

Tissue were sectioned longitudinally at 6, 10 or 12 μm using a Reichert Jung 2050 microtome. The sections were relaxed on a stretch plate in an albumin mixture and allowed to dry (Appendix A). The slides were dried overnight in an oven at 30°C. Thereafter, the sections were stained and mounted as outlined in Figure 2.7.

Sections were routinely stained using Harris haematoxylin and counterstained with Eosin (Appendix A). Stained sections were dehydrated and cover slips permanently attached using DPX mounting medium.

Micrographs were taken using a Nikon (DXM 1200F) camera attached to a Nikon E800 compound microscope. Oncomiracidia were removed from the infected Mulerian ducts and kidneys and measured in length and all the photomicrographs were taken using the 40-X objective lens and saved as JPEG files. A stage micrometer was used to calibrate the microscope.

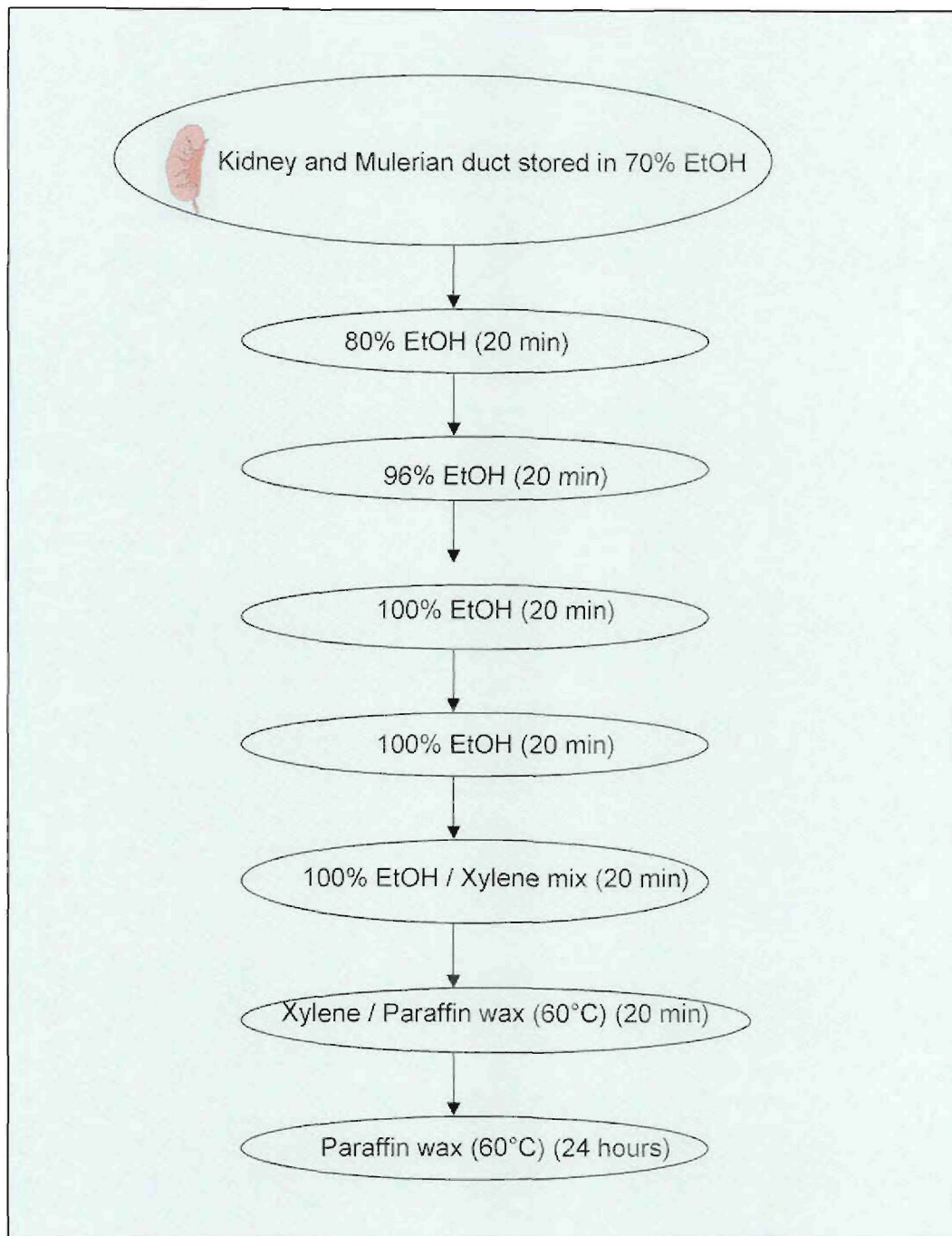


Figure 2.6: Schematic diagram indicating the tissue preparation prior to histological sectioning.

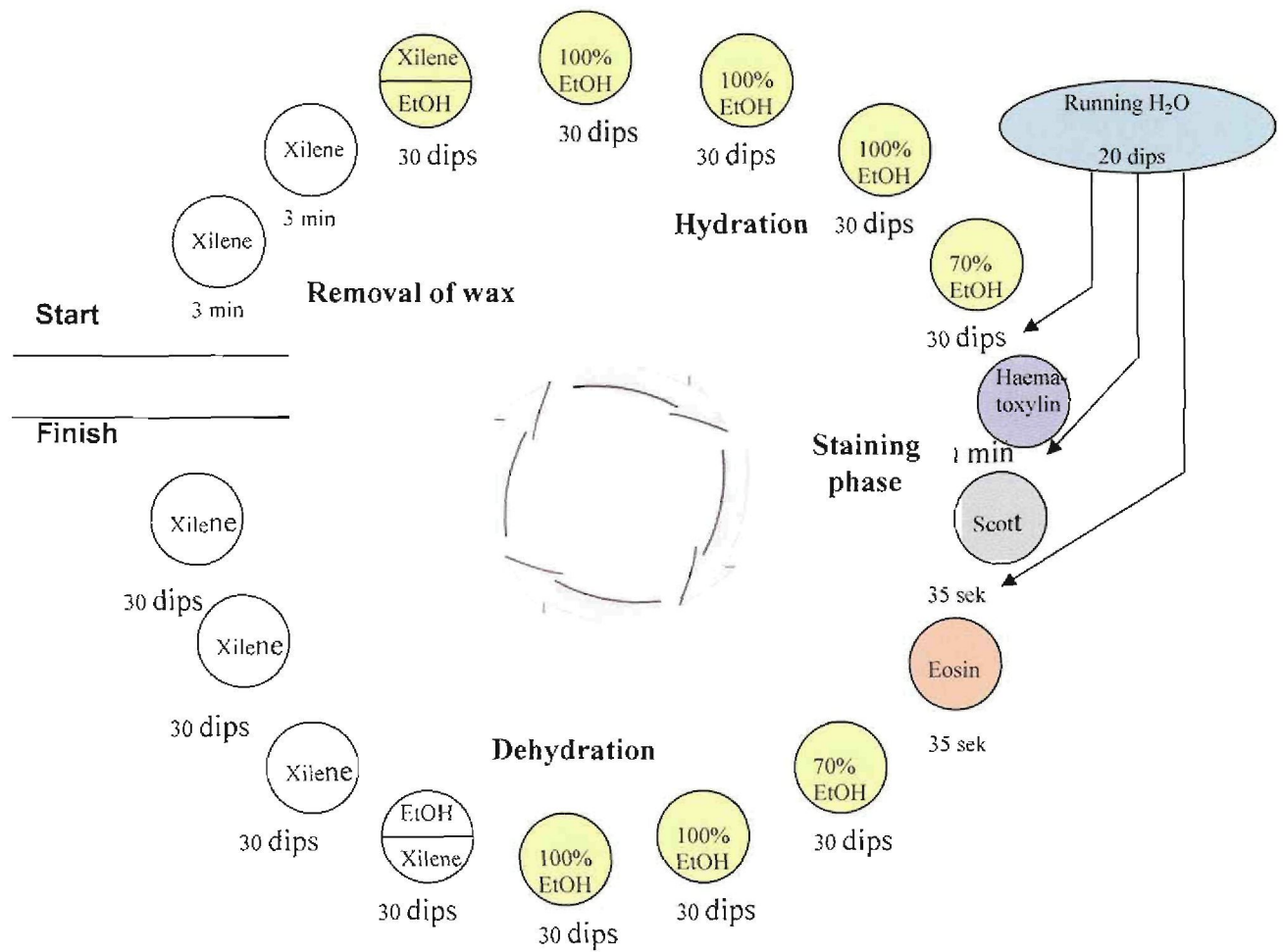


Figure 2.7: Schematic diagram outlining the staining procedures for histological sections.

2.5 DNA extraction and amplification

The DNA was extracted from *Eupolystoma* sp 1 from *Amietophrynus garmani* and *E. vanasi*. Parasites that were stored in 95% ethanol according to the protocol by Bentz *et al.* (2001; 2006), Verneau *et al.* (2002) and Verneau (personal communication)³. Each specimen's DNA was obtained by drying the parasite and adding 90 µl of water and Proteinase K. The mixture was placed under 55°C for one hour and subsequently placed under 100°C for one minute. A Wizard® DNA Clean-Up System was used for the purification of the DNA (Figure 2.8). A Nano-drop spectrophotometer was used to determine the density of the DNA and the impurities. For 18S rDNA, Polymerase chain reaction (PCR) the experiments were performed on standard procedures by Bentz *et al.* (2001) and Sinappah *et al.* (2001) by using 18 F3, 18 RG or the IR 5, 18 RG primer combination. As Dr Verneau already has a bank of polystome DNA representative samples were sent to him for DNA extraction and sequencing in order to verify the identity of parasites.

³ Dr Olivier Verneau, Laboratoire de Biologie Animale, UMR 5555 CNRS-UP, Université Perpignan, 66860 Perpignan Cedex, France

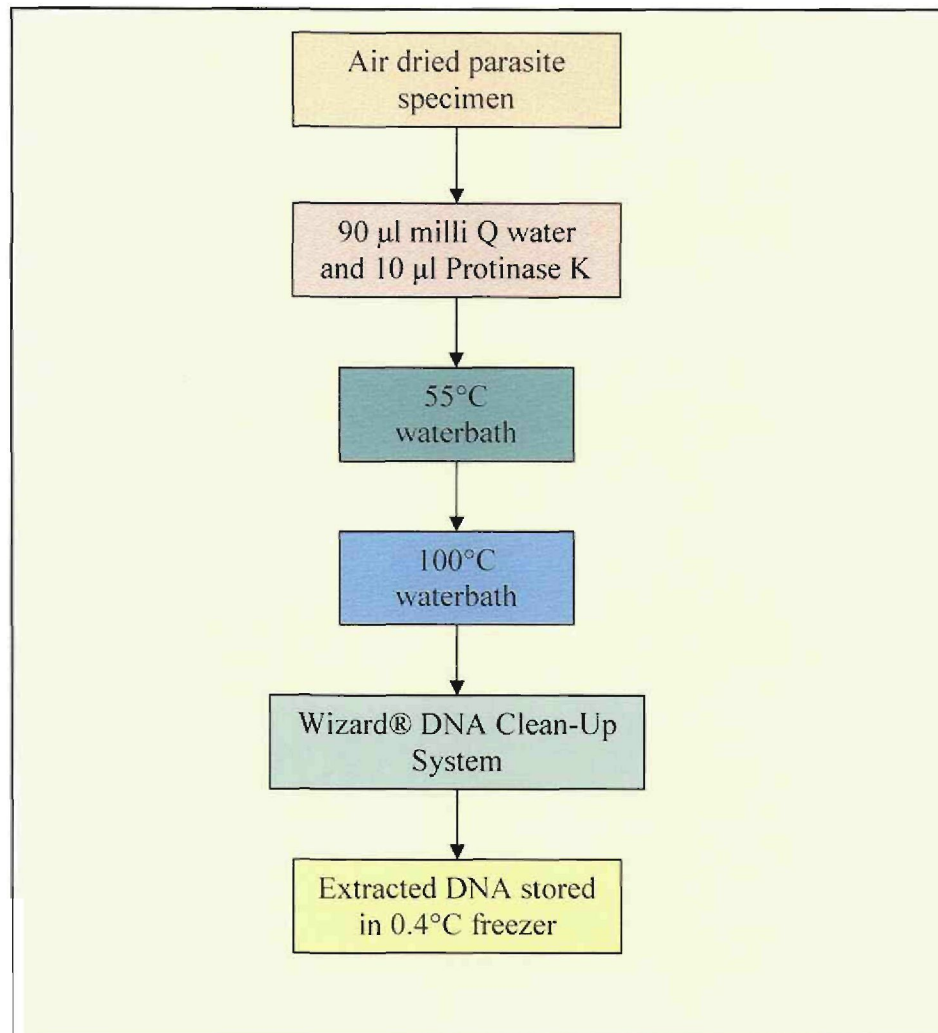


Figure 2.8: Schematic diagram outlining the DNA extraction procedure using the Wizard® DNA Clean-Up system.

2.6 Morphometric body measurements

Different morphometric body measurements that were made of the genus *Eupolystoma* are indicated in Figure 2.9. Sixteen different measurements were made of the parasite. The measurements that were taken with the four times objective are the total body length, greatest body width and haptor length and width. Other measurements including the length and width of the oral sucker, ovary, posterior suckers, eggs and pharynx were taken with the 10 times objective. Depending on the size of the parasite, the 40 or 60 times objective were used for the measurement of the genital bulb width and the genital spine length. The amount of genital spines and eggs *in utero* were also noted.

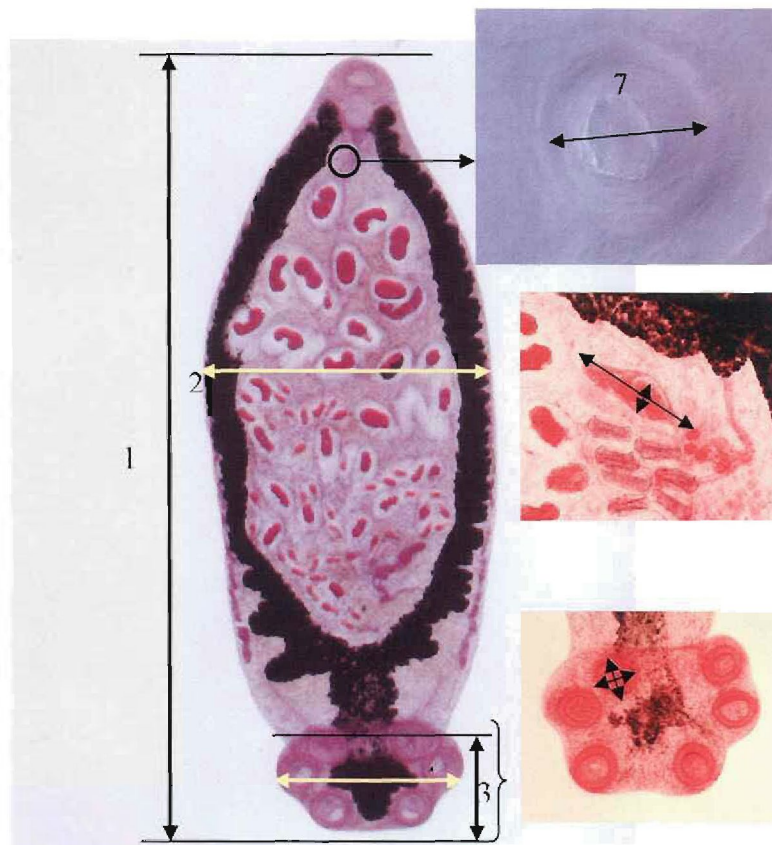


Figure 2.9: Morphometric body measurements that were made of the genus *Eupolystoma*. The measurements of each specimen included the: 1. Body length, 2. Greatest width, 3. Haptor length and 4. width and Oral sucker width, 5. Pharynx length and width, 6. Ovary length and width, 7. Genital bulb width, 8. Number of genital spines, 9. Genital spine length, 10. Sucker 1, 2 and 3 diameter and 10. Number of eggs *in utero*.

2.7 Morphometric marginal hooklet measurements

Measurements of the hooklets were taken based on the protocol for the genus *Polystoma* as developed by Du Preez and Maritz (2005). The compound microscope was connected to a standard personal computer (PC) and the measurements were taken using the EclipseNet® analyser software program. Only hooklet C1 was used (median, posterior most between sucker pair 1) (Figure 2.10). For each hook, 13 different measurements were captured (Figure 2.11). These measurements allowed for the elimination of all the insignificant parameters, thus retaining only the best parameters. By doing this, the highest classification potential was achieved. For practical reasons, Figure 1.2 is copied (Figure 2.11).



Figure 2.10. Micrograph of marginal hooklets for an *Eupolystoma* oncomiracidium with hooklet C1 indicated.

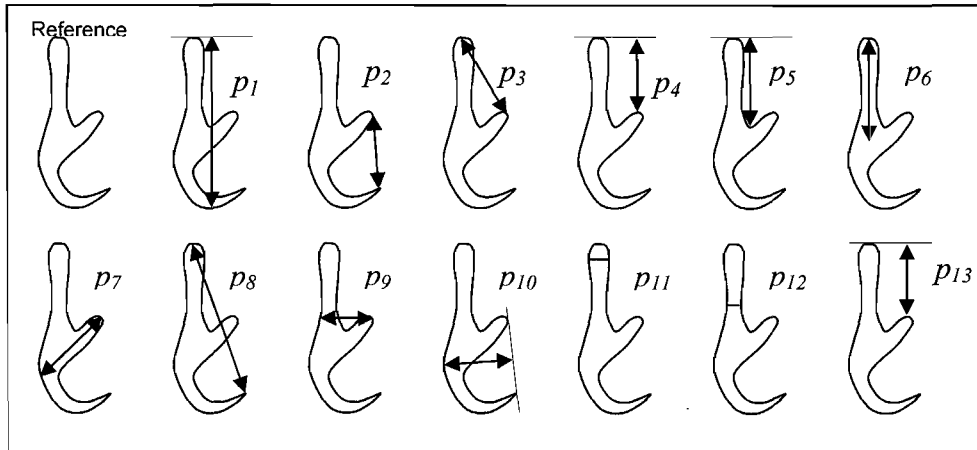


Figure 2.11: Measurements of marginal hooklets of the *Eupolystoma* species used for determining the classification potential of the genus.

Du Preez & Maritz (2005) evaluated marginal hooklet measurements for the genus *Polystoma* and reported that a combination of measurements provided the highest classification potential. The best combination was found to be (P1 x P9) and (P1 x P2).

In order to find a good classification of the species of *Eupolystoma*, a classification potential function was developed with the software Matrix Laboratory®. This function was labelled $P(N_{\text{cor}}, N_{\text{mis}}, N_{\text{mult}}, N_{\text{not}})$. The function was altered in such a manner that it increases when N_{cor} (the number of points correctly classified) increases and decreases when any of N_{mis} (number of points misclassified), N_{mult} (multiple classification) or N_{not} (classification potential) increases. With the function $P \propto N_{\text{cor}} - N_{\text{mis}} - 12N_{\text{mult}}$ it is assumed that the cost of misclassification is the same as the gain in correct classification and the cost of multiple classification is only half of that of the misclassification. The number of points not classified was ignored (Du Preez & Maritz, 2005).

Du Preez & Maritz (2005) set criteria for ideal measurements. Measurements should ideally be:

- Easy measurable

- Objectively measurable
- Repeatable
- Independent from each other
- Representative
- Non-negative (as referred to in Figure 1.1)

Based on these criteria nine parameters were chosen to the criteria of “good parameters” (Figure 2.12).

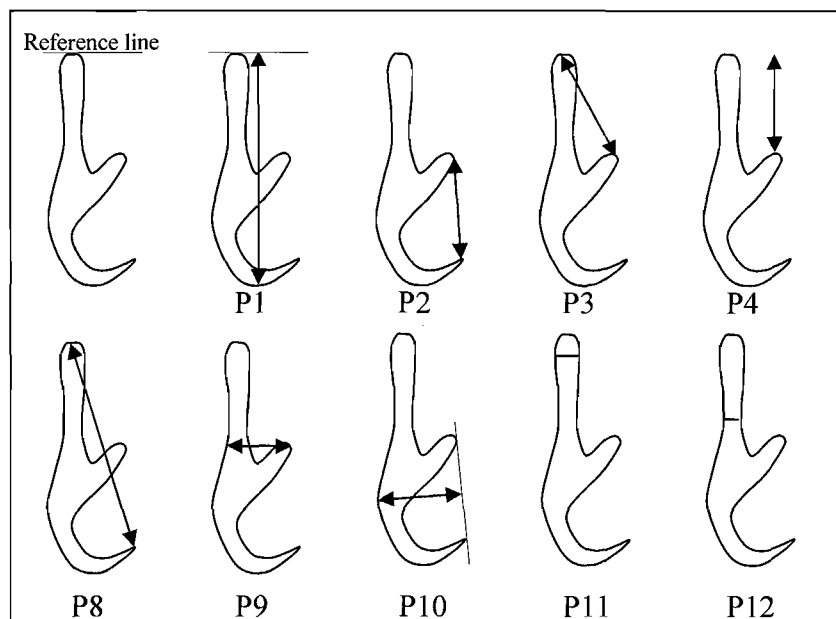


Figure 2.12: The nine parameters chosen for the genus *Eupolystoma*. Parameters P5, P6, P7 and P13 were removed.

2.8 Statistical Analysis

Statistical analyses were performed using the software program Statistica®. Data were tested for normality. The student T-test was performed to determine meaningful differences between the different species of *Eupolystoma*, especially *E. vanasi* and *Eupolystoma* sp 1. This was performed in accordance with the molecular data that was obtained of the two species. Graphs were drawn from the data of all nine parameters of each species using Matrix' Laboratory®. The graphs were adapted from the latter software, by saving them as JPEG images.

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Chapter 3: Results

3.1 Infection levels

3.1.1. *Eupolystoma* sp 1 in *Amietophrynus garmani*

In a pilot study during August 1984, Du Preez (personal communication⁴) collected 84 live *Amietophrynus garmani* from Berg-en-Dal restcamp in the Kruger National Park, South Africa. He noted that the eggs of *Eupolystoma* sp 1 are situated anterior in the uterus were larger with a more advanced level of development than the eggs situated more posteriorly. He eggs described the eggs as thin walled with a thickening on the one side.

A second excursion to Berg-en-Dal restcamp was undertaken on 2005-12-06. Twenty eight adult *A. garmani* were collected. Fifteen frogs died as a result of heat stress and were frozen for later inspection. Five frogs were infected with an unknown *Eupolystoma* sp. (*Eupolystoma* sp 1) that this yielded a prevalence of 17.86%. None of the frozen frogs had any parasites. Where the infection was high, parasites were always located in the bladder as well as the Mulerian ducts, which were enlarged (Figure 3.1).

⁴ Dr L.H du Preez, School of Environmental Sciences and Development, North-West University, Potchefstroom Campus, South Africa

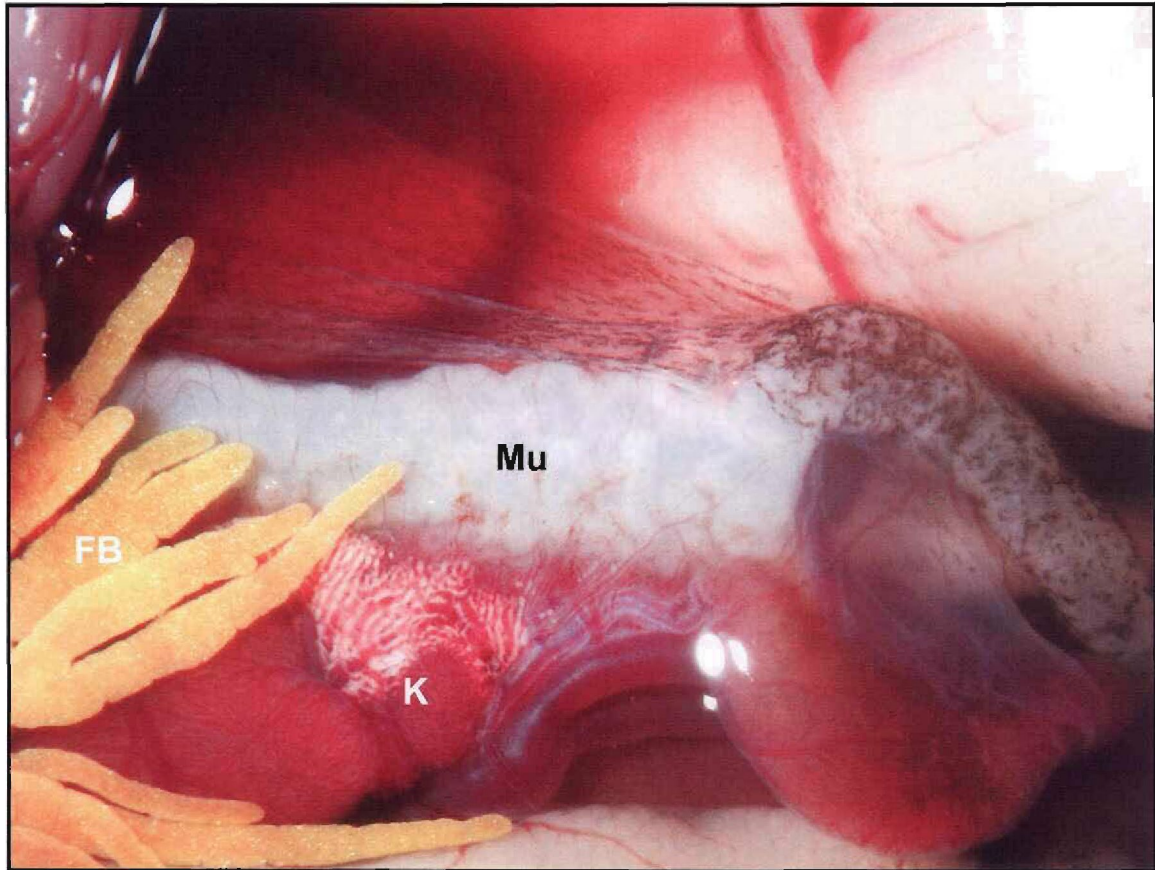


Figure 3.1: The swollen Mulerian ducts of a dissected *Amietophrynus garmani* that is infected with *Eupolystoma* sp 1 (Mu: Mulerian duct, K: Kidney, FB: Fatbodies).

The dissection of 56 frogs after the third fieldwork expedition (2006-10-03) revealed one frog infected with 48 parasites giving a prevalence of 1.78%. Two *A. garmani* specimens died due to heat stress.

A fourth field expedition on 2007-09-18 yielded a drastic decline in the population size of *A. garmani* due to the removal of vegetation that surrounded Berg-and-Dal rest camp's swimming pool. Only three individual frogs were found

during the first spring rain and only two males were collected. Neither of the two were infected.

On average 13 large adult parasites (4204 μm in total length), 20 medium sized parasites and 23 small parasites (2814 μm in total length) were found in infected *Amietophrynus garmani*.

3.1.2. *Eupolystoma anterorchis* in *Amietophrynus pantherinus*

Despite a sample size of 113 *Amietophrynus pantherinus* from in and around the type locality in Cape Town, not a single host was found to be infected.

3.2 Assessment of the morphology of *Eupolystoma* species

For the two new species, *Eupolystoma* sp 1 found in *Amietophrynus garmani* (Figure 3.2 & 3.3) and *Eupolystoma* sp 2 parasitic in *Poyntonophrynus hoeschi* (Figure 3.4 & 3.5), a full detailed species description will be included.

3.2.1 *Eupolystoma* sp 1 from *Amietophrynus garmani*

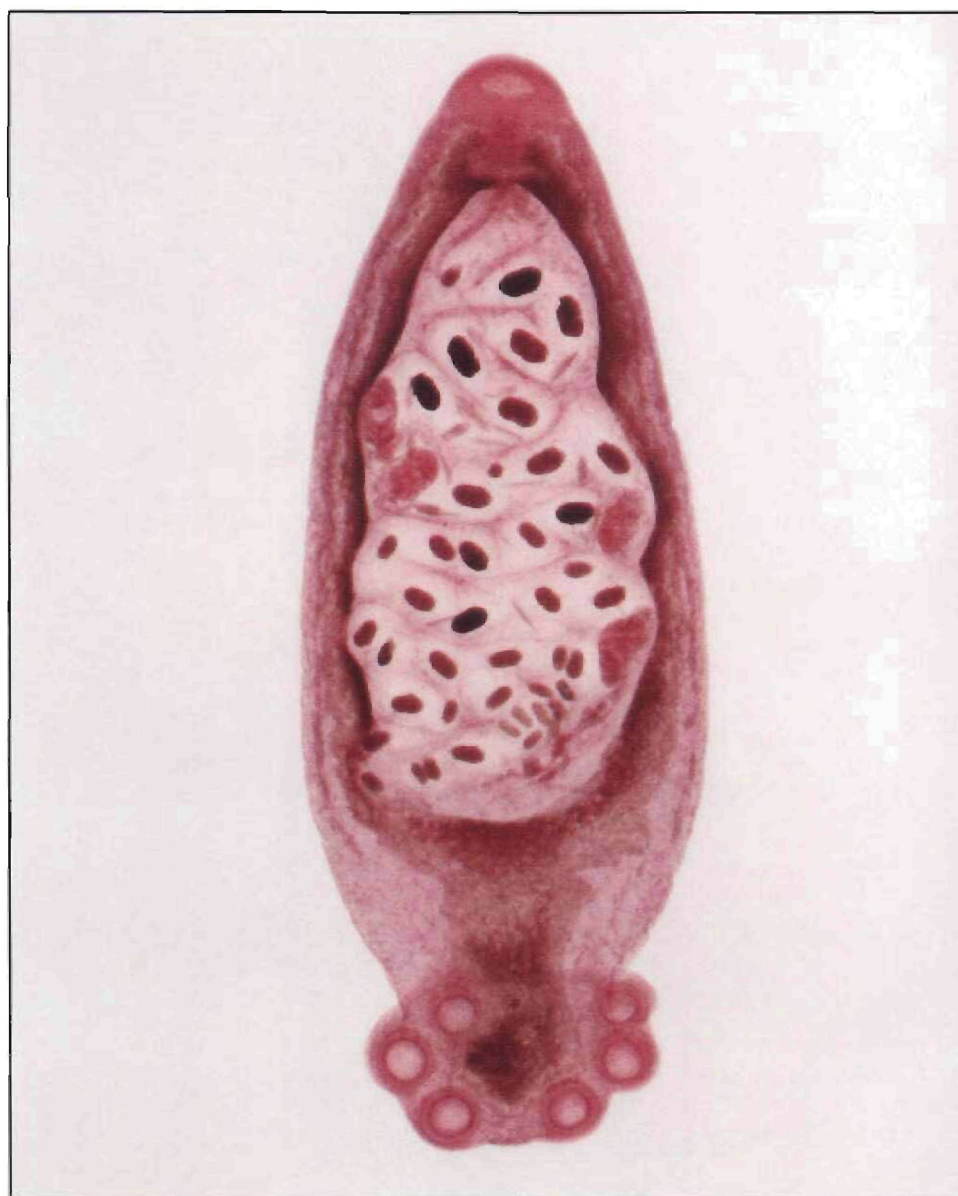


Figure 3.2: A micrograph of *Eupolystoma* sp 1 parasitic on *Amietophrynus garmani*.

In the light that this species represents a new species the description will be presented in more detail.

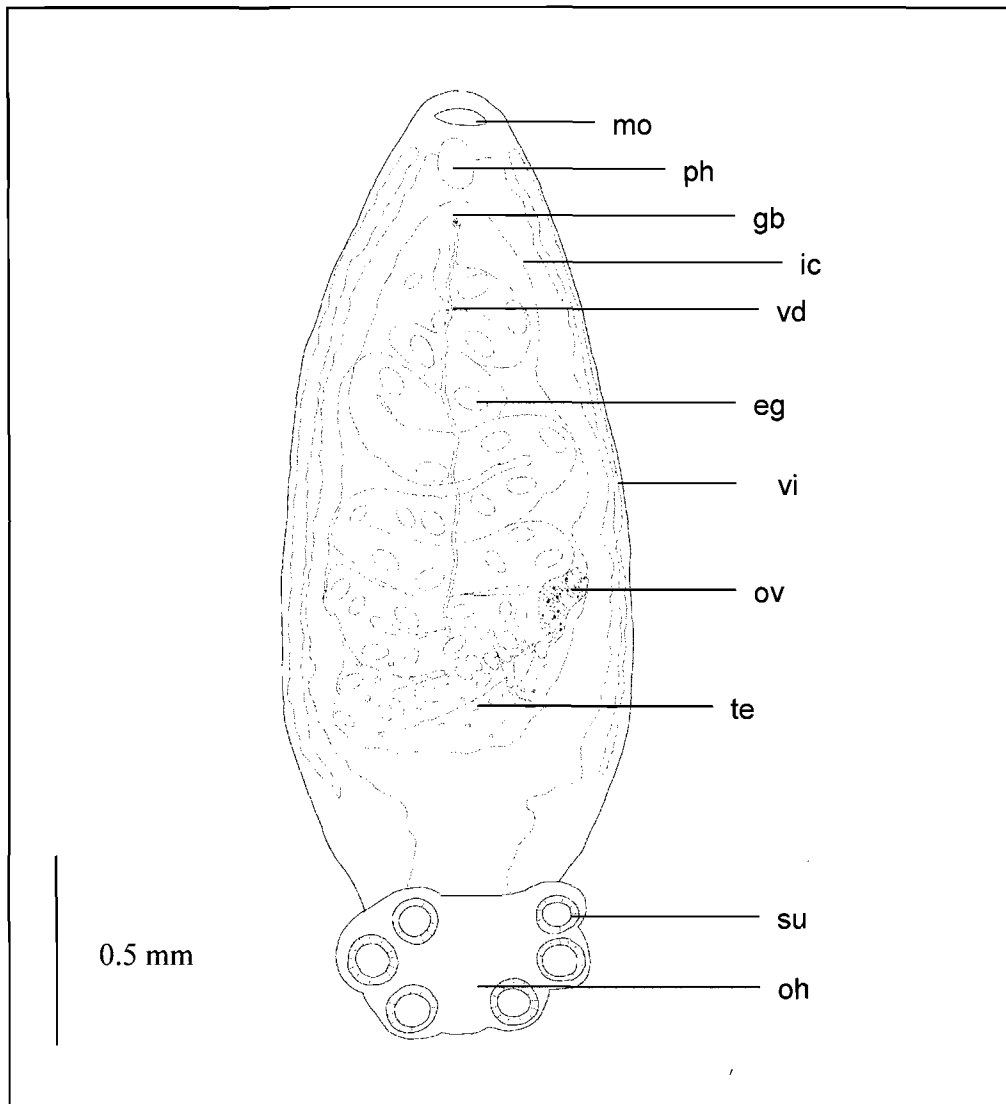


Figure 3.3: *Eupolystoma* sp 1 from *Amietophrynus garmani*. Ventral view of the Holotype. Annotations: eg, egg; gb, genital bulb; ic, intestinal caecum; mo, mouth; oh, opisthaptor; ov, ovary; ph, pharynx; su, sucker; te, testis; vd, vas deferens; vi, vitelline follicles. Scale-bar: 0.5 mm.

Specimens studied

The description is based on 41 sexually mature worms and 42 oncomiracidia. Other specimens included 184 medium sized parasites and 150 small parasites. A holotype (PL 060103) and six paratypes (PL 060103 B12-13) will be deposited in the Parasitic Worm Collection of the National Museum, Bloemfontein, South Africa.

Type host

Amietophrynus garmani (Meek, 1879) sexually mature male (AL 051206 A1) was deposited in the Amphibian Collection, North-West University, Potchefstroom Campus, Department Zoology, Potchefstroom, South Africa.

Type locality

Around swimming pool, concentrated more in the rocky areas at Berg-en-Dal rest camp, Kruger National Park, South Africa (25.29 036 S, 31.29 408 E) (Figure 2.4).

Site

Urinary bladder, kidneys and Mulerian ducts.

Description

Description was based on the reproductive adults' measurements in micrometers where the means of the 41 species were used. Larval marginal hooklets were based on the oncomiracidia that were found in the Mulerian ducts and from eggs that were released from the uterus of the holotype, paratypes and other adult specimens.

Adult parasite

Total length in μm : 3431 (2815 - 4205), greatest width 1356 (1075 - 1677), haptor length 545 (486 - 615), width 879 (103 - 1080); haptor length to body length ratio 15.88%. Six haptorial suckers, mean diameter 185 (159 - 214) and no hamuli are present. Mouth is sub-terminal and ventral. False oral sucker 322

(39 - 375) and pharynx length 167 (142 – 220); pharynx width 143 (113 -170). Intestine bifurcate has small lateral diverticula and is confluent. No median anastomoses are present.

The follicular testis is diffused and dispersed ventrally, medially, and posteriorly in a small area anterior to the haptorial anastomosis. The genital atrium and the seminal vesicle are present and are median ventrally posterior to intestinal bifurcation; the genital bulbus has five spines. The length of the spines could not be measured due to the fixation of the bulbus in an upright position. A small ovary is present on the same side as the genito-intestinal canal, sinistral in posterior 10% of the intercaecal field, length 266 (209 – 323); width 73 (50 - 86). The tubiform uterus is extensive and occupies most of the intercaecal area. Anterior to the genital atrium, the uterus occupies the majority of the intercaecal space lateral and anterior to the ovary. The uterus contains up to 58 eggs, egg capsule length 129 (101 – 161), width 76 (58 - 89). Eggs develop intra uterine with the oldest eggs fully embryonated. Eggs not operculate. Vitellaria confined to two lateral fields.

Oncomiracidium

Ciliated larvae were noted, as well as unciliated larvae. Both the ciliated and unciliated larvae had a narrow cylindrical body and a circular cup-shaped opisthaptor bearing 16 marginal hooklets of equal length 36 (34-37). Hamulus primordia are absent.

Differential diagnosis

Eupolystoma sp 1 differs from the other members of the *Eupolystoma* genus through a combination of characters. It has five genital spines where *E. vanasi* has four, *E. alluaudi* eight to nine spines, *E. anterorchis* six to eight, *E. chauhani* six and *E. rajai* eight. Marginal hooklets are larger than all the other *Eupolystoma* sp 1; this is demonstrated by the combination of hooklet measurements in the African species. The ovary is very narrow and smaller than all other known *Eupolystoma* species. The testis follicles are small, post-ovarian

and do not reach the outer perimeter of the gut (as with the other *Eupolystoma* spp.). The vitelline is confined to two thin fields starting at the lateral side of caecal union, next to the gut and ends lateral, near the pharynx, being more extensive than the vitelline of all the other *Eupolystoma* spp.

Table 3.1: Body measurements of *Eupolystoma* sp 1 parasitic in *Amietophrynus garmani*.

Measurement	Present study			
	Average (µm)	Number	STD	KV
Body length	3431 (2815 - 4205)	16	349.8	10.2
Greatest width	1356 (1075 - 1677)	16	152.3	11.2
Haptor length	545 (486 – 615)	16	39.3	7.2
Haptor width	879 (103 – 1080)	16	219.5	25.0
Oral sucker	322 (39 - 375)	16	81.7	25.4
Pharynx length	167 (142 – 220)	16	18.8	11.3
Pharynx width	143 (113 -170)	16	12.2	8.5
Ovary length	266 (209 – 323)	16	40.4	15.2
Ovary width	73 (50 - 86)	16	11.3	15.4
Gen bulb width	55 (42 – 62)	16	5.0	9.1
N Gen spines	5	8		
Gen spine length	none	none	none	none
Sucker 1A	208 (178 – 231)	15	17.6	8.5
Sucker 2A	191 (170 - 225)	13	17.5	9.2
Sucker 3A	172 (148 - 198)	15	13.3	7.8
Sucker 1B	191 (153 – 218)	15	16.2	8.5
Sucker 2B	180 (162 - 205)	13	15.8	8.8
Sucker 3B	170 (142 – 205)	15	16.7	9.8
N Eggs in utero	44 (29-58)	15	8.2	18.5
Egg length	129 (101 – 161)	16	19.9	15.4
Egg width	76 (58 - 89)	16	11.0	14.5

3.2.2 *Eupolystoma* sp 2 from *Poyntonophrynus hoeschi*

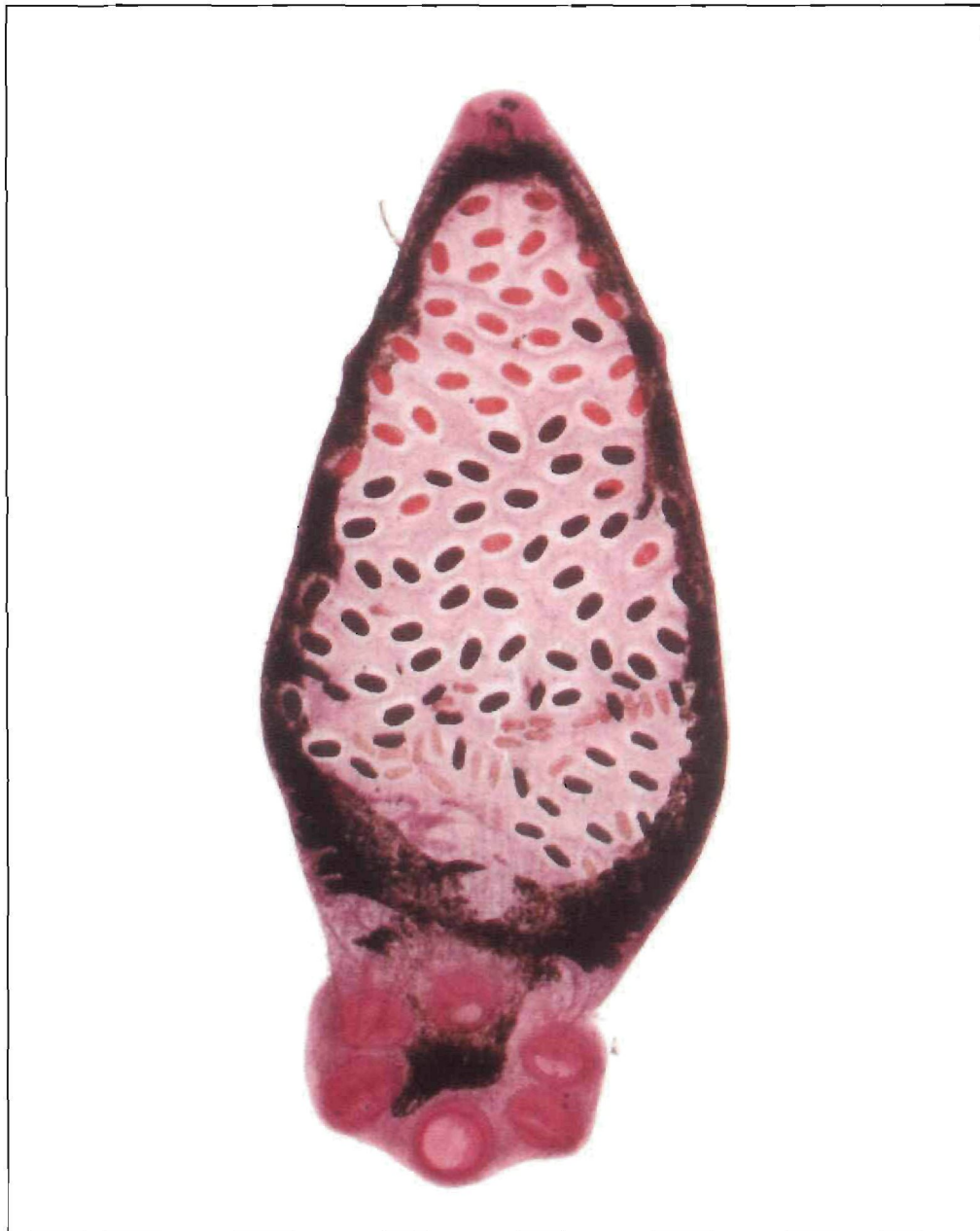


Figure 3.4: A new *Eupolystoma* species, *Eupolystoma* sp 2 that is parasitic in *Poyntonophrynus hoeschi*.

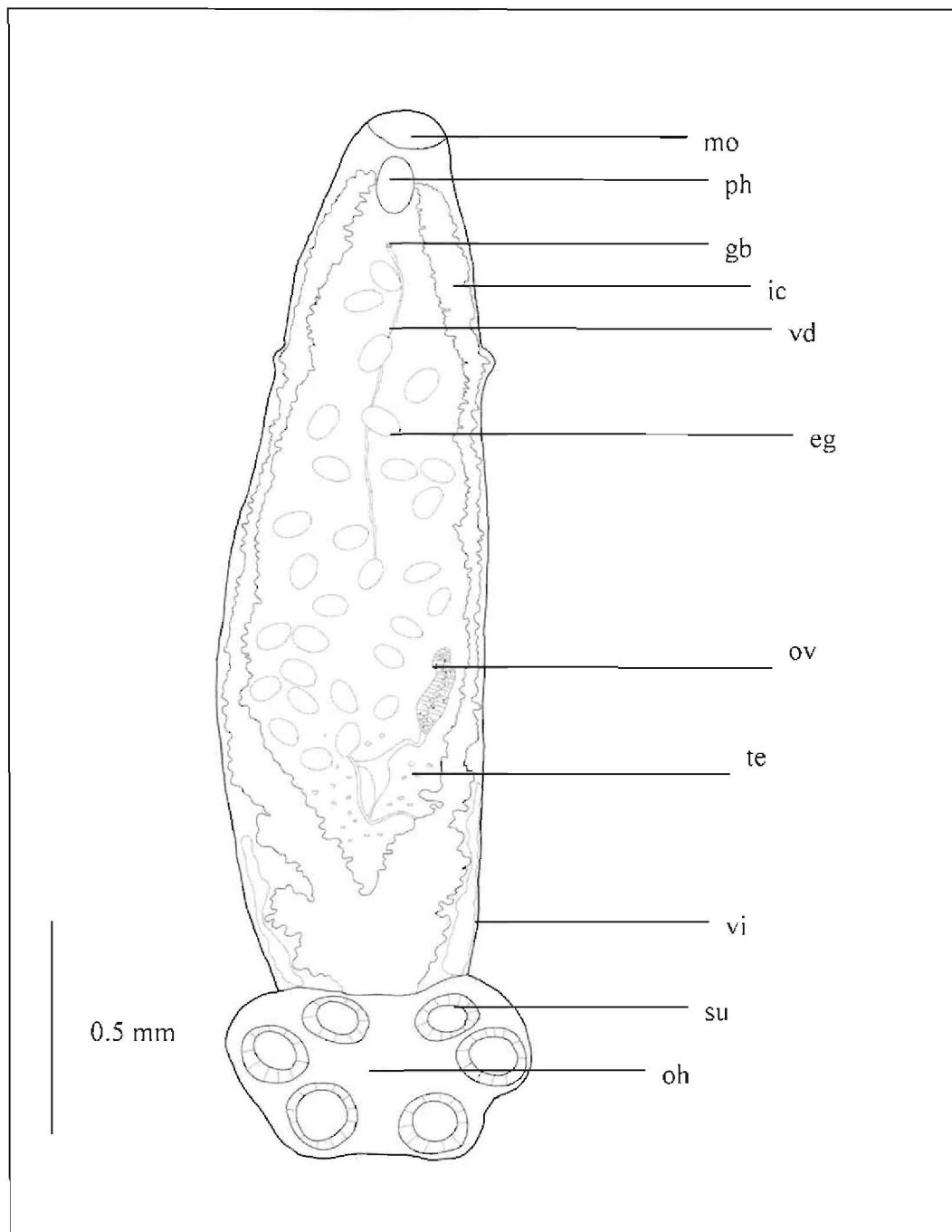


Figure 3.5: *Eupolystoma* sp 2 from *Poyntonophrynus hoeschi*. Ventral view of the Holotype. *Annotations:* eg, egg; gb, genital bulb; ic, intestinal caecum; mo, mouth; oh, opisthaptor; ov, ovary; ph, pharynx; su, sucker; te, testis; vd, vas deferens; vi, vitelline follicles. *Scale-bar:* 0.5 mm.

Specimens studied

Description of the species is based on two sexually mature trematodes and three oncomiracidia. Other species specimens included four medium sized parasites 3844 (3528-4208) and 22 small parasites 839 (721-971). Holotype (PL940119A1) and paratype (PL940119A2) will be deposited in the Parasitic Worm Collection of the National Museum, Bloemfontein, South Africa.

Type host

Specimens were collected from two *Poyntonophrynus hoeschi* (NMB A5832 and NMB A5833), sexually mature males that were deposited in the Amphibian Collection, North-West University, Potchefstroom Campus, Department Zoology, Potchefstroom, South Africa.

Site

Present in the urinary bladder.

Description

Description of the species was based on the reproductive adults' measurements (μm) where the averages of 36 specimens were used. Larval hooklet measurements of the oncomiracidia found in the urinary bladder and from eggs *in utero* of the holotype, paratypes and other specimens were used for description.

Adult parasite

Total length in μm : 2170 (586-6523), greatest width 613 (126-2496), haptor length 500 (156-1267), width 666 (235-1573); haptor length to body length ratio 21.48%. Six haptorial suckers, mean diameter 201 (99-373) and no hamuli are present. Mouth is situated sub-terminal and ventral. False oral sucker 204 (90-401) and pharynx length 126 (50-228); pharynx width 100 (53-214). The intestine is bifurcate and does not indicate any lateral diverticula. No branches

have been observed either. The caeca is situated posterior in the body of the parasite but anterior to haptoral anastomosis.

The testis is follicular, diffused and restricted in a small area anterior to the haptorial anastomosis. The seminal vesicle and genital atrium are situated median, ventrally posterior to intestinal bifurcation. On the same side as the genito-intestinal canal, the ovary are present. In the posterior of the body, the uterus occupies most of the intercaecal space lateral and anterior to the ovary. The uterus is filled with masses of eggs where some of the eggs are not fully embryonated. The eggs do not possess the usual yellow coloration (like the *Polystoma* genus); instead they are a light yellow and are transparent. If the eggs hatch, the casing collapses. The eggs also collapse as a result of the fixating procedures. The vitellaria are formed by two lateral follicles in the posterior half of the body (Figure 3.4 & 3.5).

Oncomiracidium

Ciliated larvae that had a narrow cylindrical body and circular cup-shaped opisthaptor bearing 16 marginal hooklets of equal length 32 (31-33). Hamulus primordia as well as hamuli are absent.

Differential diagnosis

Eupolystoma sp 2 differs from other members of the *Eupolystoma* genus by various characteristics. Of these characteristics, *Eupolystoma* sp 2 has four to seven genital spines where *Eupolystoma* sp 1 has five, *E. vanasi* has four, *E. alluaudi* eight to nine, *E. anterorchis* six to eight, *E. chauhani* six and *E. rajai* eight. The testes are also the most posterior and halve compressed to the posterior side of the parasite however it also does not reach the outer perimeter of the gut. The ovary is also present on the same side as the genito-intestinal canal and the vitellaria consist of two lateral follicles present in the posterior halve of the body.

Table 3.2: Body measurements of *Eupolystoma* sp 2. parasitic in *Poyntonophrynus hoeschi*.

Measurements	Present study			
	Average (µm)	Number	STD	KV
Body length	2170 (586 - 6523)	23	1607.1	74.1
Greatest width	613 (126 - 2496)	24	582.3	95.0
Haptor length	500 (156 – 1267)	20	286.5	57.3
Haptor width	666 (235 – 1573)	20	349.9	52.5
Oral sucker	204 (90 - 401)	23	91.2	44.7
Pharynx length	126 (50 – 228)	12	46.8	37.1
Pharynx width	100 (53 - 214)	12	42.5	42.5
Ovary length	244 (21 – 278)	3	38.6	15.8
Ovary width	95 (66 - 126)	3	30.4	32.0
Gen bulb width	59 (40 - 81)	3	20.7	35.0
N Gen spines	5 (4-7)	5	1.5	30.4
Gen spine length	36 (31 – 41)	5	3.6	10.1
Sucker 1A	199 (100 – 335)	21	77.4	38.9
Sucker 2A	190 (70 - 393)	18	89.4	47.1
Sucker 3A	220 (126 – 415)	7	106.4	48.2
Sucker 1B	198 (96 – 328)	20	80.4	40.6
Sucker 2B	186 (78 – 392)	18	87.3	47.0
Sucker 3B	212 (121 – 436)	7	111.2	52.5
N Eggs in utero	65 (3-118)	3	58.0	89.2
Egg length	156 (128 -183)	6	24.6	15.8
Egg width	96 (70-113)	6	16.9	17.7

3.2.3 *Eupolystoma vanasi* parasitic in *Schismaderma carens*



Figure 3.6: Micrograph of *Eupolystoma vanasi* from *Schismaderma carens*.

General structure typical for the genus *Eupolystoma* is as follows: haptor small, hamuli absent and placement of marginal hooklets as for polystomes in general. The intestine consists of two gut caeca with a haptoral anastomosis. The caeca with small diverticula, but no median anastomoses. The vitellaria are confined in the posterior half of the body and in two lateral fields. There is a genito-intestinal canal present and it joins the intestinal caecum posterior to the ovary. The ovary is small, sinistral and posterior in a position approximately 36% from the anterior end of the parasite. The testes are follicular and posteriormost in the body; in a position just anterior to the haptoral anastomosis. A prominent vas deferens connects the testis to the genital bulbus. A genital bulbus with four genital spines is present.

It was also found by du Preez *et al.* (2002) that the vitellaria forms two discrete lateral fields of follicles that is lateral to the intestine, in the posterior half of the body. The genito-intestinal canal did prove to be prominent (as discussed by du Preez *et al.* 2002) and on the same side as the ovary. The genito-intestinal canal also joined the intestinal caecum posterior to the ovary. Extending into the hind body, the uterus binds into the anterior side of the genital atrium, occupying most of the intercaecal space lateral and anterior to the ovary.

The testis consists of several follicles, which are restricted to a small area anterior to the haptoral anastomosis. The testis also diffuses ventrally, medially and posteriorly. A seminal vesicle is present. The genital atrium is present median, ventral and posterior to intestinal bifurcation. A genital bulbus is found anterior just below the oral sucker consisting of four spines. The ovary is situated on the left side in the posterior half of the intercaecal field (Figure 3.6). Average measurements are indicated in Table 3.3.

Table 3.3: Measurements for *Eupolystoma vanasi* from *Schismaderma carens*.

	Du Preez <i>et al.</i> (2002)	Present study		
Measurement	Average (µm)	Number	Standard deviation	Coefficient of variance (KV)
Body Length	5978 (3755 - 7733)	39	967.4	16.2
Body width	2356 (1505 - 3275)	37	434	18.4
Haptor length	836 (564 - 1128)	38	149	18
Haptor width	1448 (1077 - 1924)	38	236	16.3
Oral sucker	222 (160-290)	13	40	18.0
Pharynx length	206 (165 - 270)	38	22	10.5
Pharynx width	166 (131 - 230)	38	23	14.0
Ovary length	250 (185 - 470)	32	69	27.7
Ovary width	121 (100 - 150)	8	16	13.3
n Genital spines	4 (4-4)	8	none	none
Gen. spine length	32 (29 - 33)	8	1	3.6
Sucker Diameter	227 (160 - 285)	97	22	9.7
Eggs in utero	55 (38 - 81)	29	10	18.0
Egg length	129 (136 -150)	none	none	none
Egg width	83 (79 - 89)	none	none	none

3.2.4 *Eupolystoma anterorchis* from *Amietophrynus pantherinus*

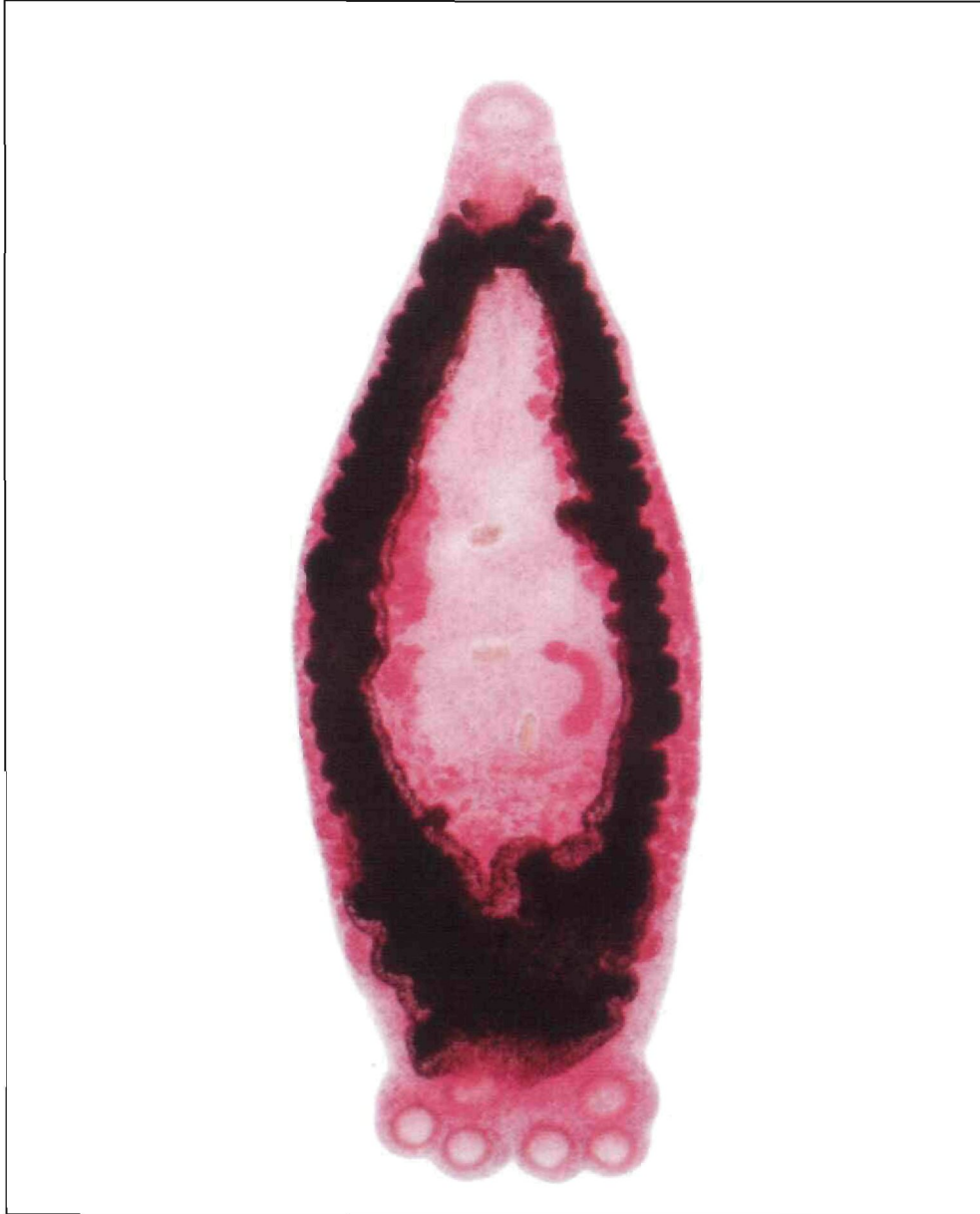


Figure 3.7: A microphotograph image of *Eupolystoma anterorchis* indicating the different morphological features.

In comparison to other *Eupolystoma* species, *E. anterorchis* morphologically differ in the formation of the testis, intestine, and vitellaria. The intestinal pattern is an established characteristic of the genus *Eupolystoma* due to the fact that it is more or less ring-shaped with an unbranched caeca (Tinsley, 1978).

Tinsley (1978) observed 20 sample specimens where a total average length of 4.32 mm was recorded and the greatest width averaged at 1.72 mm. Six haptorial suckers with a mean diameter of 0.26 mm that were arranged around the outer margin of the haptor and 16 marginal hooklets with no hamuli were also noted (Tinsley, 1978). It was also stated that all 16 hooklets were in similar size and shape. This was later proven to be incorrect.

The specific description also included the oral sucker diameter average (0.44 mm) and pharynx diameter (0.21 mm). A bucco-intestinal canal is present where the intestine bifurcates directly posterior to the pharynx of the species. The lateral caeca joins together in the posterior body and forms a large, single diverticulum that extends into the opisthaptor. In most cases, the form of the intestinal caeca varies. Some of these examples are without lobes or branches where others indicate prominent diverticula of variable lengths; usually they are short, but in exceptional cases it forms transverse intercaecal anastomosis in the post-ovarian region (Tinsley, 1978).

Distributed from the posterior caecal union, medially leading towards the ovary and laterally along and around the lower the ventro-lateral surfaces of the intestinal caeca, the testes are visible. It has also been found that the testes are elongated and distributed laterally into at least a third of the body and are often projected towards vaginal level; this is entirely unique to *Eupolystoma anterorchis*. The vas deferens can also be observed originating in the post-ovarian area where it passes forward dorsally in the lateral line, opening at the ventral genital atrium or genital bulbus with eight hooklets (0.024 ± 0.001). The follicular vitellaria forms two masses that is lateral and concentrated more

posterior however it can extend along the intestine to the pharyngeal level. The uterus occupies the entire intercaecal field lateral where eggs can be observed and is usually situated anterior to the ovary. A vagina is also present. *Eupolystoma anterorchis* is parasitic in the urinary bladder, kidneys and Mulerian ducts of Anura (Tinsley, 1978) (Figure 3.7). Average measurements of the previous study as well as the contemporary study are included in Table 3.4.

Table 3.4: Morphometric body measurements of *Eupolystoma anterorchis* from *Amietophrynus pantherinus*.

	Tinsley (1978)	Present study			
Measurements	Average (µm)	Average (µm)	Number	STD	KV
Body length	4320 (3390-4760)	4483 (4001 – 5053)	7	407.2	9.1
Greatest width	1720 (1450-1860)	1692 (1628 - 1776)	6	58.8	3.5
Haptor length	790 (550-830)	759 (597 – 886)	4	128.0	16.9
Haptor width	1310 (1070-1330)	1258 (1199 – 1376)	6	66.4	5.3
Oral sucker	440 (360-480)	390 (346 - 420)	6	27.2	7.0
Pharynx length	230 (200-240)	237(220 - 255)	6	11.5	4.8
Pharynx width	210 (170-210)	194 (173 – 207)	6	12.7	6.6
Ovary length	470 (360-560)	612 (526 – 713)	5	77.5	12.7
Ovary width	120 (90-160)	130 (114 – 155)	6	15.0	11.5
Gen bulb width	24	none	1	none	none
N Gen spines	6 - 8	none	3	none	none
Gen spine length	none	none	1	none	none
1A	All suckers: 260 (220-270)	263 (237 – 274)	5	14.1	5.4
1B	none	237 (206 – 288)		28.5	12.0
2A	none	242 (214 – 267)	5	20.7	8.6
2B	none	237(209 – 257)		15.6	6.6
3A	none	218 (194 – 233)	5	15.7	7.2
3B	none	211 (159 – 238)		27.4	13.0
N eggs	none	1 (0 - 3)	6	1.4	136
Egg length	none	none	none	none	none
Egg width	none	none	none	none	none

3.2.5 *Eupolystoma alluaudi* from *Amietophrynus maculatus*



Figure 3.8: A microphotograph indicating *Eupolystoma alluaudi* from *Amietophrynus maculatus*.

The characteristics of *Eupolystoma alluaudi* (as described by de Beauchamp, 1913) includes the minimalization of the adult parasite body in the anterior part, a haptor without hamuli, marginal digestive caecums uniting by large haptorial anastomose excluding any transverse anastomose, a posterior located ovary and the major expansion of the uterus with a high number of eggs (de Beauchamp, 1913). This accounts for all the *E. alluaudi* from their respective hosts (Figures 3.8 – 3.10). Average measurements for the different morphological species of *E. alluaudi* are included in Tables 3.5 – 3.7.

Table 3.5: Morphometric body measurements of *Eupolystoma alluaudi* from *Amietophrynus maculatus*.

	Beverley-Burton (1962), Tinsley, (1978); (1975)	Present study			
Measurements	Average (µm)	Average (µm)	Number	STD	KV
Body length	(2700-3850)	2023 (1638 – 2616)	49	330.6	16.3
Greatest width	(1030-1580)	1114 (897 - 1293)	51	132.5	11.9
Haptor length	(560-650)	428 (356 – 575)	16	80.0	18.7
Haptor width	(720-920)	662 (558 – 917)	42	138.1	20.9
Oral sucker	none	173 (148 – 207)	50	18.9	10.9
Pharynx length	none	109 (76 – 139)	35	19.8	18.2
Pharynx width	none	86 (64 – 100)	45	16.7	19.4
Ovary length	none	295 (256 – 325)	23	31.5	10.7
Ovary width	none	62 (48 - 78)	29	12.4	20.1
Gen bulb width	none	none	25	none	none
N Gen spines	8 - 9	none	23	none	none
Gen spine	none	none	1	none	none
1A	none	189 (147 – 252)	48	35.6	18.9
1B	none	164 (141 – 194)	7	19.8	12.1
2A	none	171 (137 – 245)	36	37.7	22.1
2B	none	174 (137 – 221)		30.6	17.6
3A	none	none	18	none	none
3B	none	none	none	none	none
N eggs	none	49 (35-73)	50	11.5	23.5
Egg length	none	none	none	none	none
Egg width	none	none	none	none	none

3.2.6 *Eupolystoma alluaudi* from *Amietophrynus regularis*



Figure 3.9: A microphotograph of *Eupolystoma alluaudi* from *Amietophrynus regularis*.

Table 3.6: Morphometric body measurements of *Eupolystoma alluaudi* from *Amietophrynus regularis* as revised by Beverley-Burton (1962), Tinsley (1978a) and Tinsley (1975).

	Beverley-Burton (1962), Tinsley, (1978); (1975)	Present study			
Measurements	Average (µm)	Average (µm)	Number	STD	KV
Body length	(2700-3850)	2693 (2367 – 3368)	7	362.8	13.4
Greatest width	(1030-1580)	1196 (190 - 1693)	8	513.5	42.9
Haptor length	(560-650)	611 (417 – 978)	7	177.7	29.1
Haptor width	(720-920)	872 (673 -1161)	9	152.4	17.5
Oral sucker	none	209 (128 – 244)	5	42.3	20.2
Pharynx length	none	132 (95 - 173)	4	28.4	21.5
Pharynx width	none	104 (76 – 128)	6	17.5	16.9
Ovary length	none	294 (263 – 313)	3	27.0	9.2
Ovary width	none	82 (78 - 85)	3	3.6	4.4
Gen bulb width	none	41 (36 – 44)	7	2.9	7.0
N Gen spines	8 - 9	7 (5-8)	3	1.5	21.9
Gen spine length	none	24 (24 – 24)	1	none	4.2
Sucker 1A	none	203 (176 – 239)	9	20.9	10.3
Sucker 2A	none	184 (152 – 224)	9	29.3	15.9
Sucker 3A	none	167 (132 – 187)	4	25.5	15.3
Sucker 1B	none	193 (175 - 213)	9	13.3	6.9
Sucker 2B	none	180 (155 - 231)	9	22.1	12.3
Sucker 3B	none	148 (117 – 166)	4	22.0	14.9
N Eggs <i>in utero</i>	none	93 (50 - 124)	10	29.7	31.9
Egg length	none	118 (95 - 141)	8	44.3	37.6
Egg width	none	56 (41 - 71)	8	21.4	38.3

3.2.7 *Eupolystoma alluaudi* from *Nectophrynoides malcomi*

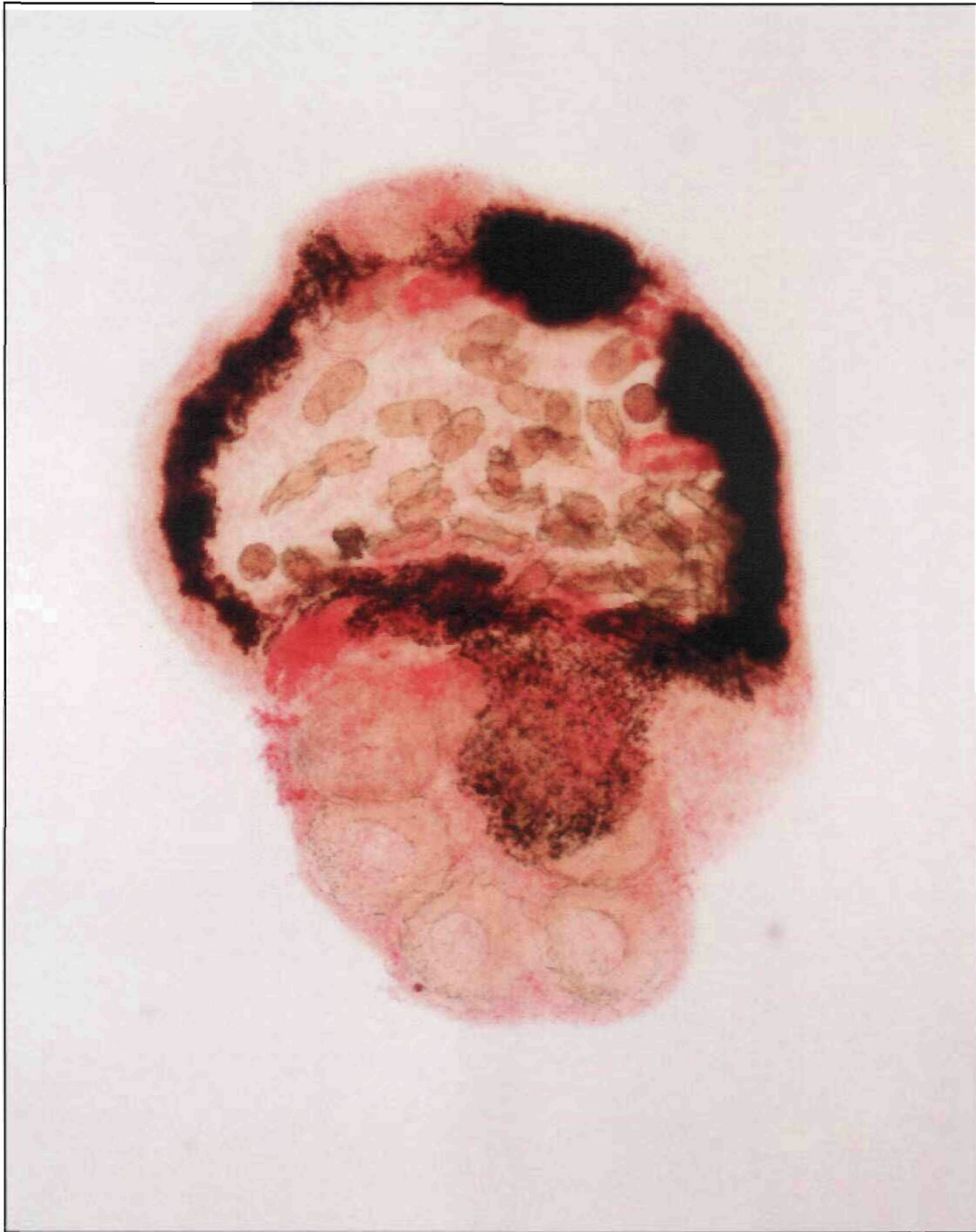


Figure 3.10: *E. alluaudi* from *Nectophrynoides malcomi* indicating the different morphological features (Specimen distorted due to the fixation process).

Table 3.7: Body measurements of *Eupolystoma alluaudi* from *Nectophrynoides malcomi*.

	Beverley-Burton (1962), Tinsley, (1978); (1975)	Present study			
Measurements	Average (µm)	Average (µm)	Number	STD	KV
Body length	(2700-3850)	670	none	none	none
Greatest width	(1030-1580)	520	none	none	none
Haptor length	(560-650)	230	none	none	none
Haptor width	(720-920)	360	none	none	none
Oral sucker	none	none	none	none	none
Pharynx length	none	none	none	none	none
Pharynx width	none	none	none	none	none
Ovary length	none	none	none	none	none
Ovary width	none	none	none	none	none
Gen bulb width	none	none	none	none	none
N Gen spines	8 – 9	none	none	none	none
Gen spine length	none	none	none	none	none
Sucker 1A	none	255	none	none	none
Sucker 2A	none	280	none	none	none
Sucker 3A	none	280	none	none	none
Sucker 1B	none	none	none	none	none
Sucker 2B	none	none	none	none	none
Sucker 3B	none	none	none	none	none
N Eggs in utero	none	33	none	none	none
Egg length	none	none	none	none	none
Egg width	none	none	none	none	none

3.2.8 *Eupolystoma rajai* from an unknown *Rana* species

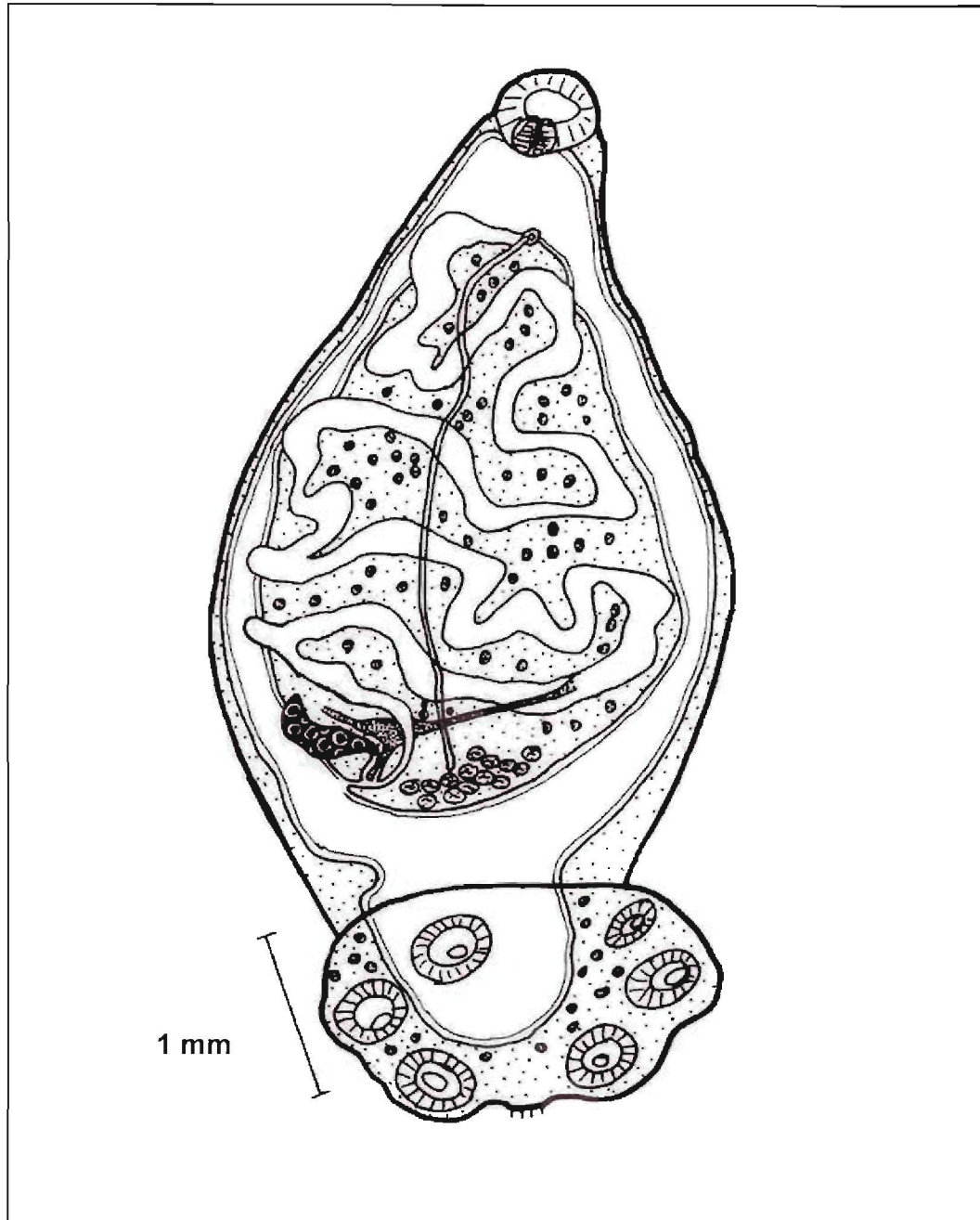


Figure 3.11: A sketch of *Eupolystoma rajai* an unknown *Rana* species indicating the different morphological features (Inserted from Prudhoe & Bray, 1982).

The adult parasites had the generic characteristics of an oval body and a haptor. The haptor that has been referred to as being anterior, was sucker-like and no eyes were observed where the posterior haptor was disc-like and bared three pairs of suckers like. The larger haptorial hooks were absent with the adult parasite, but eight pairs of hooklets were observed on the ventral surface of the posterior haptor, six were situated between the two anterior haptorial suckers, four between the two posterior haptorial suckers and one at the base of each haptorial sucker. The sub-terminal mouth projects onto the anterior haptor and leads to a funnel type of opening to the pharynx. The pharynx that was observed, was very well developed and globular. An almost absent oesophagus and the intestine branches just behind the pharynx. Intestinal caeca elongates laterally throughout the body and merge in front of the posterior haptorial suckers. No prehaptorial anastomosis was noticed from the caeca (Kaw, 1950).

The post-equatorial gonads are found in the inter-intestinal area in front of the fused intestine. Numerous follicular testes are median and post-ovarian and the vas deferens is slender and long dilating into a wider portion at the distal end representing the vesicula seminalis. It was also observed that the vesicula seminalis connects with a narrow tube to the genital atrium. The cirrus has eight hooks (Kaw, 1950).

The anterior part of the sinistral, elongated ovaries is partially dorsally overlapped by the right intestinal caecum. The oviduct is dorsal to the vitelline receptacle. A small duct, the genito-intestinal canal arises on the ovarian side of the right intestinal caecum and opens into the oviduct just behind the vitelline receptacle. These vitelline follicles are scattered throughout the body from the intestinal branching up to the posterior haptor. Transverse vitelline ducts merge to form a receptacle that is sub-median situated between the ovary and the testes. The vaginae and ducts communicate through these transverse ducts. Thin oval eggs in the uterus are developed fully and intercaecal extending in the body from the testes up to the intestinal bifurcation. The genital pore is median

and located ventrally to the intestinal bifurcation (Kaw, 1950) (Figure 3.11). Measurements made by Kaw (1950) are included in Table 3.8.

Table 3.8: Body measurements of *E. rajai* as measured by Kaw (1950).

	Kaw (1950)	Present study			
Measurements	Average (µm)	Average (µm)	Number	STD	KV
Body Length	2810 (2400-3220)	none	none	none	none
Body width	1590 (890-1400)	none	none	none	none
Haptor length	620 (580-660)	none	none	none	none
Haptor width	1090 (940-1240)	none	none	none	none
Oral sucker	315 (289-350)	none	none	none	none
Pharynx length	145 (120-170)	none	none	none	none
Pharynx width	150	none	none	none	none
Ovary length	280	none	none	none	none
Ovary width	80	none	none	none	none
Gen width	49 (44-54)	none	none	none	none
N Genital spines	8	none	none	none	none
Gen. spine length	27	none	none	none	none
Sucker Diameter	Anterior: 160(150-170) x 210(200-220) Middle: 190(180-200) x 225(220-230) Posterior: 205(200-210) x 250	none	None	none	none
Eggs in utero		none	none	none	none
Egg length	163.5 (157-170)	none	none	none	none
Egg width	90 (81-99)	none	none	none	none

3.2.9 *Eupolystoma chauhani* from an unknown *Bufo* species

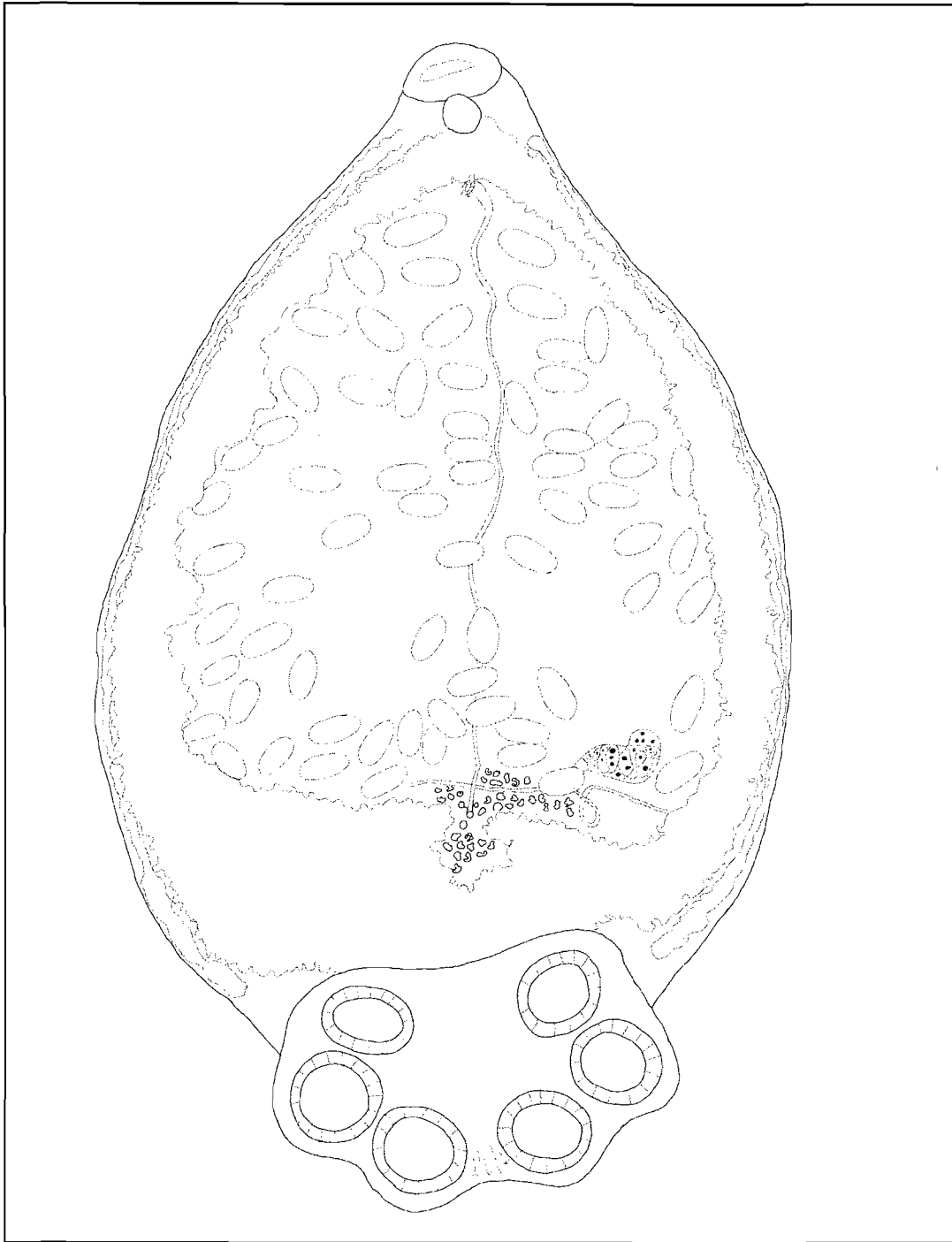


Figure 3.12: A drawing of *Eupolystoma chauhani* from an unknown *Bufo* species indicating the different morphological features.

According to Pandey (1969) *Eupolystoma chauhani* was described as a spindle-shaped parasite with pointed ends. The anterior end was more sharply pointed than the posterior end (because of the oral sucker at the anterior end). The haptor is shaped disc-like and has a set of three paired suckers that are cup shaped and more or less circular and equal in size. On the haptor, six larval, T-shaped hooks with a long blade are found between the last pair of suckers.

A mouth leads into the well-developed muscular pharynx and a prepharynx and an oesophagus are absent. The intestinal caeca lacks a diverticulum and a caecal union, located at the anterior border of the opisthaptor, has also been observed. A branch in the middle of the posterior anastomosis extends into the opisthaptor posteriorly where it moves upwards toward the last pair of suckers (Pandey, 1969).

The testes are located posterior to the ovary and are follicular. The numerous follicles can be noted in front of the caecal union and are restricted to the sinistral half of the body. The vas deferens is elongated and extends into the wider seminal vesicle. Six genital spines were observed, but referred to “a cirrus coronet”. The genital pore is median and located posterior to the branched intestine (Pandey, 1969).

The ovary is club-shaped and in a lateral position located to the caecal union. The uterine coils occupies all the available inter caecal space and opens into the genital pore. Follicular vitellaria elongates laterally along the intestinal caeca from the pharyngeal level to the posterior body end. The median vitelline duct that is formed by the union of two transverse vitelline ducts opens at the ootype. The eggs are large and thin shelled; some of these eggs are embryonated while others have well developed oncomiracidia (Pandey, 1969) (Figure 3.12). Average measurements made by Pandey (1969) are included in Table 3.9.

Table 3.9: Body measurements of *Eupolystoma chauhani* as measured by Pandey (1969).

	Pandey (1969)	Present study			
Measurements	Average (μm)	Average (μm)	Number	STD	KV
Body Length	3745 (3240-4250)	none	none	none	none
Body width	1475 (1200-1750)	none	none	none	none
Haptor length	1250 (1210-1350)	none	none	none	none
Haptor width	1385 (1320-1450)	none	none	none	none
Oral sucker	255 (210-300)	none	none	none	none
Pharynx length	85 (80-90)	none	none	none	none
Pharynx width	65 (60-70)	none	none	none	none
Ovary length	none	none	none	none	none
Ovary width	none	none	none	none	none
N Genital spines	6	none	none	none	none
Gen. spine length	none	none	none	none	none
Sucker Diameter	135 (120-150)	none	none	none	none
Eggs in utero	none	none	none	none	none
Egg length	75 (70-80) (embryonated) 80 (70-90)(larval body)	none	none	none	none
Egg width	25 (20-30) (embryonated) 35 (30-40)(larval body)	none	none	none	none

3.3 Species identification

3.3.1 Evaluation of marginal hooklet measurements

Measurements (Figure 3.13) obtained for the 261 specimens of the genus *Eupolystoma* examined, were entered into the software program Matrix Laboratory®. Of all possible combinations the 10 best results were evaluated. The results included the Classification Potential (CP) that had a maximum of 50.50, the Scale factor (Sca) (Max 1.60) and the products of the measurements that concluded in the specific CP and Sca (Table 3.10). For practical reasons, Figure 2.11 is copied (Figure 3.13).

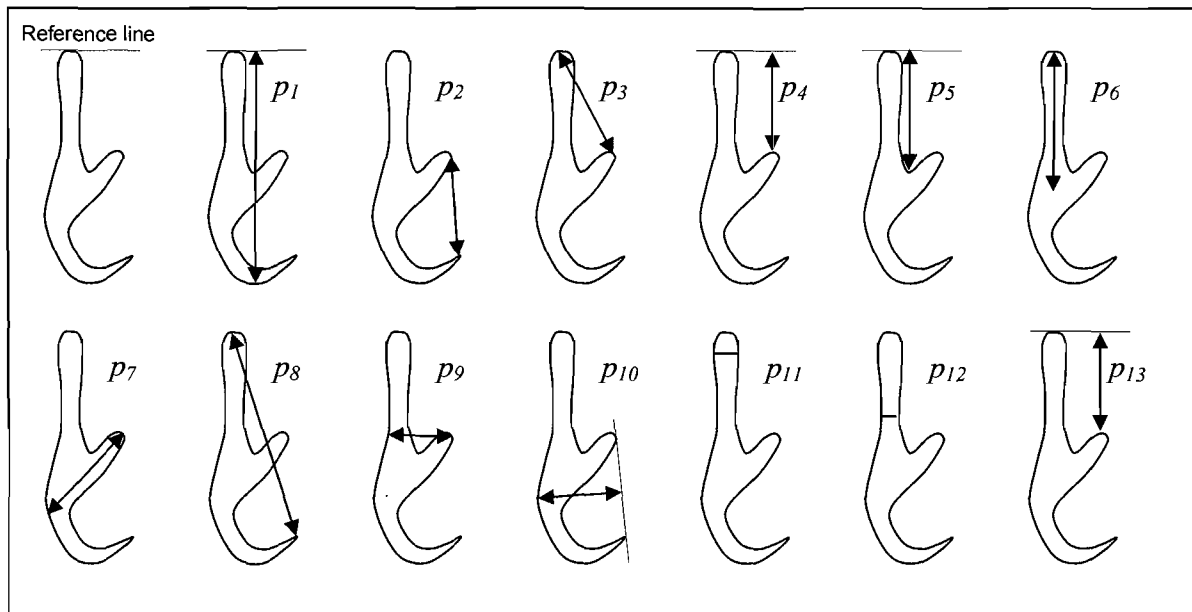


Figure 3.13: The 13 different measurements that were taken of the marginal hooklets for all the species that was available of the genus *Eupolystoma* (Du Preez & Maritz, 2005).

Table 3.10: Table of the 10 best classification potentials (CP) and scale factors (SCa) for 13 different measurement products of the species of *Eupolystoma*.

	X-axis		Y-axis			
	PX ₁	PX ₂	PY ₁	PY ₂	CP	SCa
1	P1	P8	P6	P13	50.50	1.60
2	P1	P8	P5	P13	48.00	1.45
3	P3	P5	P4	P7	47.50	1.70
4	P1	P4	P6	P13	46.50	1.70
5	P1	P5	P7	P13	46.00	1.70
6	P3	P8	P4	P7	45.50	1.70
7	P2	P8	P7	P12	45.50	1.65
8	P6	P13	P7	P13	45.00	1.60
9	P5	P13	P7	P13	45.00	1.65
10	P3	P4	P4	P7	45.00	1.65

When plotted according to the best Classification Potential (50, 50), some of the species do cluster (Figure 3.14). It was decided, however, to plot the best Scale Factor combination as well (Figure 3.15) in order to indicate the best possible combination of measurements for easy classification of the genus.

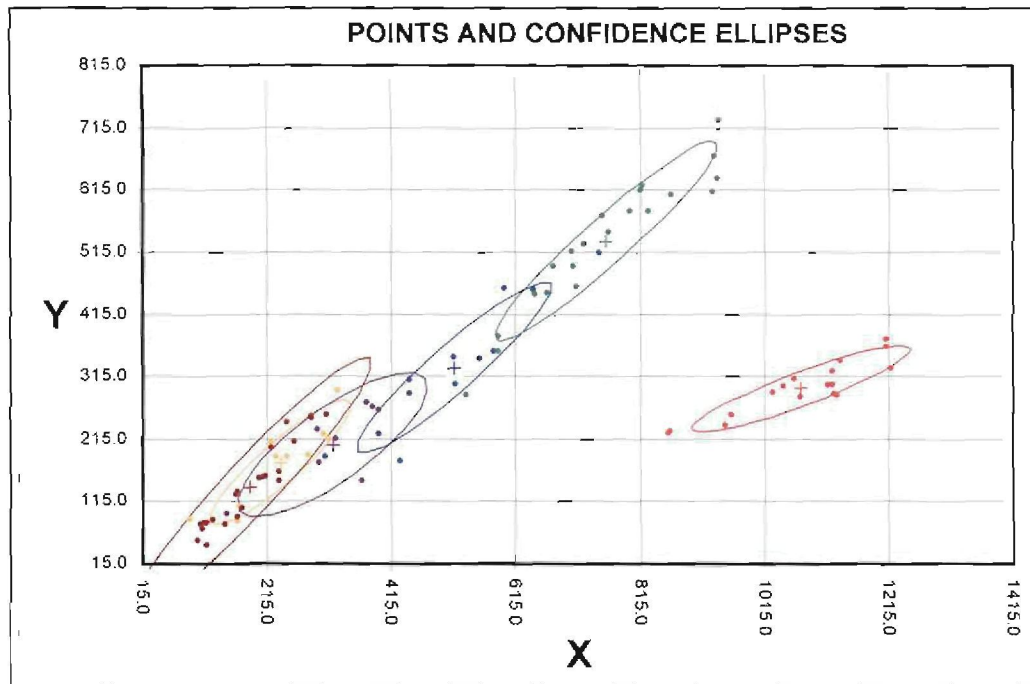


Figure 3.14: Plot of the maximum Classification Potential (50.50) for all the species of the *Eupolystoma* genus. The graph includes the products of the measurements of P1 x P8 (X-axis) and P6 x P13 (Y-axis) The scale factor for this specific combination was 1.60. Key: **A (Blue)** – *E. vanasi*, **B (Red)** – *Eupolystoma* sp 2 from *Poyntonophrynus hoeschi* **C (Green)**– *Eupolystoma* sp 1 from *A. garmani*, **D (Yellow)** – *E. alluaudi* from *A. regularis*, **E (Purple)**- *E. alluaudi* from *N. malcomi*, **F (Brown)** - *E. alluaudi* from *A. maculatus*.

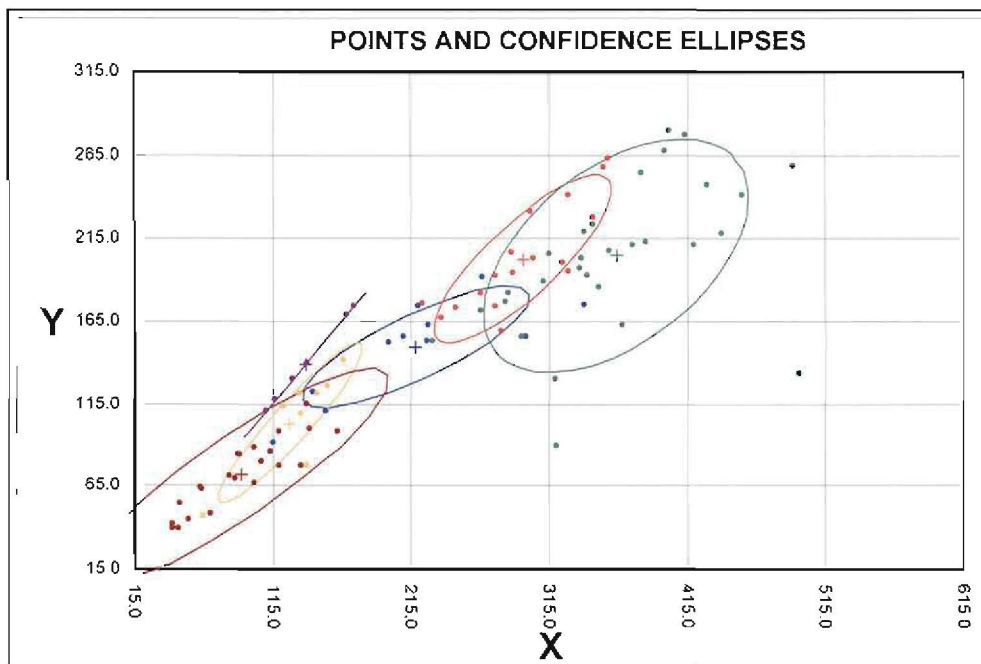


Figure 3.15: Plot for the combination of measurements that described the maximum Scale Factor (1.70). In this case, the highest Classification Potential for a 1.70 Scale Factor included the measurements P3 x P5 and P4 x P7. Key: **A (Blue)** – *E. vanasi*, **B (Red)** – *Eupolystoma* sp 2 from *Poyntonophrynus hoeschi*, **C (Green)** – *Eupolystoma* sp 1 from *A. garmani*, **D (Yellow)** – *E. alluaudi* from *A. regularis*, **E (Purple)** – *E. alluaudi* from *N. malcomi*, **F (Brown)** – *E. alluaudi* from *A. maculatus*.

On the basis of poor repeatability and lack of objective measurability, measurements P5, P6, P7 and P13 (Figure 3.16) did not comply with the criteria for good parameters defined by Du Preez & Maritz (2005). These measurements were expelled and the complete exercise rerun (Figures 3.17 & 3.18)

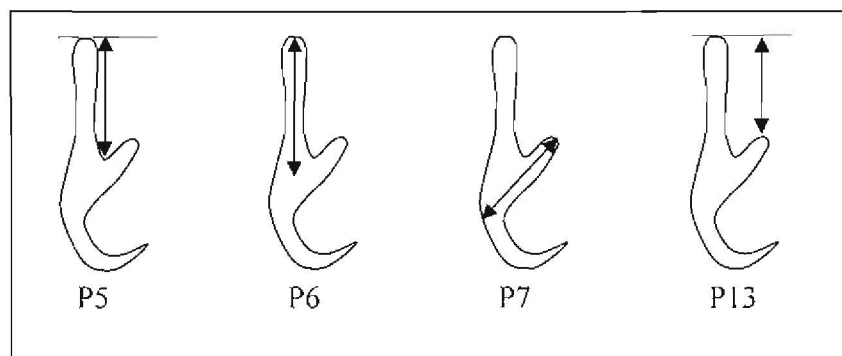


Figure 3.16: Four of the measurements that represent poor parameters for the classification of the genus *Eupolystoma* that were expelled from further analyses.

The remaining nine measurements (P1, P2, P3, P4, P8, P9, P10, P11 and P12) were entered into Matrix Laboratory® software. The best 10 results were evaluated for a more likely classification potential (Table 3.11).

Table 3.11: Table of 10 best classification potentials (CP) and scale factors (SCa) for nine measurement products of the species of *Eupolystoma*.

	X-axis		Y-axis		CP	SCa
	PX ₁	PX ₂	PY ₁	PY ₂		
1	P2	P3	P2	P5	19.50	0.60
2	P3	P4	P4	P5	18.50	0.40
3	P2	P4	P3	P5	18.50	0.50
4	P2	P6	P3	P5	18.00	0.70
5	P3	P4	P5	P6	16.50	0.50
6	P1	P3	P1	P7	16.50	0.70
7	P1	P3	P1	P7	16.50	0.60
8	3	6	7	9	16.00	0.35
9	2	5	3	5	16.00	0.55
10	1	3	2	7	16.00	0.65

In this case, the best Classification Potential was 19.50 that included the products of the parameters P2 x P3 and P2 x P5 (Figure 3.17).

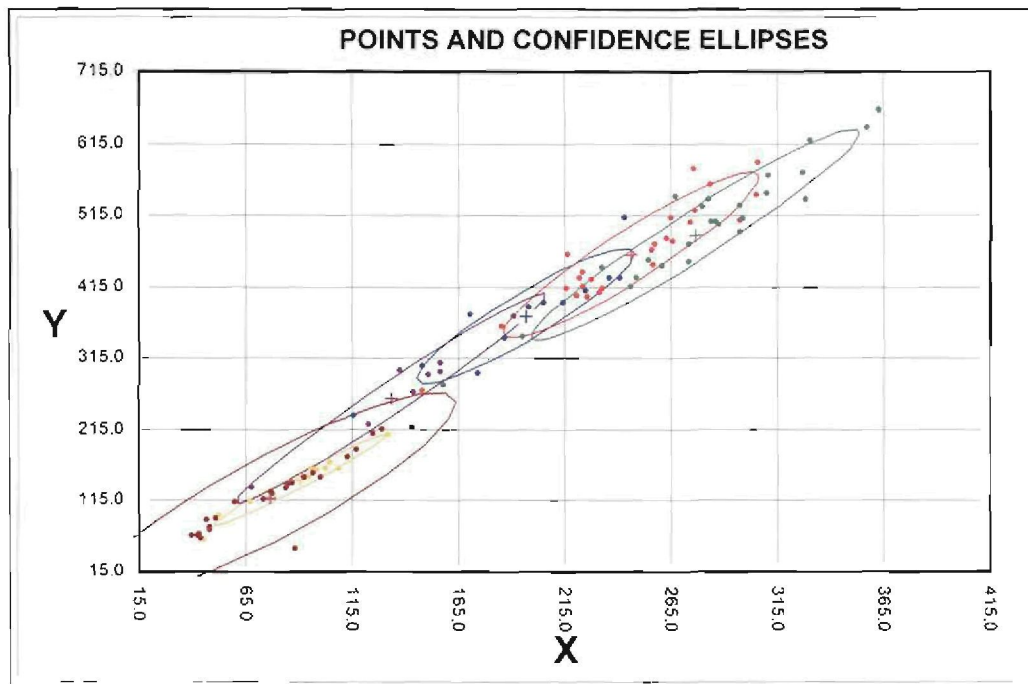


Figure 3.17: The measurement combinations that gave the best Classification Potential (CP) of 19.50 with a Scale factor (SCa) of 0.60 of the different measurements of the marginal hooklets in the genus *Eupolystoma*. Key: **A (Blue)** – *E. vanasi*, **B (Red)** – *Eupolystoma* sp 2 from *Poyntonophrynus hoeschi*, **C (Green)**– *Eupolystoma* sp 1 from *A. garmani*, **D (Yellow)** – *E. alluaudi* from *A. regularis*, **E (Purple)**- *E. alluaudi* from *N. malcomi*, **F (Brown)** - *E. alluaudi* from *A. maculatus*.

In an attempt to scatter the different species more an experimental plot of the best SCa (0.70) against the best CP (18.00) was prepared (Figure 3.18).

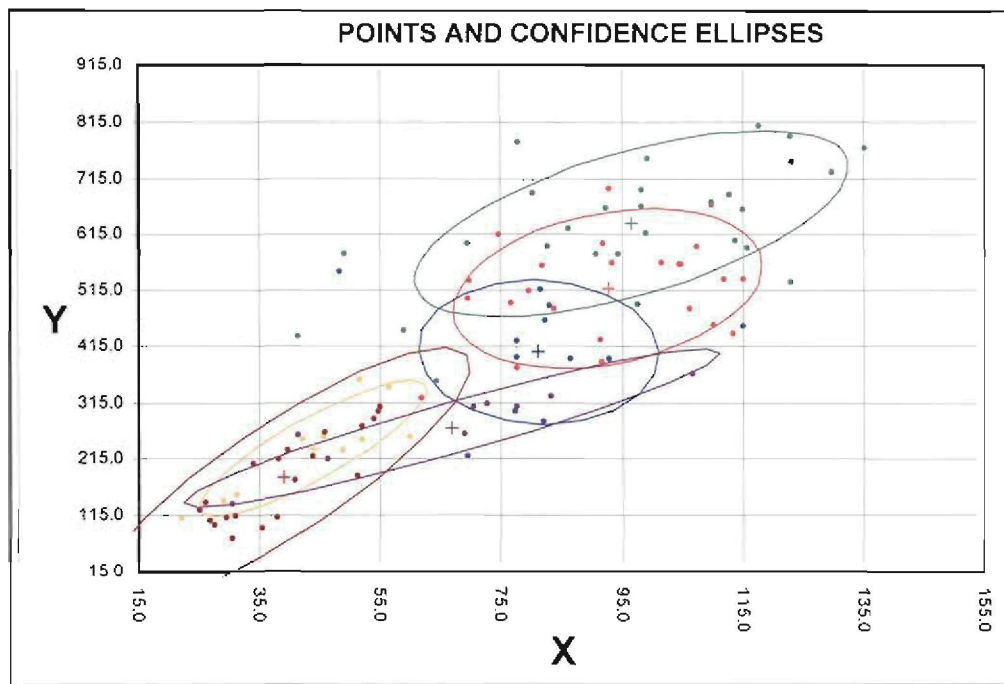


Figure 3.18: The best SCa (0.70) for the nine parameters of nine different measurements of the species of the genus *Eupolystoma*. Key: **A (Blue)** – *E. vanasi*, **B (Red)** – *Eupolystoma* sp 2 from *Poyntonophrynus hoeschi*, **C (Green)** – *Eupolystoma* sp 1 from *A. garmani*, **D (Yellow)** – *E. alluaudi* from *A. regularis*, **E (Purple)** – *E. alluaudi* from *N. malcomi*, **F (Brown)** – *E. alluaudi* from *A. maculatus*.

The products of the measurements are widely dispersed, indicating a poor classification. It was decided to plot the same measurement parameters for the genus *Eupolystoma* which were used for the classification of the genus *Polystoma* (Figure 3.19), suggesting that taxonomists can use one protocol for all the genera instead of one protocol for each genus. Du Preez & Maritz (2005) developed the protocol and the measurements that proved to give the greatest classification potential (CP = 20) and Scale factor (SCa = 0.91) was P1 x P9 and P1 x P2 for the genus *Polystoma*.

Species of the genus *Eupolystoma* did not separate out well. Du Preez & Maritz (2005) defined a protocol for the genus *Polystoma*. They proposed a plot

of $P_1 \times P_2$ against $P_1 \times P_9$. For comparison this protocol was applied to the *Eupolystoma* dataset and the plot is presented in Figure 3.20.

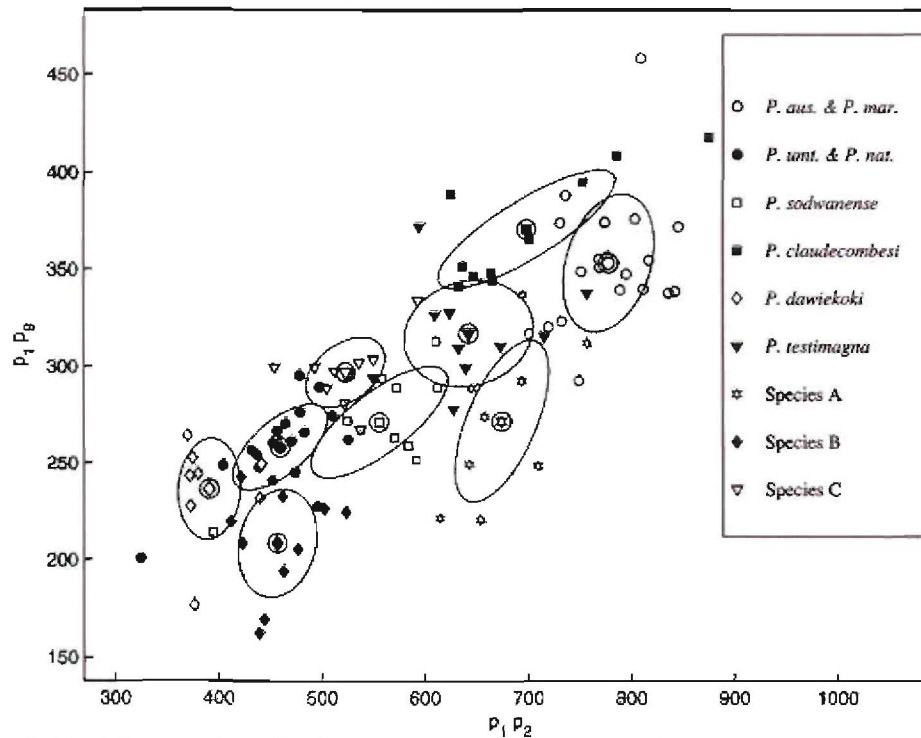


Figure 3.19: The scatter plot for the genus *Polystoma* of measurement $P_1 \times P_9$ and $P_1 \times P_2$ (Du Preez & Maritz, 2005).

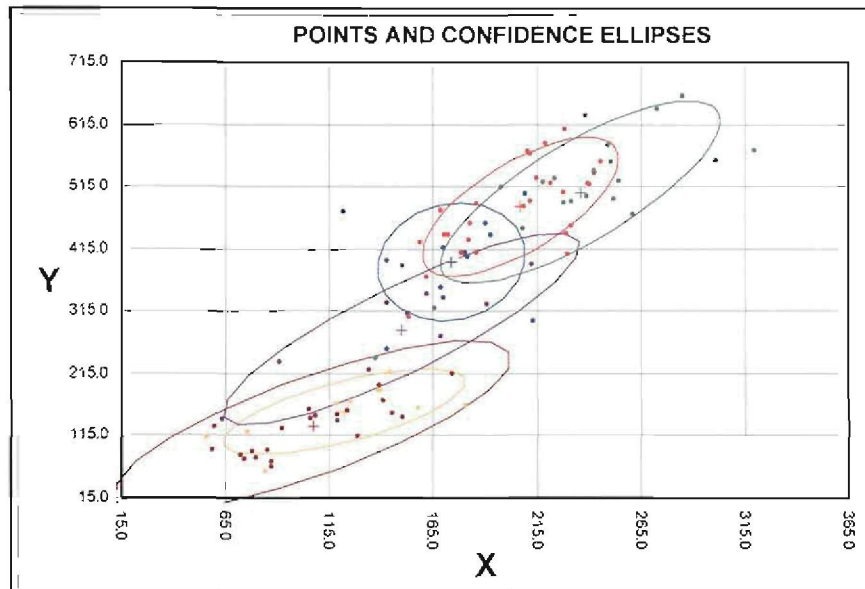


Figure 3.20: The scatter plot of the genus *Eupolystoma* of measurement according to the *Polystoma* protocol. Key: **A (Blue)** – *E. vanasi*, **B (Red)** – *Eupolystoma* sp 2 from *Poyntonophrynus hoeschi*, **C (Green)** – *Eupolystoma* sp 1 from *A. garmani*, **D (Yellow)** – *E. alluaudi* from *A. regularis*, **E (Purple)** – *E. alluaudi* from *N. malcomi*, **F (Brown)** – *E. alluaudi* from *A. maculatus*.

With Figure 3.20 the measurements P3 x P5 and P4 x P7 were used. By taking Figure 3.20 and only replacing the poor measurement parameters with good parameters, P5 can be replaced with P4 (88% dependable) and P7 with P2 (61% dependable) (Table 3.12) (Figure 3.21).

Table 3.12: The mathematically determined 13 different measurements of the marginal hooklets' dependability on each other (Du Preez and Maritz, 2005).

	p1	p2	p3	p4	p5	p6	p7	p8	p9	p10	p11	p12	p13
p1	-	90	85	85	88	89	46	99	49	61	31	25	87
p2	90	-	61	58	72	72	61	91	41	54	25	19	64
p3	85	61	-	90	85	88	31	84	53	49	31	31	89
p4	85	58	90	-	88	87	24	83	45	47	21	18	97
p5	88	72	85	88	-	96	50	87	41	38	19	13	92
p6	89	72	88	87	96	-	49	89	47	42	26	18	89
p7	46	61	31	24	50	49	-	47	52	35	20	20	28
p8	99	91	84	83	87	89	47	-	50	61	32	27	86
p9	49	41	53	45	41	47	52	50	-	75	37	49	40
p10	61	54	49	47	38	42	35	61	75	-	33	38	43
p11	31	25	31	21	19	26	20	32	37	33	-	72	18
p12	25	19	31	18	13	18	20	27	49	38	72	-	16
p13	87	64	89	97	92	89	28	86	40	43	18	16	-

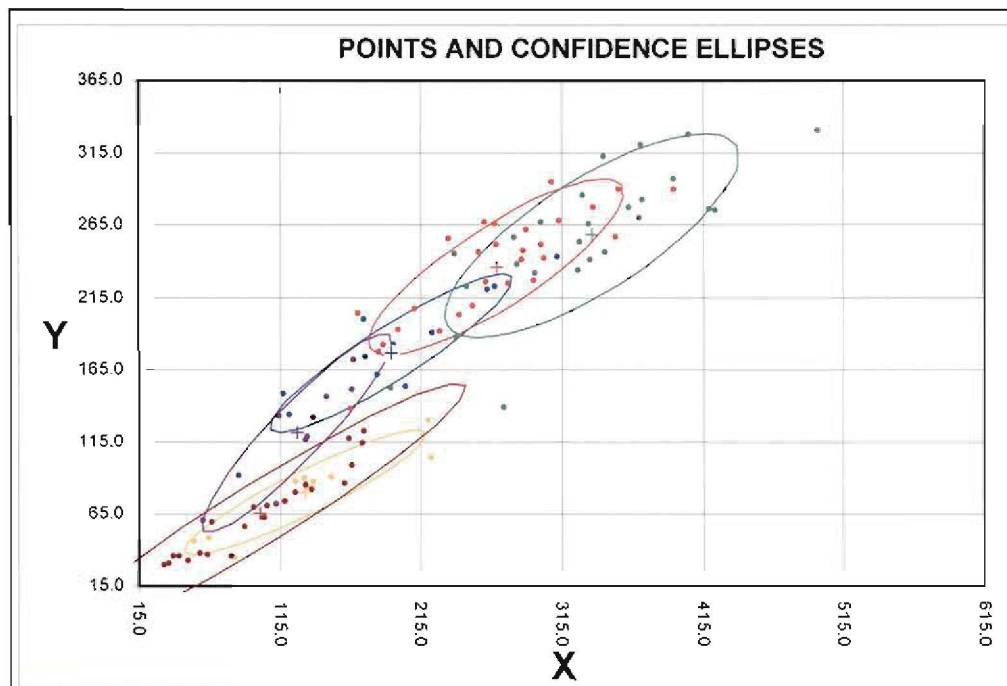


Figure 3.21: The scatter plot of the genus *Eupolystoma* with P5 replaced by P4 and P7 replaced by P2. Key: **A (Blue)** – *E. vanasi*, **B (Red)** – *Eupolystoma* sp 2 from *Poyntonophrynus hoeschi*, **C (Green)**– *Eupolystoma* sp 1 from *A. garmani*, **D (Yellow)** – *E. alluaudi* from *A. regularis*, **E (Purple)**- *E. alluaudi* from *N. malcomi*, **F (Brown)** - *E. alluaudi* from *A. maculatus*.

Additional graphs were drawn of the repetition of the measurements made of the best nine parameters using Matrix Laboratory® to elucidate the pattern that arises in the different species (Figure 3.22).

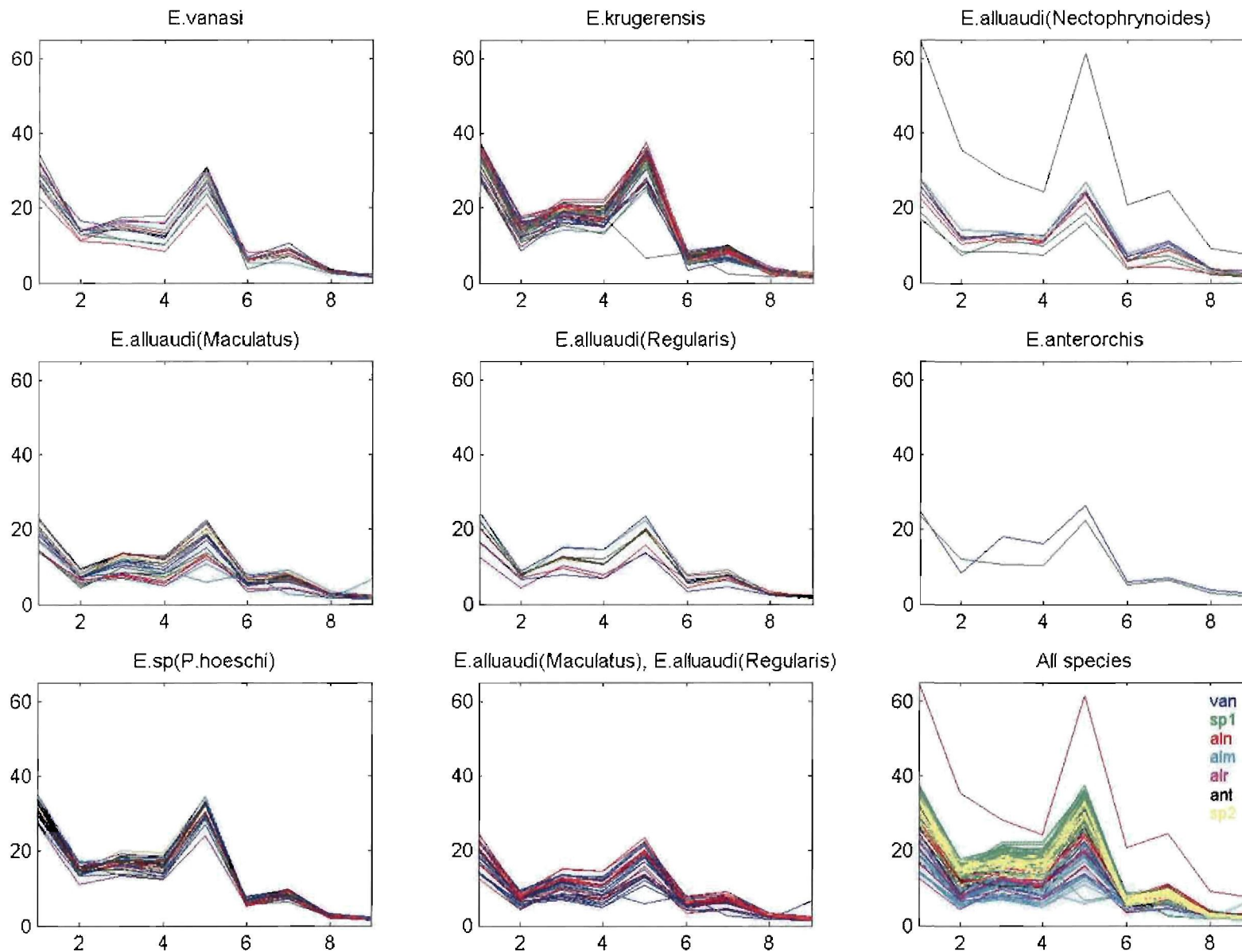


Figure 3.22: The repetition of the 9 different measurements of all of the available species in the genus *Eupolystoma*. Abbreviations: van: *E. vanasi*, sp 1: *Eupolystoma* sp 1 from *Amietophrynus garmani*. aln: *E. alluaudi* from *Nectophrynoides malcomi*. alm: *E. alluaudi* from *A. maculatus*. alr: *E. alluaudi* from *A. regularis*. ant: *E. anterorchis*. sp 2: *Eupolystoma* sp 2 from *A. hoeschi*.

3.3.2 Statistical analysis

Statistic Box and Whisker plots (Figures 3.23 - 3.31) were prepared using Statistica® to clear up any similarities or differences for *Eupolystoma* sp 1 from *Amietophrynus garmani* and *E. vanasi* from *Schismaderma carens*. The results of additional meaningful differences that were determined for these to species are also included in Table 3.13. The differences were drawn to the different measurements that were taken of the marginal hooklets of the two species starting at P1 – P13. The measurements that were seen as poor parameters (P5, P6, P7 and P13) were not included.

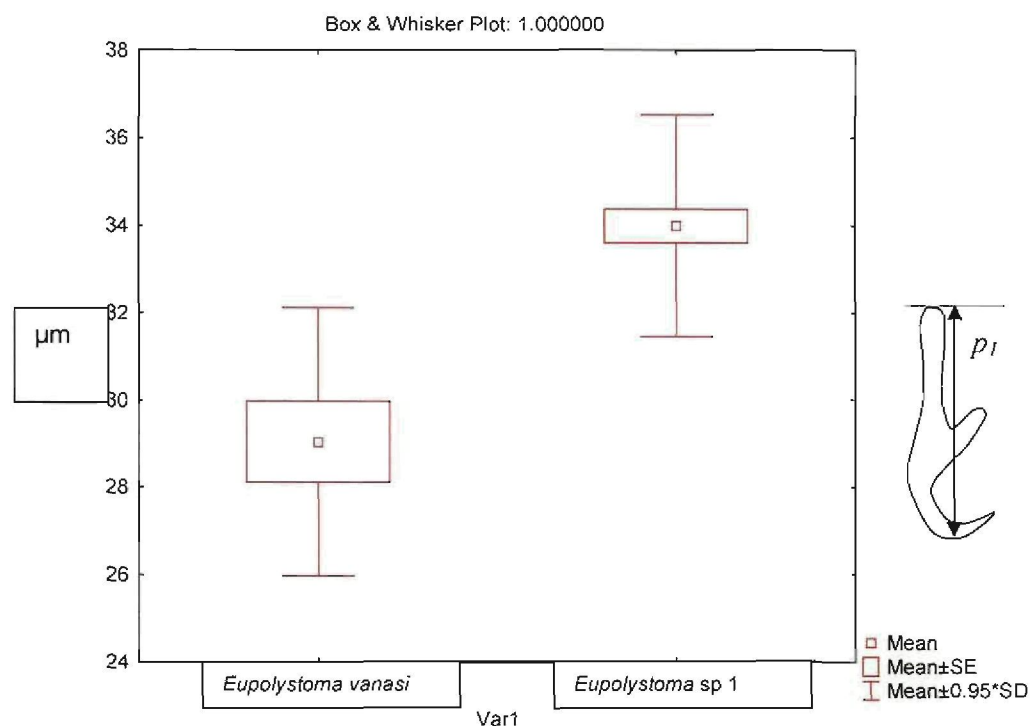


Figure 3.23: Box chart with standard deviation of measurement P1 for *E. vanasi* and *Eupolystoma* sp 1.

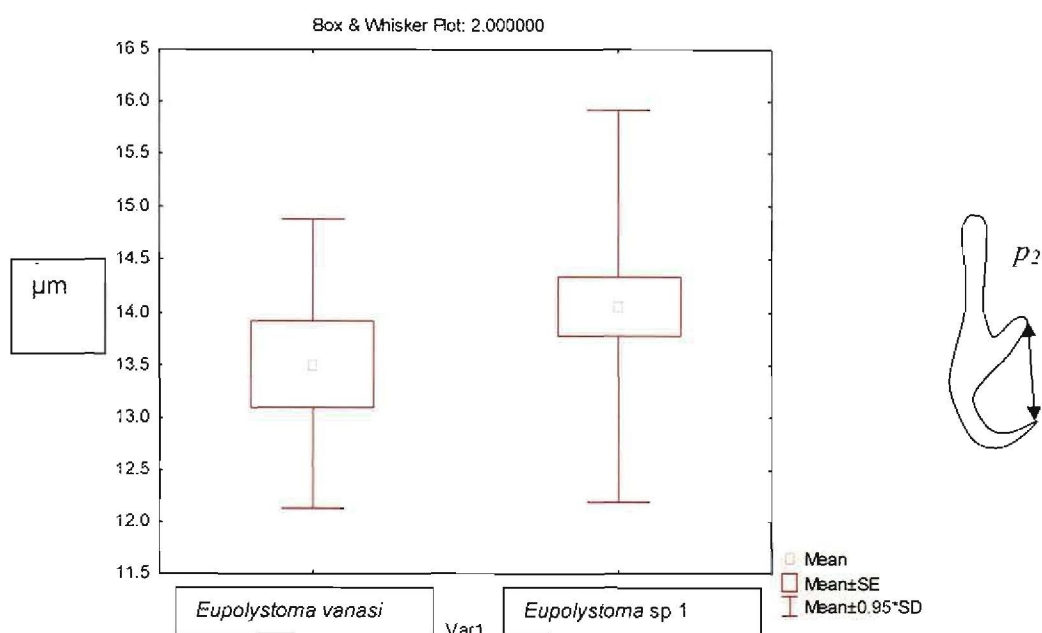


Figure 3.24: Box chart with standard deviation of measurement P2 for *E. vanasi* and *Eupolystoma* sp 1.

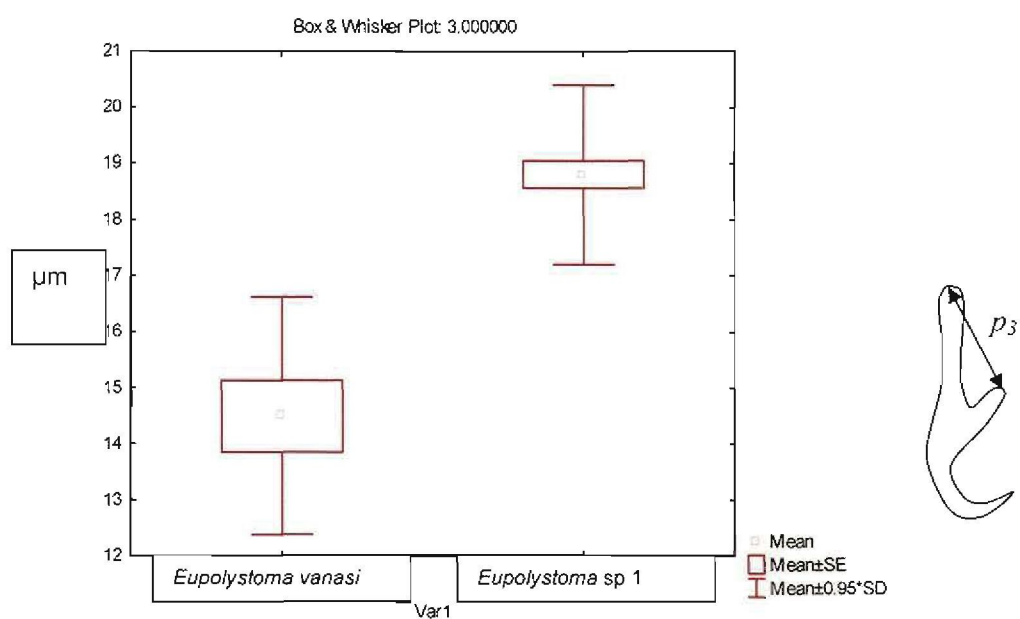


Figure 3.25: Box chart with standard deviation of measurement P3 for *E. vanasi* and *Eupolystoma* sp 1.

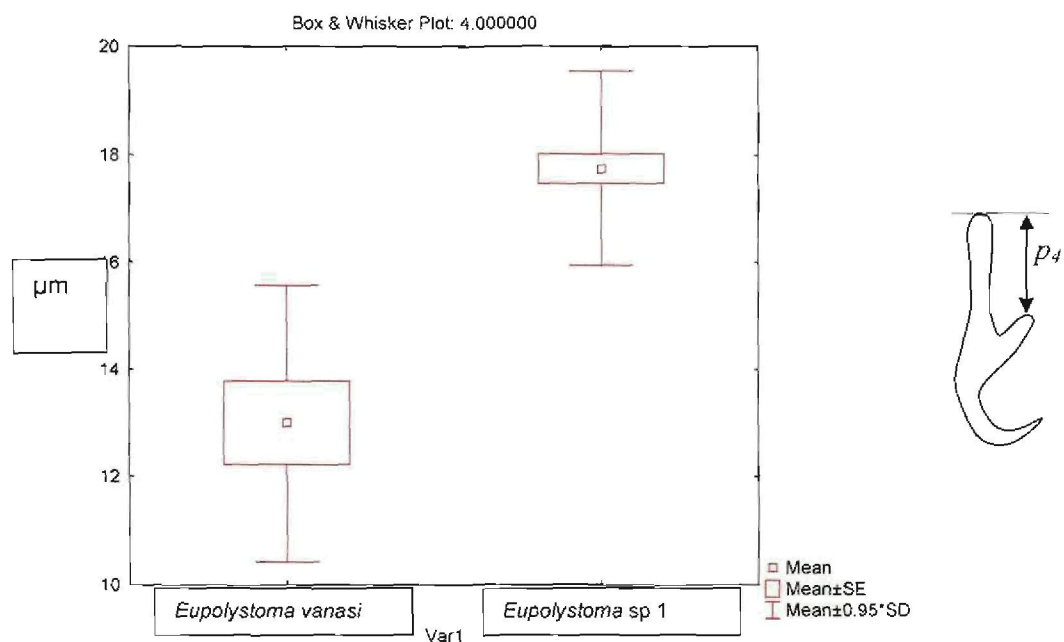


Figure 3.26: Box chart with standard deviation of measurement P4 for *E. vanasi* and *Eupolystoma sp 1*.

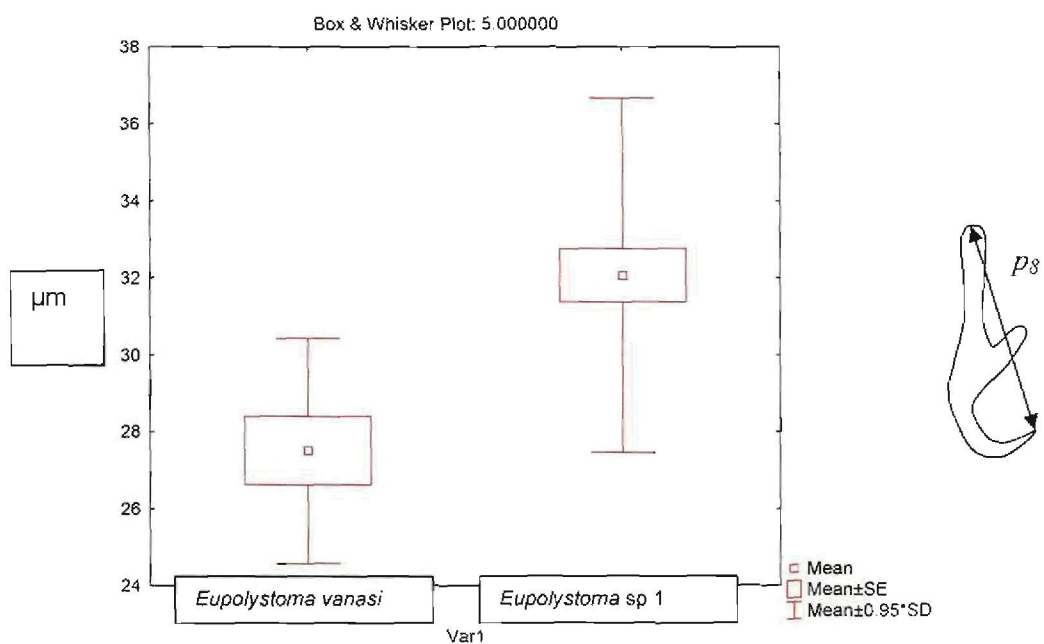


Figure 3.27: Excluding measurement P5, P6 and P7, measurement P8 of *E. vanasi* and *Eupolystoma sp 1* are presented in terms of the mean of the measurement, the standard deviation and the standard error.

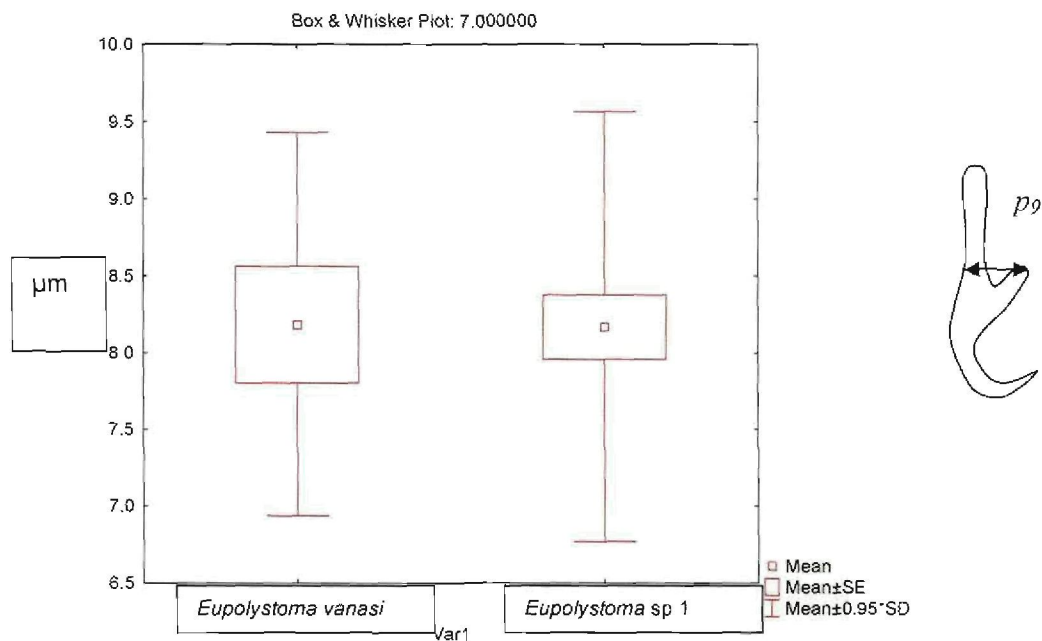


Figure 3.28: Box chart with standard deviation of measurement P9 for *E. vanasi* and *Eupolystoma* sp 1.

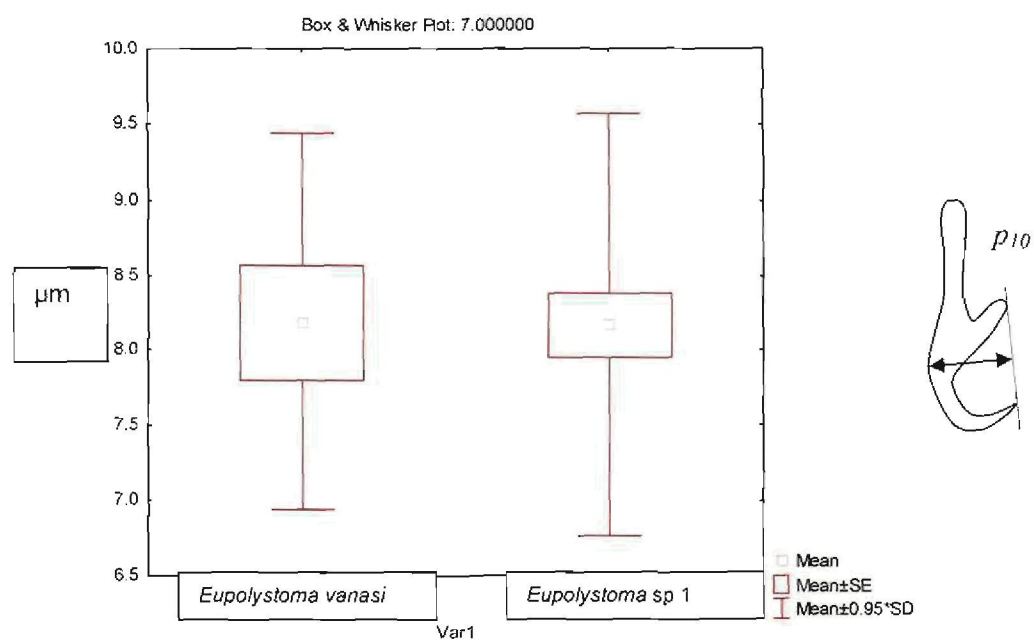


Figure 3.29: Box chart with standard deviation of measurement P10 for *E. vanasi* and *Eupolystoma* sp 1.

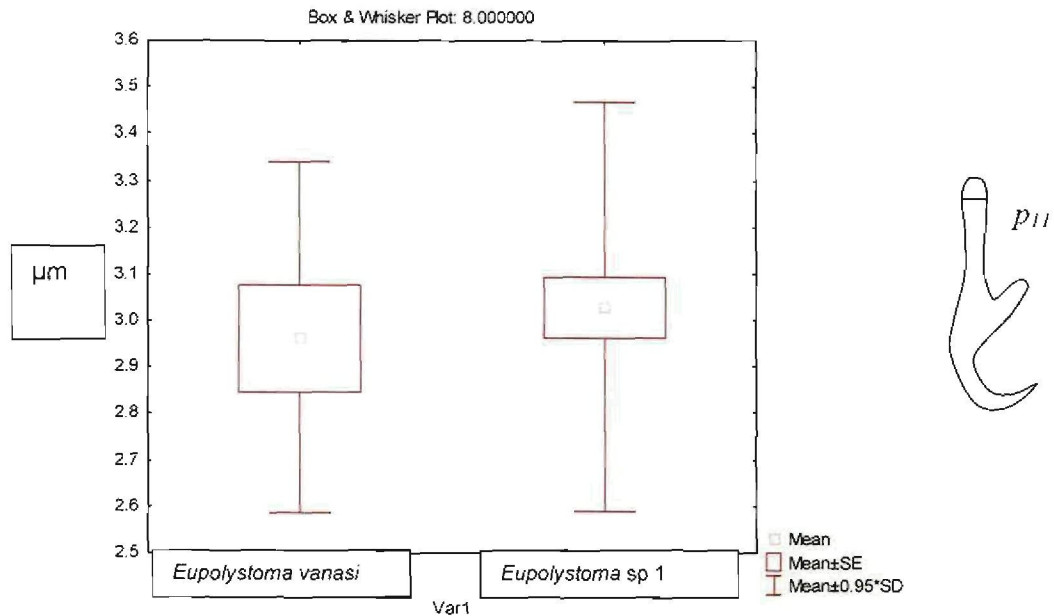


Figure 3.30: The statistical analysis of the two species *E. vanasi* and *Eupolystoma* sp 1. according to the mean, standard deviation and standard error of measurement P11.

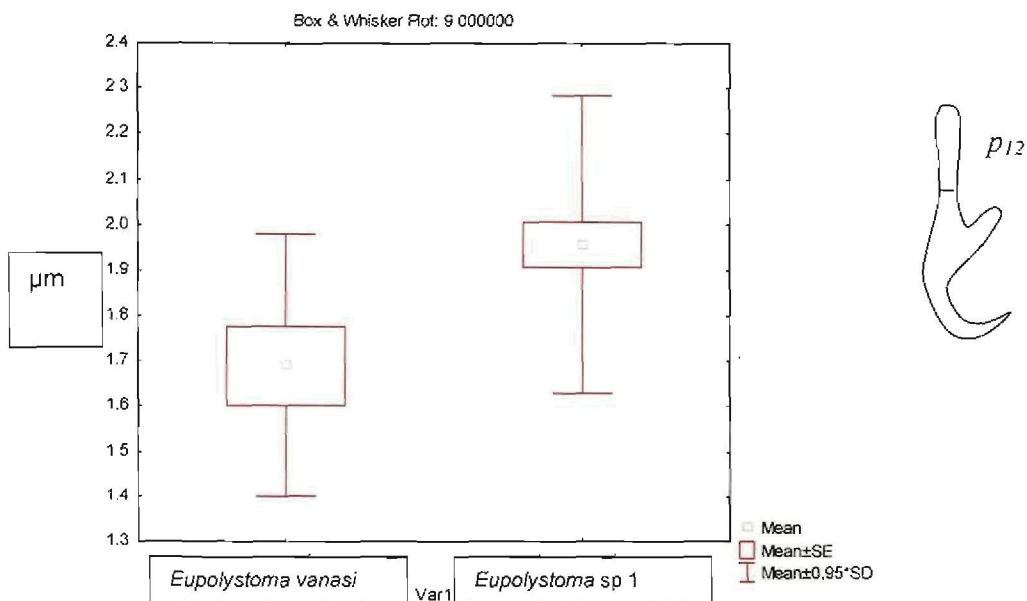


Figure 3.31: The differences of the two species of the *Eupolystoma*, *E. vanasi* and *Eupolystoma* sp 1. according to the mean, standard deviation and standard error of measurement P12.

Table 3.13: Significant differences between the different species of *Eupolystoma* excluding *E. anterorchis* for variable 1-9. Marked differences are significant when $p < 0.05$ (Highlighted).

Variable 1	{1}	{2}	{3}	{4}	{5}	{6}
<i>E. vanasi</i> {1}		0.000483	0.005688	0.000020	0.000020	0.065330
<i>Eupolystoma</i> sp 1. {2}	0.000483		0.000020	0.000020	0.000020	0.215410
<i>E. alluaudi</i> (n) {3}	0.005688	0.000020		0.000119	0.054844	0.000020
<i>E. alluaudi</i> (m) {4}	0.000020	0.000020	0.000119		0.487558	0.000020
<i>E. alluaudi</i> (r) {5}	0.000020	0.000020	0.054844	0.487558		0.000020
<i>Eupolystoma</i> sp 2 {6}	0.065330	0.215410	0.000020	0.000020	0.000020	

Variable 2	{1}	{2}	{3}	{4}	{5}	{6}
<i>E. vanasi</i> {1}		0.964330	0.027921	0.000020	0.000020	0.353777
<i>Eupolystoma</i> sp 1. {2}	0.964330		0.002386	0.000020	0.000020	0.422251
<i>E. alluaudi</i> (n) {3}	0.027921	0.002386		0.000022	0.000045	0.000046
<i>E. alluaudi</i> (m) {4}	0.000020	0.000020	0.000022		0.996443	0.000020
<i>E. alluaudi</i> (r) {5}	0.000020	0.000020	0.000045	0.996443		0.000020
<i>Eupolystoma</i> sp 2 {6}	0.353777	0.422251	0.000046	0.000020	0.000020	

Variable 3	{1}	{2}	{3}	{4}	{5}	{6}
<i>E. vanasi</i> {1}		0.000021	0.044609	0.000025	0.039554	0.031514
<i>Eupolystoma</i> sp 1 {2}	0.000021		0.000020	0.000020	0.000020	0.000542
<i>E. alluaudi</i> (n) {3}	0.044609	0.000020		0.662456	0.999998	0.000021
<i>E. alluaudi</i> (m) {4}	0.000025	0.000020	0.662456		0.532464	0.000020
<i>E. alluaudi</i> (r) {5}	0.039554	0.000020	0.999998	0.532464		0.000020

<i>Eupolystoma</i> sp 2 {6}	0.031514	0.000542	0.000021	0.000020	0.000020	
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Variable 4	{1}	{2}	{3}	{4}	{5}	{6}
<i>E. vanasi</i> {1}		0.000021	0.253636	0.000118	0.129009	0.018029
<i>Eupolystoma</i> sp 1 {2}	0.000021		0.000020	0.000020	0.000020	0.006580
<i>E. alluaudi</i> (n) {3}	0.253636	0.000020		0.514650	0.999967	0.000031
<i>E. alluaudi</i> (m) {4}	0.000118	0.000020	0.514650		0.585631	0.000020
<i>E. alluaudi</i> (r) {5}	0.129009	0.000020	0.999967	0.585631		0.000021
<i>Eupolystoma</i> sp 2 {6}	0.018029	0.006580	0.000031	0.000020	0.000021	

Variable 5	{1}	{2}	{3}	{4}	{5}	{6}
<i>E. vanasi</i> {1}		0.053563	0.102115	0.000020	0.000032	0.171401
<i>Eupolystoma</i> sp 1 {2}	0.053563		0.000027	0.000020	0.000020	0.975326
<i>E. alluaudi</i> (n) {3}	0.102115	0.000027		0.008639	0.295618	0.000073
<i>E. alluaudi</i> (m) {4}	0.000020	0.000020	0.008639		0.730746	0.000020
<i>E. alluaudi</i> (r) {5}	0.000032	0.000020	0.295618	0.730746		0.000020
<i>Eupolystoma</i> sp 2 {6}	0.171401	0.975326	0.000073	0.000020	0.000020	

Variable 6	{1}	{2}	{3}	{4}	{5}	{6}
<i>E. vanasi</i> {1}		0.937402	0.999271	0.998314	0.999921	0.971264
<i>Eupolystoma</i> sp 1 {2}	0.937402		0.853809	0.441664	0.889749	0.999860
<i>E. alluaudi</i> (n) {3}	0.999271	0.853809		1.000000	0.999992	0.908637
<i>E. alluaudi</i> (m) {4}	0.998314	0.441664	1.000000		0.999986	0.582425
<i>E. alluaudi</i> (r) {5}	0.999921	0.889749	0.999992	0.999986		0.937300
<i>Eupolystoma</i> sp 2 {6}	0.971264	0.999860	0.908637	0.582425	0.937300	

Variable 7	{1}	{2}	{3}	{4}	{5}	{6}
<i>E. vanasi</i> {1}		1.000000	0.995526	0.097912	0.557705	0.982119
<i>Eupolystoma</i> sp 1 {2}	1.000000		0.994479	0.006686	0.574401	0.852483
<i>E. alluaudi</i> (n) {3}	0.995526	0.994479		0.062979	0.290740	0.999999
<i>E. alluaudi</i> (m) {4}	0.097912	0.006686	0.062979		0.980864	0.000134
<i>E. alluaudi</i> (r) {5}	0.557705	0.574401	0.290740	0.980864		0.194253
<i>Eupolystoma</i> sp 2 {6}	0.982119	0.852483	0.999999	0.000134	0.194253	

Variable 8	{1}	{2}	{3}	{4}	{5}	{6}
<i>E. vanasi</i> {1}		0.998937	0.601746	0.132694	0.565390	0.545652
<i>Eupolystoma</i> sp 1 {2}	0.998937		0.804920	0.001563	0.342624	0.011839
<i>E. alluaudi</i> (n) {3}	0.601746	0.804920		0.002587	0.019647	0.027790
<i>E. alluaudi</i> (m) {4}	0.132694	0.001563	0.002587		0.990632	0.892483
<i>E. alluaudi</i> (r) {5}	0.565390	0.342624	0.019647	0.990632		0.999997
<i>Eupolystoma</i> sp 2 {6}	0.545652	0.011839	0.027790	0.892483	0.999997	

Variable 9	{1}	{2}	{3}	{4}	{5}	{6}
<i>E. vanasi</i> {1}		0.822861	0.813081	0.703011	0.991616	0.984094
<i>Eupolystoma</i> sp 1 {2}	0.822861		0.999971	0.999749	0.994986	0.951251
<i>E. alluaudi</i> (n) {3}	0.813081	0.999971		1.000000	0.983496	0.986351
<i>E. alluaudi</i> (m) {4}	0.703011	0.999749	1.000000		0.979334	0.909170
<i>E. alluaudi</i> (r) {5}	0.991616	0.994986	0.983496	0.979334		1.000000
<i>Eupolystoma</i> sp 2 {6}	0.984094	0.951251	0.986351	0.909170	1.000000	

3.3.3 Molecular analysis

DNA was successfully extracted from *Eupolystoma vanasi* and from *Eupolystoma* sp 1 found in *Amietophrynus garmani*. Problems were experienced with the Polymerase Chain Reaction (PCR) and the sequences did not work well. In the light that the laboratory of Dr Olivier Verneau⁵ in France specialise on the DNA analyses of polystomes it was decided to send specimens to him for analysis and for comparison with other *Eupolystoma* material in his collection.

Verneau found no differences for the 28S section after comparison of 1.4kb. For CO1 three to five differences were noted between three individuals of *E. vanasi* and nine differences between *Eupolystoma* sp 1 from *A.garmani* after comparison of 400 bp.

⁵ Dr Olivier Verneau, Laboratoire de Biologie Animale, UMR 5555 CNRS-UP, Université Perpignan, 66860 Perpignan Cedex, France

3.4 The *Eupolystoma* life cycle

Polystomatid flatworms all have direct life cycles with a close synchronisation between parasite and host. *Eupolystoma* display a life cycle that fits in with the explosive breeding strategy of its terrestrial hosts. The basic *Eupolystoma* life cycle is presented in Figure 3.32. Their brief and infrequent water visits only take place when hosts enter the water to breed. Transfer of infective stages mostly takes place during the brief period when the host is in the water (Tinsley, 1978).

Unlike most other anuran polystomes, where we usually find less than five mature polystomes in the bladder, *Eupolystoma* are sometimes found in large numbers. In the present study as many as 132 specimens of *Eupolystoma* sp 1 were found in a single *Amietophrynus garmani* specimen. The unique internal reproductive cycle of *Eupolystoma* facilitates this build up of parasite numbers. Parasites in the bladder do not represent a gradual increase in size from small to mature but clear size classes are evident (Figure 3.33). These sizes of the parasites reflect parasite breeding events and indirectly host reproductive activity. Furthermore toads have huge urinary bladders allowing them to store large volumes of water when they hibernate. These urinary bladders are highly vascularised (Figure 3.34) which allow ample feeding opportunity for the sanguinivoric parasites.

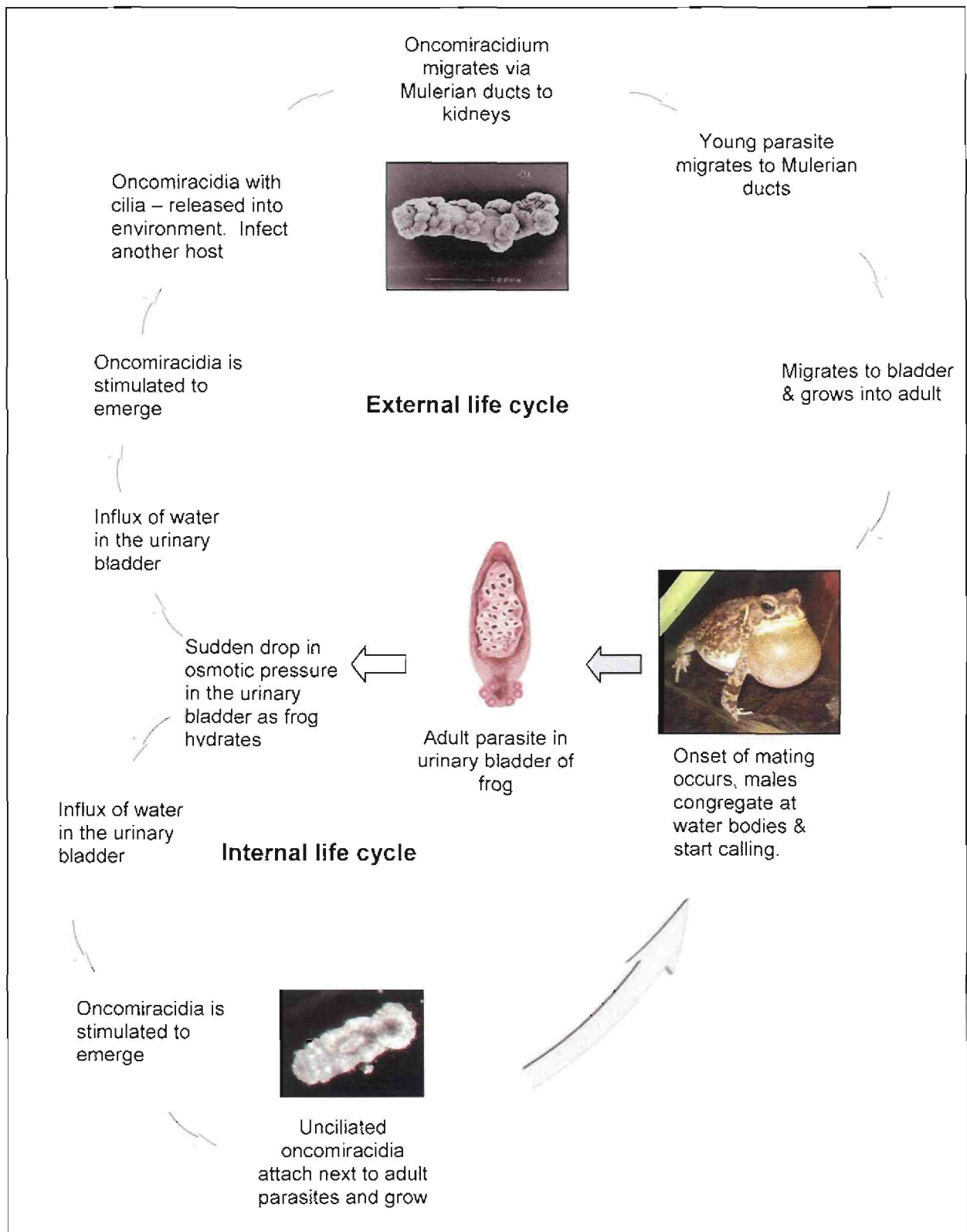


Figure 3.32: Direct life cycle of the genus *Eupolystoma* (Adapted from Tinsley, 1975; 1990).



Figure 3.33: Live *Eupolystoma* sp 1 in the bladder of *Amietophrynus garmani* indicating individuals from different size classes.

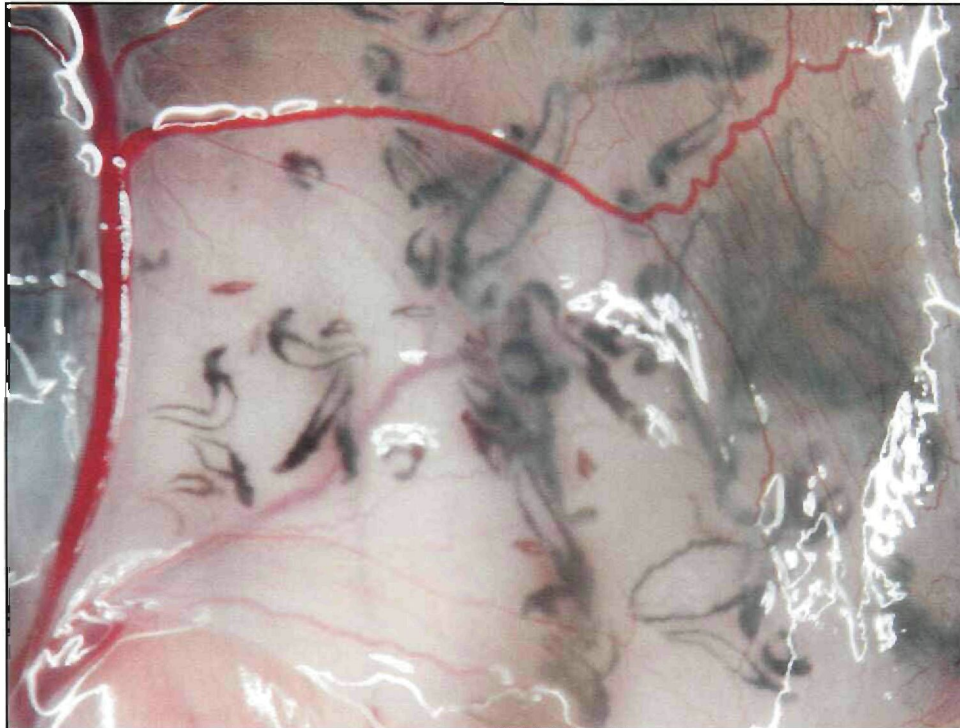


Figure 3.34: The vascularised bladder of the host that creates the perfect milieu for *Eupolystoma*.

When dissecting infected *Amietophrynus garmani*, it could be clearly seen that the Mulerian ducts were swollen in comparison with an uninfected Mulerian duct (Figures 3.35 & 3.36).

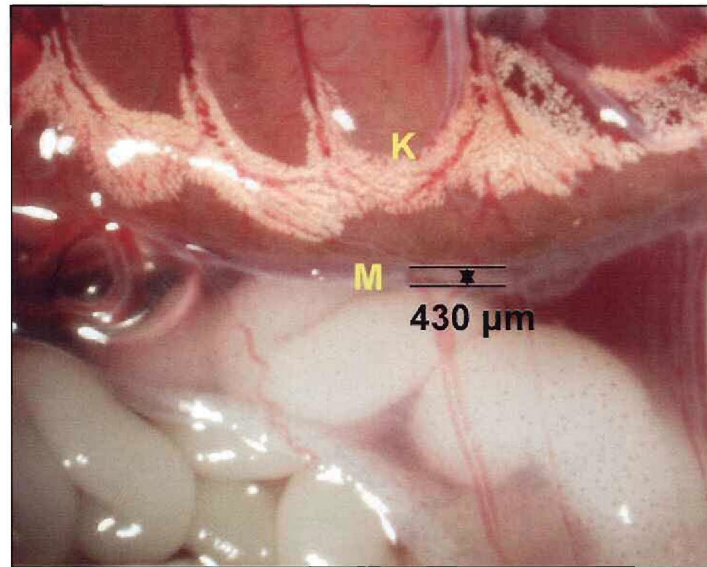


Figure 3.35: Micrograph of an uninfected Mulerian duct. Annotations: K, Kidney; M, Mulerian duct.

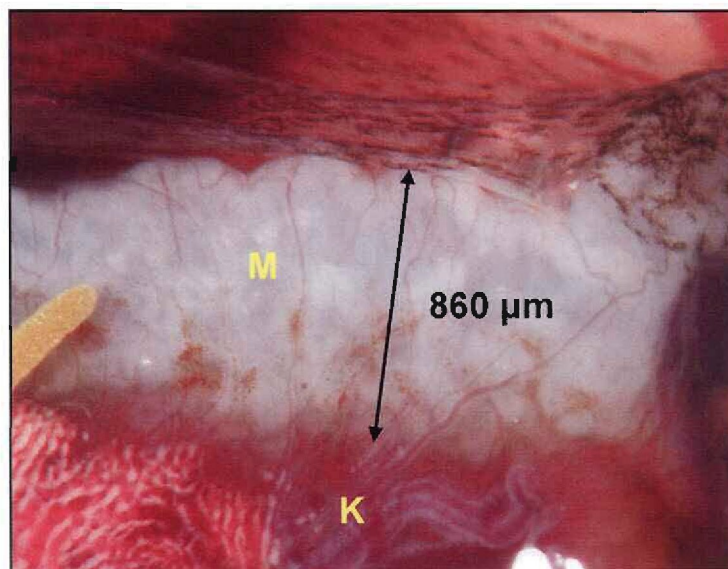


Figure 3.36: An infected Mulerian duct, filled with parasites. Annotations: K, Kidney; M, Mulerian duct.

Gross necropsy of the kidneys of *A. garmani* revealed a high level of infection (up to 15 parasites in the proximal and distal tubuli). Parasites in the proximal and distal tubuli were observed easily (Figures 3.37 – 3.40). The kidneys of host individuals with no parasites in the bladder were histologically examined and found to be infected with juvenile parasites and oncomiracidia, confirming that newly recruited parasites first migrate to the kidney.

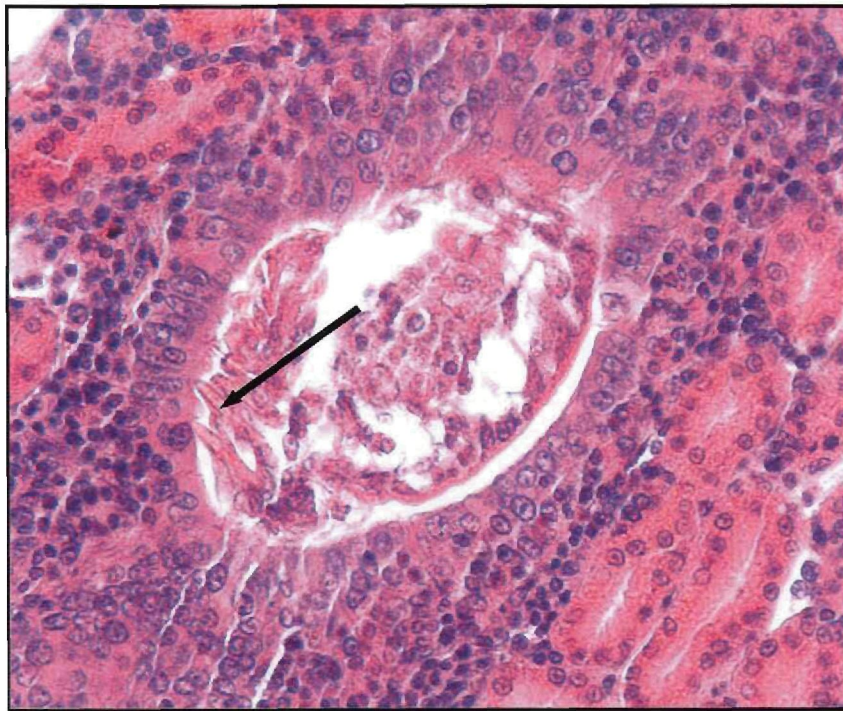


Figure 3.37: Micrograph of histological section through the kidney. The arrow indicates marginal hooklets of the parasite.

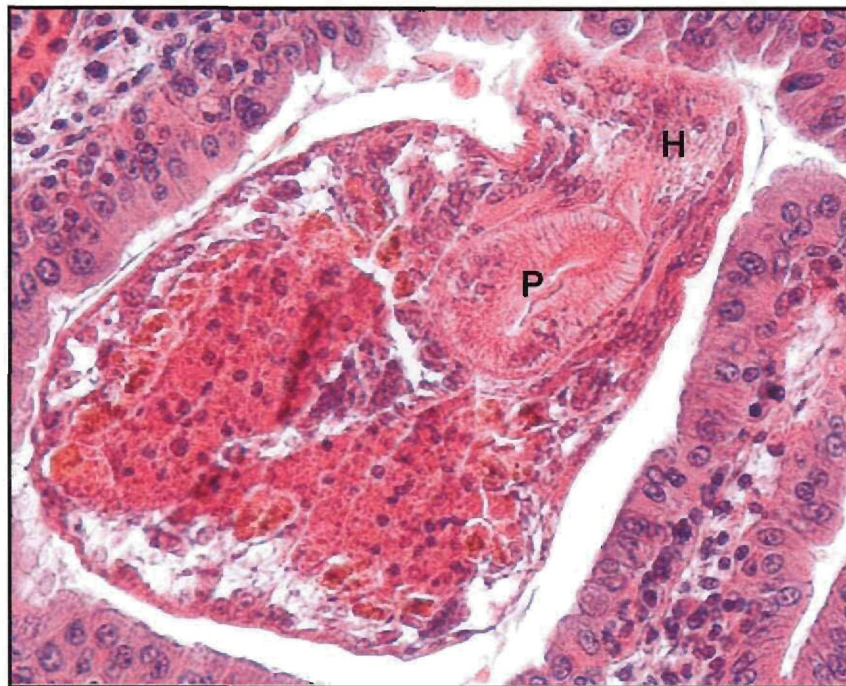


Figure 3.38: Cross section through a parasite in the kidneys. Note the muscular pharynx P and Haptor, H.

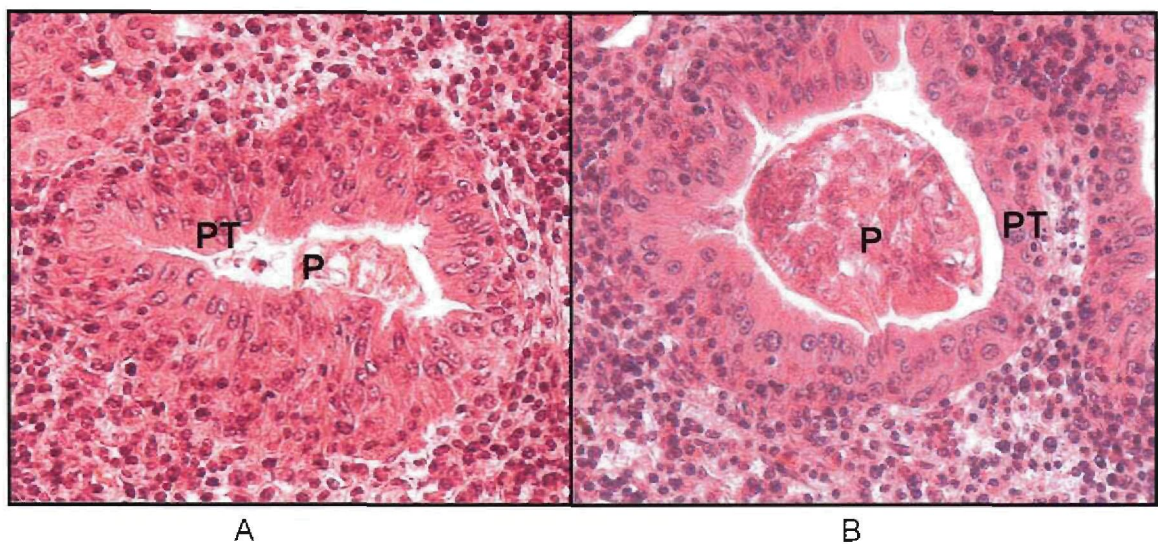


Figure 3.39 A & B: A small specimen (P) of *Eupolystoma* sp 1 in the proximal and/or distal tubuli (PT) of *Amietophrynus garmani*.

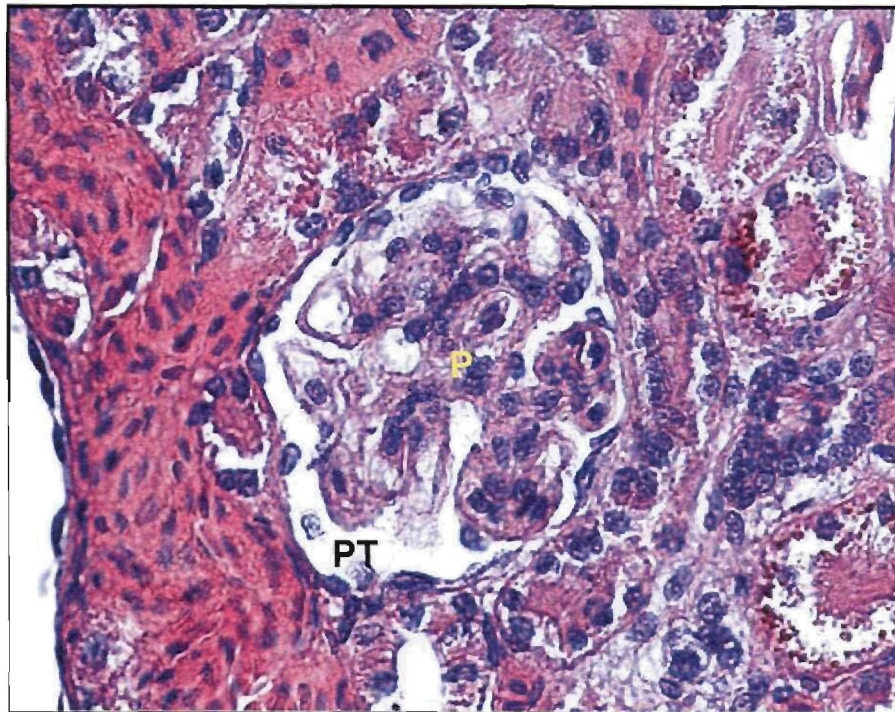
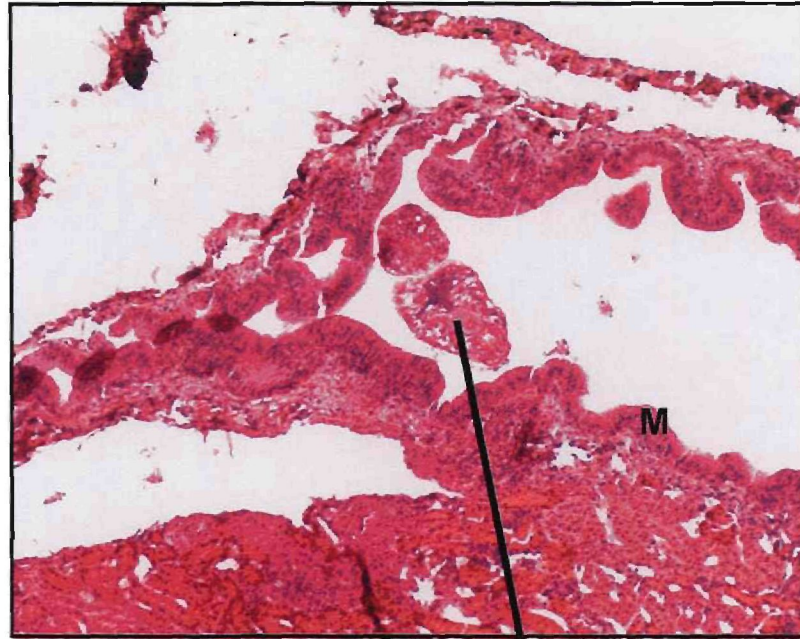


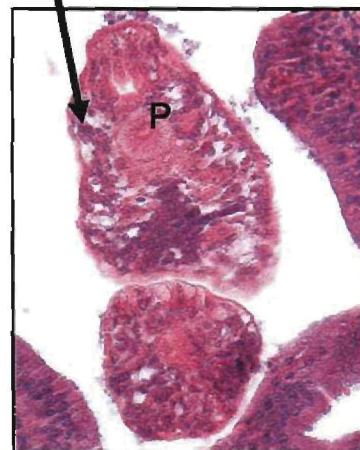
Figure 3.40: A small specimen (P) found in the proximal / distal tubuli (PT) from *Amietophrynus garmani* that yielded no parasites in the urinary bladder when the frog was dissected.

Mulerian ducts removed from *Amietophrynus garmani* that were histologically sectioned proved to be infected with an unknown *Eupolystoma* sp 1 (Figures 3.41 – 3.43). Multiple infections occurred where oncomiracidia as well as small parasites were present in the duct as seen in Figure 3.42 A & B.



A

Figure 3.41:(A) *Eupolystoma* sp.1 present in the Mulerian ducts (M) of *Amietophrynus garmani*. Enlarged, (B) the muscular pharynx (P) and the haptor of Sp. 1 can be seen.



B

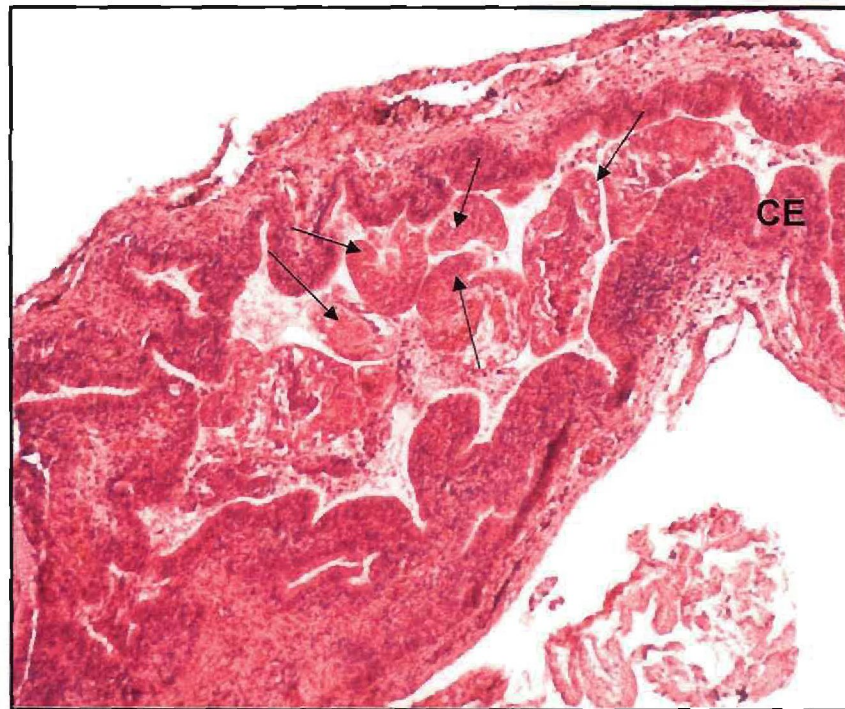


Figure 3.42: Multiple parasites (indicated with the arrows) present in the mulerian duct (M) of *Amietophrynus garmani*. Annotations: CE, Columnar epithelium.

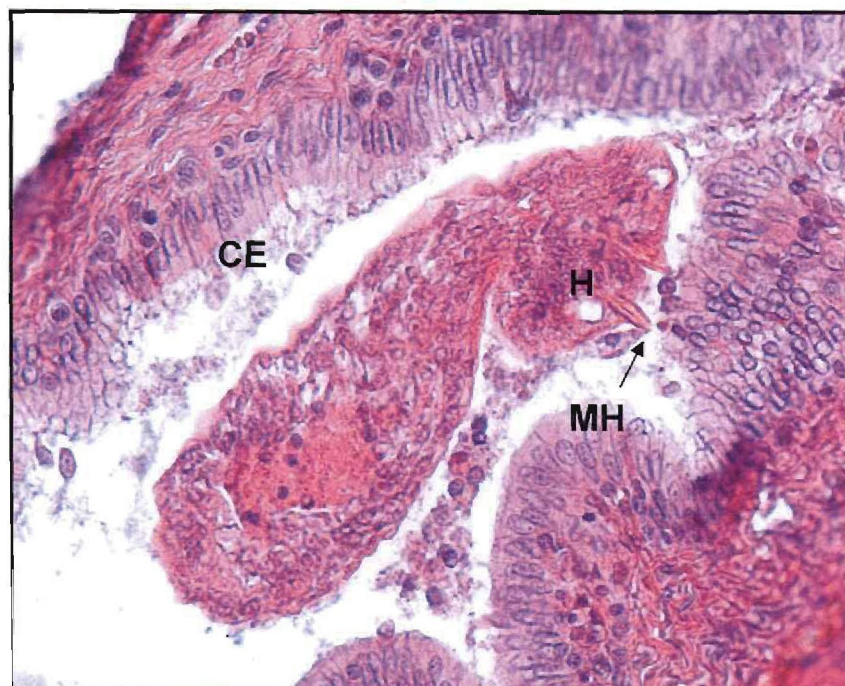
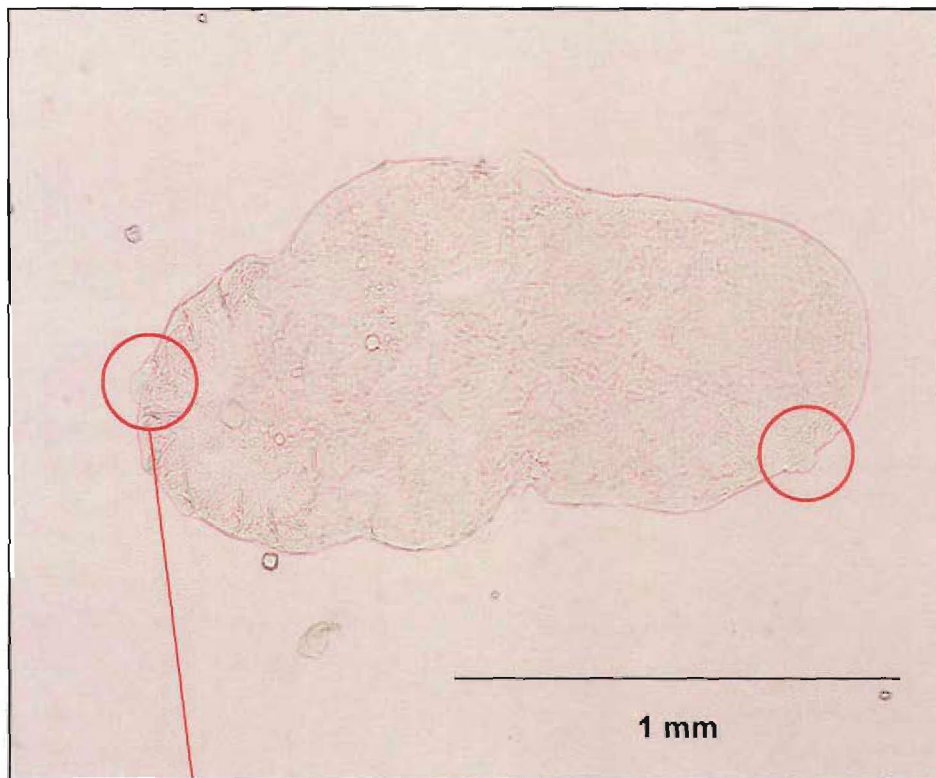


Figure 3.43: A single parasite in the Mullerian duct. MH: marginal hooklets. H: haptor. CE; columnar epithelium.

Oncomiracidia that were collected from the bladder and the Mulerian ducts were differentiated on the basis of ciliated and unciliated larvae (Figures 3.44 & 3.45)



A



B

Figure 3.44: A: Ciliated oncomiracidium that was found in the Mulerian ducts of *A. garmani*. B: Enlargement of a tuft of cilia found on the haptor of the parasite.

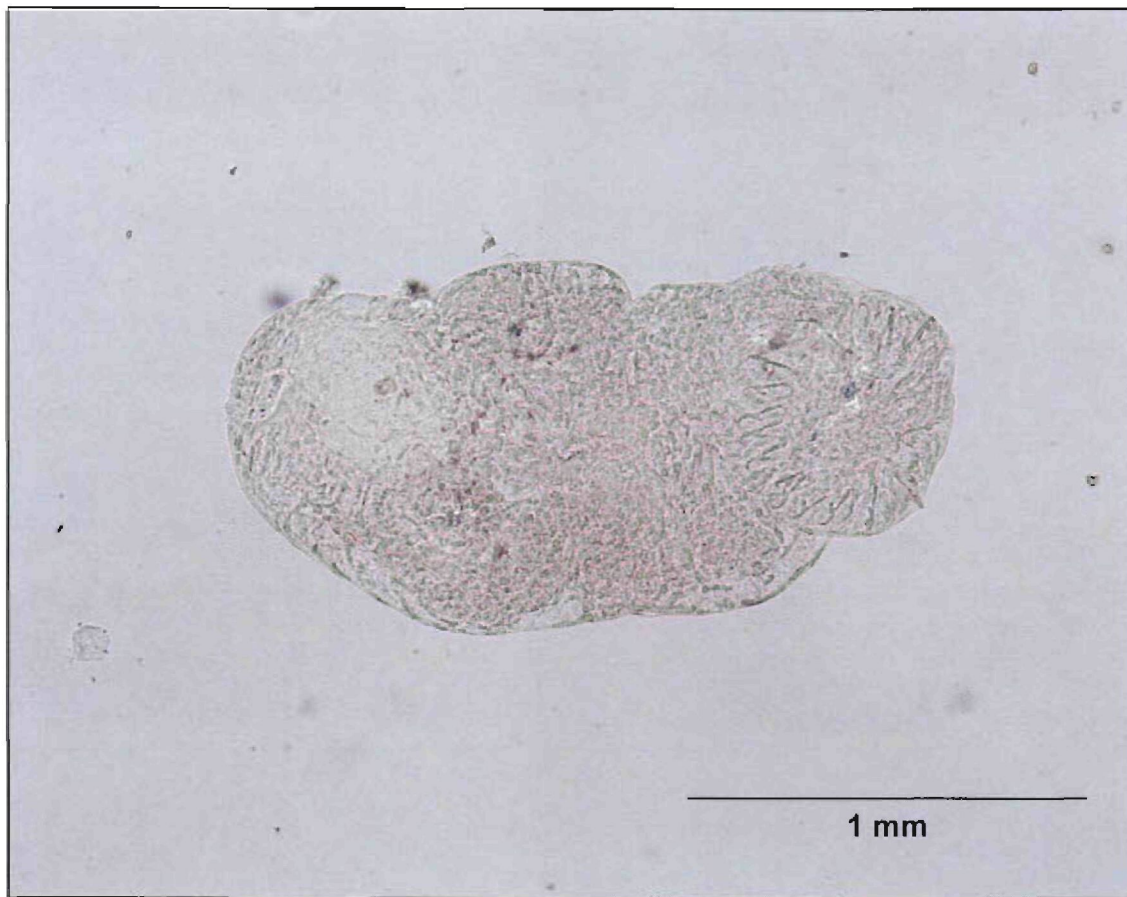


Figure 3.45: An unciliated oncomiracidia that was found in the bladder of *A. garmani*.

Chapter 4: General discussion

Chapter 4: General discussion

Anurans belong to a very old evolutionary group of amphibians that evolved around 350 million years ago. They are very well adapted to their environment and are found from permanently aquatic to harsh, arid desert environments. The anuran reproductive strategies are tied with environmental constraints. The majority of polystomes infect anuran hosts. Polystomes co-evolved with their anuran hosts and as they radiated and occupied new niches, so did their polystome parasites. Polystomes infect a wide spectrum of frogs from permanently aquatic forms to desert adapted species. They are not known from terrestrial breeders, as the infective stage can only transmit in an aquatic environment.

Protopolystoma is only known from the aquatic clawed frog *Xenopus* where the reproduction of the parasite occurs only the water, enabling the *Protopolystoma* to reproduce throughout the year (Tinsley, 1998). *Polystoma* infects a wide range of the more common frogs with a typical life cycle (Figure 4.1). At the extreme opposite is, *Pseudodiplorchis americanus* which is found in the desert adapted *Scaphiopus couchi* and which only breeds one or two nights per season. Parasite transfer has to take place during this brief period that the hosts enter the water to breed (Tinsley & Jackson, 2001).

Eupolystoma also displays an adaptation to a drier environment. Unlike most polystomes, where the infective stage of the parasite can only infect the host in the tadpole stage, the free-swimming oncomiracidium of *Eupolystoma* infects adult frogs only. *Eupolystoma* has a unique feature among polystomes, in that they produce two types of infective stages, namely ciliated and unciliated oncomiracidia. Whereas ciliated oncomiracidia infect other host individuals, unciliated oncomiracidia are responsible for auto-infection of the same host by

hatching *in utero*. This phenomenon has been observed and reported for *Eupolystoma anterorchis* by Tinsley (1990).

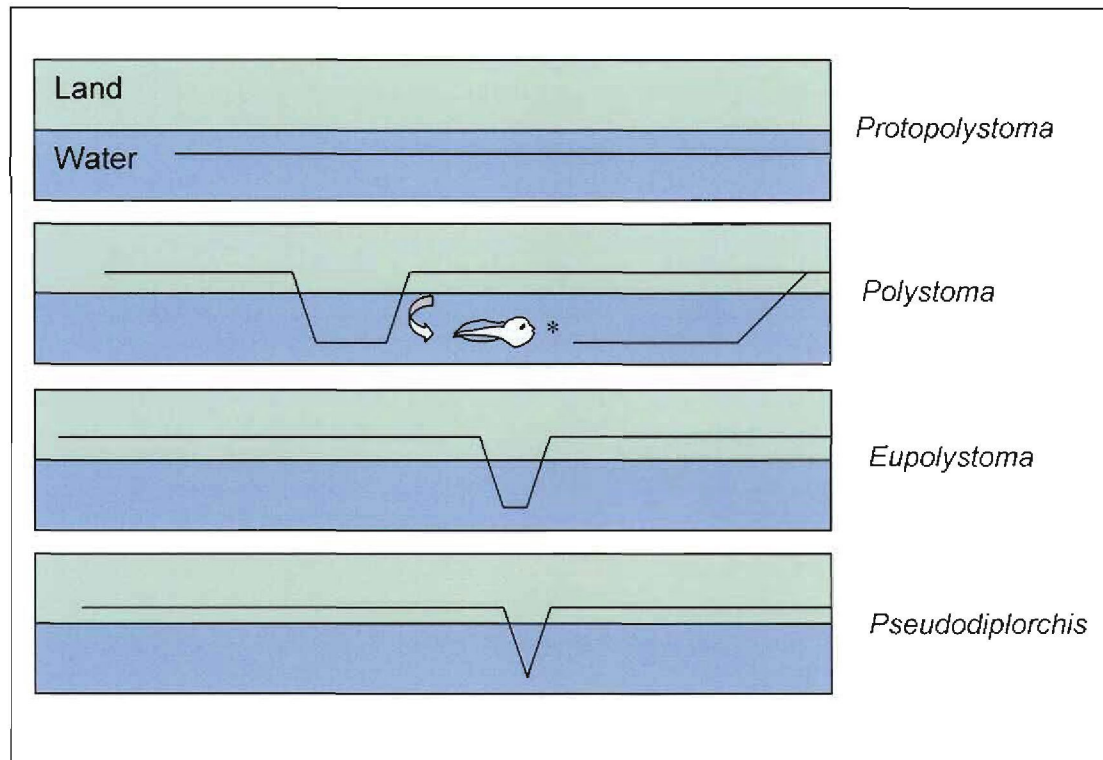


Figure 4.1: Schematic illustration of how polystomes are adapted to their environment with *Protopolystoma* in a permanently aquatic host and *Pseudodiplorchis* in an arid adapted desert frog. *Polystoma* visits the water more frequent than *Eupolystoma* and also has a neotenic cycle (*) (Adapted from Tinsley, 1990).

Eupolystoma sp 1 have been observed in both the kidneys and Mulerian ducts of *Amietophrynus garmani*. Tinsley (1978) reported the same phenomenon in *E. anterorchis* and in some specimens of *E. alluaudi* from *A. regularis* (Tinsley, 1978). In the present study, specimens with no parasites in the bladder were found to have parasites in the kidneys and Mulerian ducts, supporting the hypothesis that these originated from ciliated oncomiracidia that infected a new naive and uninfected host and confirms that ciliated oncomiracidia first migrate to the kidneys. A similar kidney phase is also known for *Protopolystoma* where the parasite first moves into the kidneys through the

Mulerian ducts of *Xenopus* and later to the urinary bladder as described by Thurston (1963). These parasites were well established 12 days after induced infection. It is also clear that the smallest parasites of *Eupolystoma* sp 1 are found in the kidneys and not in the urinary bladder, however, some small parasites are also present in the bladder. This also has been observed with *P. xenopodis*. Thurston (1964) stated that the presence of the parasites in the kidneys might be because of the richer food source. It was, however, also found that the kidney may serve as protection for the small fragile parasites against larger parasites and dessication. The development in the kidneys and Mulerian ducts has a further advantage in that the parasites develop in a protected environment that would be much safer than the urinary bladder. The bladders of especially bufonids are extremely flexible as the host has the ability to store large volumes of water in the bladder. This changing environment, with constant flushing as the frog walks, or worse; hops, would pose a severe threat of the parasites being dislodged and flushed out when urinating. The bladder might also act as a milieu for high intraspecies competition, forcing the newly hatched oncomiracidia into the kidneys and Mulerian ducts for survival.

The presence of larval parasites in the Mulerian ducts clearly causes a reaction at the tissue level. The ducts nearly doubled in diameter; it would appear that the ducts become more vascularised and for the sanguinivoric parasites, this creates the ideal feeding environment (No acquired evidence).

Dissection of *Amietophrynus pantherinus* to retrieve *E. anterorchis* revealed no parasites in the urinary bladder, kidneys or Mulerian ducts of road kills found in the Cape Peninsula. *Eupolystoma anterorchis* may be under threat, as its host is regarded as a vulnerable species, declining in population since 1978 when Tinsley (1978) did the description of the species. Theoretically, the host and the parasite are in constant dynamic interaction and the parasite population can decrease with the decreasing host population (Todar, 2002). The reduction in parasite population may also be due to a drastic change in the environment and habitat loss of its host. During the breeding season of *A.*

pantherinus, the frogs migrate to the nearest breeding ponds and pools across many tarred roads and highways in the Cape peninsula, elongating their normal sporadic breeding period and thus the exposure to traffic, likely causing the parasite to become extinct. Although unlikely, it could be that *E. anterorchis* is now extinct in the wild, as the host is under severe pressure of urbanisation and habitat loss. On the other hand, polystomes are known to be over dispersed and the possibility of under-sampling at infected sites cannot be excluded.

It has also been found that the infection levels of adult worms and their contribution to transmission are regulated by external environmental factors, especially temperature. The level of infection can also be altered by host factors (host behaviour, population density of the host) and by a range of parasite factors including intra- and inter-specific competition and variation in intrinsic characters, especially survivorship and reproductive output (Tinsley & Jackson, 2001).

Field and experimental studies suggest a high level of anuran polystome host specificity (Tinsley, 1982; Du Preez & Kok, 1997; Tinsley & Jackson, 1998). Not all genera and species display the same degree or level of host specificity. *Eupolystoma alluaudi*, for example, has been reported from at least seven anuran species representing four genera and two families. Bourgat *et al.* (1983) referred to this apparent lack of host-specificity in the case of *E. alluaudi*. This low level of host-specificity was also noted by Salami-Cadoux (1975) who experimentally infected *Bufo maculatus* with *E. alluaudi* usually infecting *B. regularis* in Togo. Indications are that *Eupolystoma* are not as host-specific as *Polystoma* and that the mechanism of host-specificity is different. Du Preez & Kok (1997) postulated that host specificity for *Polystoma* is determined by the oncomiracidium's ability to recognise only the correct host tadpole. In the case of *Eupolystoma*, the tadpole is not involved at all. It could be that the degree of host-specificity is reduced. Preliminary results indicated the latter and that *Eupolystoma* oncomiracidia can enter a substitute hosts, but that these parasites

do not survive longer than a few days (Du Preez¹ and Aisien⁶, personal communication). However, species of *Protopolystoma* and *Pseudodiplorchis* also infect only post-metamorphic stages of their respective hosts and the tadpoles are not involved in the life cycle at all but they still display a high degree of host-specificity. Rejection of parasites in foreign hosts occurs in the sites of juvenile development, in the kidneys and lungs respectively (Tinsley, 1995; Jackson & Tinsley, 1998). Thus it can also be assumed that other hosts might act as a refuge for *E. anterorchis* if the *Amietophrynus pantherinus* becomes greatly reduced.

Flatworms are usually soft bodied and during the fixation process the degree of flattening can have an enormous effect on the size of the parasite. Emphasis has thus been placed on the measurements of the more reliable hard structures such as the sclerites including genital spines marginal hooklets and hamuli.

Several authors attempted to develop taxonomic protocols. Malmberg (1970) measured the marginal hooklets of the genus *Eupolystoma*, indicating an interspecies difference. Llewellyn (1963) noticed that hooklets do vary between species and that the most posterior hooklets were larger in size than the rest of marginal hooklets of the parasite. Major contributions were made by Murith (1979) who recognised the potential of using the stability of the marginal hooklets as a taxonomic character. She studied the most posterior pair of marginal hooklets (C1) of the taxonomic classification of Monogenean species. A proposed set of measurements were taken when comparing the different polystome species and based on these measurements Murith (1979) was able to point out that various polystome species can indeed infect the same host. This discovery was surprising as polystome parasites are known to be host specific (Combes, 1966; Maeder, Euzet & Combes 1970; Maeder, 1973; Euzet, Combes

⁶ Dr M. Aisien, Zoology, University of Benin, Benin City, Nigeria.

& Batchvarov, 1974; Tinsley, 1974; Bourgat & Salami-Cadoux, 1976; Murith, 1982; Kok & Van Wyk, 1986; Kok & Du Preez, 1987; Du Preez & Kok, 1997).

Although the protocol of Murith (1979) was adopted by many researchers (Kok & Van Wyk, 1986; Kok & Seaman, 1987; Du Preez & Kok, 1992, 1993, 1995; Van Niekerk, Kok & Seaman, 1993; Du Preez & Lim, 2000; Lim & Du Preez, 2001; Du Preez, Vaucher & Mariaux, 2002; Du Preez, Tinsley & De Sa, 2003). Murith's protocol had its shortcomings. Measurement b, "e+" and "e-" (Figure 1.1) has poor repeatability and can not be objectively measured, consequently a new protocol had to be established. Du Preez & Maritz (2005) developed a new protocol for the genus *Polystoma*. They measured 13 measurements on at least 10 specimens from a number of *Polystoma* species. and through mathematical modelling they determined the combination of measurements that provided the best and most reliable classification potential using the computer software Matlab®.

An objective of the present study was to determine a taxonomic protocol for the genus *Eupolystoma* by evaluating all possible measurements and selecting the combination of measurements that gave the highest classification potential. It was shown that it is not always best to choose the greatest Classification Potential (CP) over the greatest Scale factor (SCa) (Figure 3.14). Both should be taken into consideration when attempting to plot a bi-variate graph (see Figure 3.15). The Scale factor gives an indication of how many products of the measurements will be included into the concentric ellipse; the concentric ellipse increases with SCa. The CP depends on the SCa and therefore no points will fall inside of the ellipse generated from a small SCa. As the SCa increases, more and more points are correctly classified. However, as the SCa gets too large, more of the points are misclassified. There should thus be an ideal scale factor where the CP is the largest (Figure 4.2) (Du Preez & Maritz, 2005).

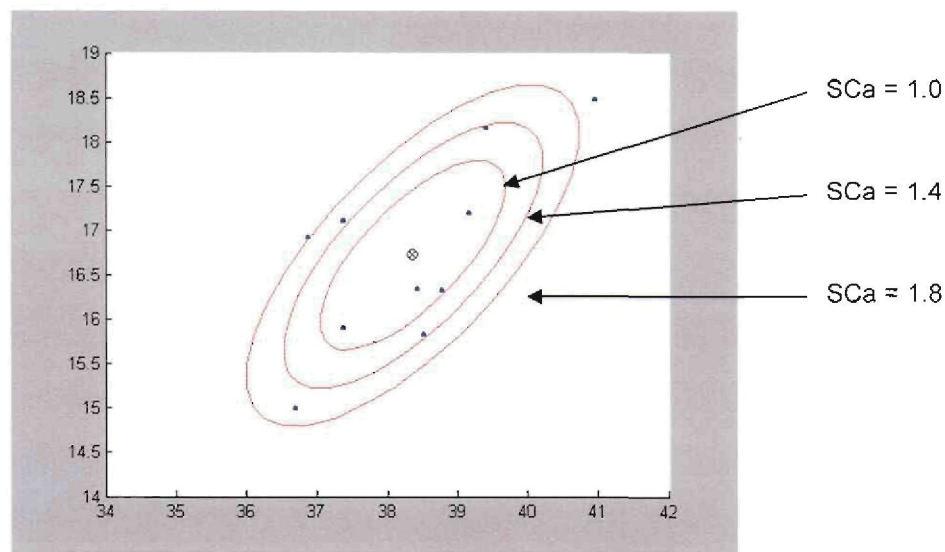


Figure 4.2: An example of the ellipses that are formed by the increasing (from 1.0 – 1.8) Scale Factor (SCa).

By determining statistical meaningful differences, large differences can be pointed out between species. According to Figures 3.2 - 3.12, large significant morphological differences can be seen between the two species *Eupolystoma vanasi* and *Eupolystoma* sp 1 especially with P1, P4 and P8.

The protocol developed by Du Preez & Maritz (2005) was successfully applied to the genus *Polystoma* (Figure 3.20). For *Eupolystoma*, the combination of measurements with the highest classification potential did not separate all species well. Some species did separate as distinct clusters. Based on clusters, it was observed that major differences between *Eupolystoma* sp 1 and *E. alluaudi* from *A. regularis* and *E. alluaudi* from *A. maculatus*. The clear difference of *E. vanasi* versus *E. alluaudi* from *A. maculatus* and *A. regularis* makes the second plot not as successful if taken in comparison with the plot from Figure 3.15.

By not eliminating the “poor” measurement parameters and only by replacing them with measurement parameters that had a reasonable

dependability, Figure 3.21 suggests a plot that can be used to differentiate between the species of *Eupolystoma* using marginal hooklets. It can clearly be observed that *Eupolystoma* sp 1 and sp 2 differ from *E. alluaudi* from *A. regularis* and from *A. maculatus* respectively. However, none of the *E. alluaudi* differ from each other on the basis of marginal hooklet measurements. This is due to the fact that host-specificity may not be applicable to the genus *Eupolystoma* in a host species-specificity way but may rather be applied to the term host family-specificity. *Eupolystoma vanasi* can also only be distinguished from *E. alluaudi* from *A. maculatus* and *A. regularis*.

Generally, the genus *Eupolystoma* can be differentiated on lack of features whose variation has been typically employed in polystomatid taxonomy (the absence of hamuli and of intestinal branching). Otherwise, characteristics such as the number of spines on the genital bulbus, the vitellaria formation and the intestinal caecal can also contribute to the taxonomy of the genus. Most important of all, the marginal hooklets are the most prominent feature for distinguishing between species.

Statistically meaningful differences of morphological features between the other species of *Eupolystoma* clearly indicate that there are differences between every single species except for the *E. alluaudi* cluster (Table 3.4). Here, *E. alluaudi* differs from the rest of the *Eupolystoma* spp. but not from each other. It has been controversial for almost a century whether *Eupolystoma* as a genus is host specific (Van Niekerk, 1992). Carefully planned host-specificity experiments might provide these answers but since the hosts are explosive breeders; correct timing would be essential.

Based on the various plots of marginal hooklet measurements of the *Eupolystoma alluaudi* complex, it is evident that *E. alluaudi* from *A. maculatus* and *E. alluaudi* from *A. regularis* are the same species based on morphological marginal hooklet measurements. Within the *alluaudi* complex *E. alluaudi* from *Nectophrynoides malcomi* cluster out as a separate species.

Although molecular evaluation did not give a conclusive answer whether *E. vanasi* and *Eupolystoma* sp 1 are the same or different species, morphological differences were observed. Morphological measurements can not be ignored and must be taken into account when studying and describing new polystomes.

Before evolutionary studies, biology consisted almost exclusively out of taxonomy. Naturalists of the 18th and 19th century identified animals, discovering patterns and making sense how the organisms evolved through time (O'Neil, 2007). Empirical patterns of a taxonomic range is important for identifying parasites to construct an orderly classification system, allowing new specimens to be classified with greater ease.

Recommendations for further studies would definitely include a more detailed molecular description of the *Eupolystoma* genus. The difference that was previously found between *Eupolystoma* sp 1 from *Amietophrynus garmani* and *E. vanasi* cannot illuminate that they are different species making further molecular research inevitable. By doing 18 S and 28 S analyses, species could be differentiated on a 500 bp and 1000 bp level. A phylogenetic cladogram would also give evolutionary evidence for this genus; determining different speciations.

More material for the *alluaudi* cluster is inevitably needed for a greater classification of the species; material that was not available for *E. alluaudi* included *E. alluaudi* from *Pyxicephalus adspersus*, and *E. alluaudi* from *Bufo taitanus*. Analysing these two species may drastically alter the results. Host-specificity experiments will help to determine the extent of preferences for the genus *Eupolystoma*. This would also determine if the genus is species-specific or family-specific and help to determine the *E. alluaudi* cluster.

In order to resolve the problems associated with the speciation of *E. rajai* and *E. chauhani* one would acquire more specimens. *Eupolystoma anterorchis* may be found if yearly seasonal data could be compared to see whether the parasite depends on the hosts' life cycle.

Although the protocol developed by Du Preez and Maritz (2005) does not provide the highest mathematical classification potential for *Eupolystoma*, it does provide relatively accurate separation of species. As this protocol complies with the set of criteria of a good parameter, it will most likely separate species for all polystome genera. Because of its availability, we therefore propose that the Polystoma protocol be used for *Eupolystoma*.

Chapter 5: References

Chapter 5: References

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Appendix A

Albumin mixture

Adhesive for histological sections.

The albumin concentrate mixture is prepared as follow:

- Egg white from one egg beaten to froth and allowed to stand overnight.
- Pour into measuring cylinder.
- Add equal volume of glycerin.
- Add one crystal of tymol to the mixture to prevent fungal growth.
- Keep refrigerated.

Prepare the working solution as follows:

- Three drops of albumin concentrate
- Add 50 ml of aqua dist and store at 4 °C.

Alum Carmine

- 100 ml aqua dist.
- Add 7 g alum carmine (or 4 g alum & 3 g carmine) and boil till dissolved (15 min).
- Leave to cool
- Add 100 ml of 70% EtOH solution.
- Leave to stand for a few days and filter.
- Biological tissue can be transferred to the stain from 50% or 70% EtOH.
- If the tissue is overstained, it can be destained by rinsing briefly in acid alcohol (Du Preez & van Wyk, 2007).

Acetocarmine

Stem solution

- Add 55 ml aqua dist in a glass beaker
- Add 45 ml acetic acid and heat the mixture, do not boil.
- Add 2 g carmine powder in small quantities.
- Stir after adding each quantity addition or use a magnetic stirrer.
- Allow to cool.
- Chill in refrigerator, and filter to remove undissolved carmine.
- Keep in an amber bottle at room temperature (Du Preez & van Wyk, 2007).

Working solution

- Take 5 ml aqua dist and add drop by drop of the stem solution till it has the light orange colour of orange Fanta soft drink.

Harris haematoxylin

For staining of histological sections.

The following is needed for the staining:

- 5 g of Haematoxylin
 - 50 mL of 95% EtOH
 - 100 g Alum, Potassium
 - 2.5 g Mercuric Oxide
-
- Dissolve haematoxylin in the EtOH and the Alum in the water by heating the two substances separately.
 - Placed two substances together when fully dissolved.
 - Bring to boil and add mercuric oxide. Continue to boil the mixture until it becomes a rich dark purple color.
 - Cool beaker with solution in water bath.
 - Add 40 ml Glacial Acetic Acid
 - When a golden sheen can be observed in the mixture, the solution is ready to filter. Be sure that the golden sheen does appear otherwise the stain would be inferior (Anon, 2007).

Appendix B

Marginal Hooklet measurements (P1-P13)

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13
<i>E. alluaudi</i> from <i>Amietophrynus maculatus</i>													
D1	22.79	9.42	13.26	12.36	10.54	12.54	9.25	22.1	7.63	8.45	2.75	1.81	11.35
D8	14.63	4.95	8.57	7.41	7.24	8.78	8.55	12.92	5.97	7.73	2.73	1.46	6.58
D10	13.94	6.5	7.18	5.51	6.5	8.47	6.99	13.37	4.23	4.45	2.1	1.3	3.35
D11	16.94	6.76	10.85	8.31	8.39	9.75	10.11	17.01	7.57	9.1	3.34	2.47	7.32
D13	14.61	5.28	8.17	6.07	5.85	7.57	8.85	12.75	5.08	7.29	2.87	1.63	5.54
D14	14.25	5.52	8.67	6.76	7.35	8.38	9.31	13.04	5.61	8.43	2.75	1.97	6.09
D18	20.42	7.22	11.63	10.17	9.23	11.51	7.82	18.31	5.28	6.83	2.08	1.58	8.04
D19	17.41	7.33	8.23	8.11	8.44	9.6	5.92	15.12	3.41	4.07	1.74	1.48	5.89
D23	20.35	7.69	12.01	11.08	9.42	11.47	7.75	19.16	5.15	6.23	2.67	1.97	8.46
D27	14.37	6.38	7.95	5.53	6.79	7.97	8.03	13.82	5.94	7.68	2.47	1.83	5.82
D30	13.96	5.9	6.75	4.94	6.27	7.35	7.77	10.85	5.19	6.13	2.27	1.37	4.6
D31	18.44	6.83	11.37	9.08	8.91	10.94	7.29	18.04	4.98	6.32	2.45	1.87	8.39
D32	22.51	8.76	13.37	13	10.58	12.68	7.64	21.33	6.17	7.08	2.61	2.13	11.68
D38	23.2	9.5	13.58	12.88	11.92	13.39	7.58	22.63	5.77	6.3	2.88	1.69	11.17
D41	19.96	7.41	11.7	10.77	8.69	10.57	8.2	18.64	5.93	7.41	2.82	1.88	8.32
D44	14.67	4.41	9.87	8.16	8.43	10.14	8.64	13.82	5.94	7.65	2.19	1.58	5.57
D49	20.44	7.34	13.66	11.78	8.74	10.81	8.35	19.95	7.09	7.75	2.83	2.45	10.05
D52	18.71	7.71	11.46	9.21	7.85	9.35	9.13	5.83	8.03	2.5	1.69	6.72	17.68
D63	14.01	6.36	7.52	4.79	5.61	7.83	8.65	12.2	5.57	6.81	2.78	1.91	6.33
D67	20.44	7.56	12.79	10.79	9.37	12.67	7.11	20.36	6.05	6.47	2.31	1.59	10.29
D70	21.02	8.17	13.86	11.98	9.78	11.78	6.44	21.49	6.68	6.52	2.63	1.37	11.25
D74	19.16	7.43	10.33	9.36	8.47	8.7	7.35	17.11	5.51	5.91	2.67	1.98	7.58
<i>E. alluaudi</i> from <i>A. regularis</i>													
C3	16.74	6.69	7.79	6.9	8	9.97	6.93	13.8	3.31	4.5	2.45	1.48	6.02
C10	24.89	7.51	12.57	12.06	10.29	12.25	7.38	19.66	5.6	6.42	2.63	1.87	11.25
C11	20.51	7.79	12.56	10.65	9.31	10.87	7.7	20.25	7.67	8.39	3.26	1.83	10.26

C12	22.8	7.13	15.26	14.58	11.4	13.76	8.36	22.33	7.9	9.1	2.69	2.03	9.58
C20	12.72	4.37	10.42	7.9	5.61	7.28	7.63	13.47	6.62	7.34	2.25	1.65	6.23
C33	20.79	8.12	11.85	10.66	9.3	11.02	6.8	19.41	6.01	6.77	2.72	1.48	9.56
C34	20.7	8.01	12.75	10.91	10.14	11.96	7.15	19.93	5.7	6.35	2.59	2.04	9.26
C36	24.59	8.81	15.01	14.65	11.2	13.37	8.2	23.54	5.84	7.66	2.36	1.82	12.33
C38	22.39	8.4	12.44	10.63	11.19	12.57	9.53	19.91	6.18	7.91	2.65	1.63	9.46
C40	17.01	7.02	9.52	6.82	8.6	10.08	7.93	15.89	4.45	6.9	2.85	2.47	6.76
<i>E. anterorchis</i> from <i>A. pantherinus</i>													
A	24.98	8.27	17.98	16.07	11.35	15.11	7.81	26.15	5.97	6.85	3.62	2.53	14.69
F	23.74	12.11	10.61	10.36	9.64	13.04	10.34	22.34	5.01	6.33	2.83	1.84	9.57
<i>E. alluaudi</i> from <i>Nectophrynoides malcomi</i>													
B5onko8	27.33	11.94	13.12	12.62	11.8	14.04	9.92	24.79	6.98	9.36	3.93	2.1	10.69
B5onko7	17.05	8.24	8.27	7.33	7.76	8.85	6.3	16.16	3.69	6.02	2.2	1.29	5.8
B5onko4	26.04	12.6	10.91	10.48	12.34	14.14	11.52	23.6	5.48	8.68	3.69	2.03	6.24
onko3	27.85	14	13.63	12.28	12.21	13.42	11.47	26.7	7.62	11.17	3.89	2.17	10.2
onko2	24.37	11.22	12.82	10.52	10.58	13.33	10.34	23.78	6.92	10.92	3.62	2.74	9.06
onko1	27.62	12.41	12.59	11.74	11.7	14.31	10.27	24.83	5.85	8.88	3.81	2.19	10.5
adult	65.03	35.49	28.35	24.35	23.72	31.33	26.68	61.46	20.7	24.42	9.12	7.31	25.88
B5E5	25.95	11.97	12.6	10.97	9.71	11.47	10.32	24.36	5.88	10.37	3.26	2.14	7.25
B5E3	18.84	7.37	11.47	9.77	9.24	10.96	8.08	18.62	6.29	7.16	2.88	1.69	8.19
B5E1	22.62	10.32	11.87	11.22	11.79	13.09	6.86	21.45	4.02	4.16	2.28	1.67	10.69
B5 onko1	27.62	12.41	12.59	11.74	11.7	14.31	10.27	24.83	5.85	8.88	3.81	2.19	10.5
B5 onko2	24.37	11.22	12.82	10.52	10.58	13.33	10.34	23.78	6.92	10.92	3.62	2.74	9.06
B5 onko3	27.85	14	13.63	12.28	12.21	13.42	11.47	26.7	7.62	11.17	3.89	2.17	10.2
B5 onko4	26.04	12.6	10.91	10.48	12.34	14.14	11.52	23.6	5.48	8.68	3.69	2.03	6.24
B5 onko7	17.05	8.24	8.27	7.33	7.76	8.85	6.3	16.16	3.69	6.02	2.2	1.29	5.8
B5 onko8	27.33	11.94	13.12	12.62	11.8	14.04	9.92	24.79	6.98	9.36	3.93	2.1	10.69
<i>E. vanasi</i> from <i>Schismaderma carens</i>													
vanasi 1	30.27	16.64	14.61	11.95	15.57	19.11	13.56	30.78	6.91	10.5	2.59	2.36	11.49
vanasi 2	34.6	13.69	17.59	17.74	19.34	21.67	9.84	31.11	3.52	7.06	2.61	1.41	16.48

vanasi 3	26.16	11.44	15.24	13.36	14.45	16.94	13.02	25.64	8.13	8.63	3.36	1.77	11.99
vanasi 4	29.73	13.49	15.87	14.07	14.25	17.36	10.85	28.98	6.1	8.62	2.99	1.37	11.91
vanasi 5	32.56	13.99	16.88	15.83	17.64	19.76	9.83	30.51	5.83	7.21	3.35	1.79	14.79
vanasi 6	30.18	13.79	14.89	13.17	14.75	18.48	11.37	28.47	5.62	7.61	2.86	1.96	12.02
vanasi 7	29.16	14	14.15	12.39	14.8	17.8	12.56	27.6	6.18	7.82	2.53	1.49	10.92
vanasi 8	27.71	12.76	14.63	12.6	13.59	16.02	12.03	26.81	6.08	8.6	3.01	1.79	10.73
vanasi 9	26.35	12.72	11.67	10.45	12.36	14.96	11.65	23.88	6.45	9.42	3.59	1.87	9.31
vanasi 10	22.83	11.11	10.4	8.23	11.13	13.49	11.03	20.96	6.25	8.64	2.76	1.73	8.81
vanasi 11	26.96	14.67	11.64	10.06	13.14	15.92	10.99	25.54	5.28	5.3	2.43	1.44	7.03
vanasi 12	31.85	13.72	16.37	16.02	16.26	18.77	11.95	29.75	6.05	8.76	3.46	1.29	15.25
<i>E. krugerensis</i> from <i>A. garmani</i>													
PL060103B17	33.53	16.03	17.65	15.94	17.84	21.08	12.91	33.42	7.22	9.27	3.12	1.91	14.68
PL060103B18	35.4	13.97	21.27	19.73	20.65	23.14	11.05	35.14	7.11	9.76	2.95	2.45	17.65
PL060103B19	37.27	17.69	20.52	18.08	20.93	25.19	13.65	37.39	7.65	9.66	3.49	2.58	16.9
PL060103B20	33.84	14.52	18.84	17.38	18.06	20.83	12.61	32.68	6.83	9.38	3.13	1.81	15.74
PL060103B21	37.66	16.99	21.02	19.27	21.64	24.81	12.48	37.47	7.23	9.31	3.32	2.1	17.84
PL060103B22	35.74	14.78	19.35	18.74	19.87	22.85	11.33	34.11	6.25	8.44	2.65	1.85	17.98
PL060103B23	35.48	16.39	18.98	17.4	18.87	21.51	11.94	34.75	7.02	9.22	2.71	1.98	15.69
PL060103B24	36.42	17.27	19.12	18.03	19.93	23.73	14.09	35.8	6.53	8.77	2.89	1.87	16.9
PL060103B25	33.99	15.36	19.35	17.27	18.12	21.33	10.73	34.32	6.4	8.44	3.2	1.87	15.38
PL060103B27	34.72	15.05	19.87	18.71	18.86	21.71	11.29	33.78	7.31	8.8	3.13	1.98	16.91
PL060103B29	32.82	13.61	19.15	18.04	17.62	20.57	10.93	32.54	6.33	8.93	3.58	2.09	15.1
PL060103B30	33.59	16.51	16.17	14.8	17.67	21.24	12.29	32.72	7.45	9.86	3.99	2.21	13.98
PL060103B31	32.33	15.42	18.44	15.36	16.87	19.96	12.29	32.72	7.38	9.24	2.95	2.42	13.58
PL060103B32	37.52	15.26	20.3	19.4	20.38	24.81	14.27	35.67	8.51	9.88	3.06	2.26	17.13
PL060103B70	33.76	14.47	16.09	15.35	17.62	19.69	11.49	30.42	6.74	6.25	3.03	2.09	14.68
PL060103B72	35.34	13.32	20.53	20.64	19.55	22.6	13.57	33.72	7.38	10	3.41	2.04	17.16
PL060103B73	37.73	14.77	22.22	22.36	22.11	24.96	11.56	36.25	7.98	8.93	3.12	2.09	20.02
PL060103B74	28.67	11.13	14.19	13.65	16.26	18.64	11.2	24.84	5.77	6.67	3.31	2.24	11.44
PL060103B76	31.45	12.34	15.83	15.13	16.77	18.67	11.32	27.94	4.77	7.89	3.24	1.55	12.67
PL060103B77	35.54	14.47	19.85	18.64	20.08	23.29	14.35	34.65	5.55	7.81	2.79	2.21	16.74

PL060103B78	35.34	13.32	20.53	20.64	19.55	22.6	13.57	33.72	7.38	10	3.41	2.04	17.16
PL060103B79	28.19	8.49	16.84	16.29	17.49	20.82	9.51	25.54	4.86	6.48	2.71	1.58	14.78
PL060104B1	33.72	13.26	18.53	17.62	18.72	21.16	12.69	31.2	7.11	7.63	3.55	2.43	16.26
PL060104B2	33.17	13.33	18.64	18	18.37	20.81	10.68	31.98	5.21	7.37	2.09	1.1	16.35
PL060104B3	33.78	14.11	18.05	16.38	18.71	21.86	12.39	32.02	6.42	7.78	3.56	2.09	15.58
PL060105A2	34.12	15.73	17.77	16.92	17.95	21.23	7.66	33.36	5.27	8.51	2.33	2.09	15.93
PL060105A22	37.08	15.89	20.57	18.59	20.41	23.03	11.33	36.07	7.75	8.81	2.52	1.37	16.77
PL060105A3	37.41	14.85	21.47	21.65	23.07	25.67	6.14	36.17	5.24	6.25	3.01	2.17	20.9
PL060105A4	35.71	15.13	17.6	17.46	18.21	20.53	5.1	32.88	3.24	5.81	2.99	1.87	16.47
PL060105B1	35.07	12.39	18.6	18.68	19.8	21.74	8.69	30.61	4.93	7.72	3.15	1.94	17.21
PL060105B8	35.09	13.67	20.45	20.2	19.58	22.18	9.49	34.32	5.46	8.06	3.47	1.91	18.36
PL060105C1	34.27	14.99	18.72	17.52	18.63	22.32	9.8	33.22	6.14	8.25	2.21	1.49	17.05
PL060105C2	31.62	12.95	18.67	17.04	17.03	20.39	10.74	31.7	6.98	8.23	2.63	1.81	15.07
PL060105C3	35.18	13.48	20.1	19.66	18.63	21.45	9.3	33.28	5.9	8.57	2.82	2.26	17.45
PL060105C6	27.7	9.71	17.55	15.22	16.21	18.89	10.2	26.85	6.77	7.92	3.71	2.24	12.96
PL060105C7	30	12.1	16.99	15.19	14.53	17.55	9.88	27.86	8.15	9.74	3.22	2.26	13.02
PL060105C8	26.99	11.54	15.44	13.19	14.13	16.88	11.15	25.98	7.25	9	2.91	1.54	9.92
PL060116A2	29.09	9.51	17.95	16.43	16.26	18.84	7.86	27.3	6.86	8.69	2.83	1.75	14.25
PL060116A3	33.16	14.11	18.46	17.95	18.55	22.21	8.38	32.12	5.25	5.59	2.75	1.13	16.89
PL060117B39	36.76	15.27	19.02	18.66	19.78	22.45	10	35.03	7.23	7.58	3.12	1.5	16.77
PL060117B40	36.57	15.83	18.83	18.45	20.58	23.4	10.27	34.78	5.78	6.74	3.53	2.3	16.77
PL060117B41	33.95	13.53	18.75	17.68	22.17	9.44	32.27	6.53	8.12	2.34	1.56	2.3	16.77
PL060117B42	36.05	15.95	18.88	18.56	19.41	22.4	9.28	34.69	5.59	6.8	3.57	2.04	18.39
PL060117B43	34.52	14.35	19.29	19.34	18.49	19.42	10.86	33.1	6.53	9.4	3.47	2.04	16.77
PL060117B44	34.38	14.08	20.2	16.87	19.01	22	8.8	34.14	5.59	7.91	2.43	1.13	16.63
PL060117B46	34.12	13.37	18.46	18.48	19.73	22.06	10.51	31.9	5.21	6.2	2.85	1.87	17.07
PL060117B48	35.66	15.09	20.47	18.71	20.72	23.65	13.79	35.32	6.03	8.96	3.56	1.78	17.01
PL060117B49	31.65	12.46	19.55	17.54	17.42	20.74	10.83	31.8	5.37	7.3	2.52	2.35	14.62
PL060117B50	31.5	10.88	15.9	16.39	18.04	20.52	10.87	27.03	6.15	9.94	2.86	1.87	13.92

Marginal Hooklet Measurements (without P5, P6 and P7)

	P1	P2	P3	P4	P8	P9	P10	P11	P12
<i>E. vanasi</i> 1	30.27	16.64	14.61	11.95	30.78	6.91	10.5	2.59	2.36
<i>E. vanasi</i> 2	34.6	13.69	17.59	17.74	31.11	3.52	7.06	2.61	1.41
<i>E. vanasi</i> 3	26.16	11.44	15.24	13.36	25.64	8.13	8.63	3.36	1.77
<i>E. vanasi</i> 4	29.73	13.49	15.87	14.07	28.98	6.1	8.62	2.99	1.37
<i>E. vanasi</i> 5	32.56	13.99	16.88	15.83	30.51	5.83	7.21	3.35	1.79
<i>E. vanasi</i> 6	30.18	13.79	14.89	13.17	28.47	5.62	7.61	2.86	1.96
<i>E. vanasi</i> 7	29.16	14	14.15	12.39	27.6	6.18	7.82	2.53	1.49
<i>E. vanasi</i> 8	27.71	12.76	14.63	12.6	26.81	6.08	8.6	3.01	1.79
<i>E. vanasi</i> 9	26.35	12.72	11.67	10.45	23.88	6.45	9.42	3.59	1.87
<i>E. vanasi</i> 10	22.83	11.11	10.4	8.23	20.96	6.25	8.64	2.76	1.73
<i>E. vanasi</i> 11	26.96	14.67	11.64	10.06	25.54	5.28	5.3	2.43	1.44
<i>E. vanasi</i> 12	31.85	13.72	16.37	16.02	29.75	6.05	8.76	3.46	1.29
<i>E. 1sp</i> 1	33.53	16.03	17.65	15.94	33.42	7.22	9.27	3.12	1.91
<i>E. 1sp</i> 2	35.4	13.97	21.27	19.73	35.14	7.11	9.76	2.95	2.45
<i>E. 1sp</i> 3	37.27	17.69	20.52	18.08	37.39	7.65	9.66	3.49	2.58
<i>E. 1sp</i> 4	33.84	14.52	18.84	17.38	32.68	6.83	9.38	3.13	1.81
<i>E. 1sp</i> 5	37.66	16.99	21.02	19.27	37.47	7.23	9.31	3.32	2.1
<i>E. 1sp</i> 6	35.74	14.78	19.35	18.74	34.11	6.25	8.44	2.65	1.85
<i>E. 1sp</i> 7	35.48	16.39	18.98	17.4	34.75	7.02	9.22	2.71	1.98
<i>E. 1sp</i> 8	36.42	17.27	19.12	18.03	35.8	6.53	8.77	2.89	1.87
<i>E. 1sp</i> 9	33.99	15.36	19.35	17.27	34.32	6.4	8.44	3.2	1.87
<i>E. 1sp</i> 10	34.72	15.05	19.87	18.71	33.78	7.31	8.8	3.13	1.98
<i>E. 1sp</i> 11	32.82	13.61	19.15	18.04	32.54	6.33	8.93	3.58	2.09
<i>E. 1sp</i> 12	33.59	16.51	16.17	14.8	32.72	7.45	9.86	3.99	2.21
<i>E. 1sp</i> 13	32.33	15.42	18.44	15.36	32.72	7.38	9.24	2.95	2.42
<i>E. 1sp</i> 14	37.52	15.26	20.3	19.4	35.67	8.51	9.88	3.06	2.26
<i>E. 1sp</i> 15	33.76	14.47	16.09	15.35	30.42	6.74	6.25	3.03	2.09

E. 1sp 16	35.34	13.32	20.53	20.64	33.72	7.38	10	3.41	2.04
E. 1sp 17	37.73	14.77	22.22	22.36	36.25	7.98	8.93	3.12	2.09
E. 1sp 18	28.67	11.13	14.19	13.65	24.84	5.77	6.67	3.31	2.24
E. 1sp 19	31.45	12.34	15.83	15.13	27.94	4.77	7.89	3.24	1.55
E. 1sp 20	35.54	14.47	19.85	18.64	34.65	5.55	7.81	2.79	2.21
E. 1sp 21	35.34	13.32	20.53	20.64	33.72	7.38	10	3.41	2.04
E. 1sp 22	28.19	8.49	16.84	16.29	25.54	4.86	6.48	2.71	1.58
E. 1sp 23	33.72	13.26	18.53	17.62	31.2	7.11	7.63	3.55	2.43
E. 1sp 24	33.17	13.33	18.64	18	31.98	5.21	7.37	2.09	1.1
E. 1sp 25	33.78	14.11	18.05	16.38	32.02	6.42	7.78	3.56	2.09
E. 1sp 26	34.12	15.73	17.77	16.92	33.36	5.27	8.51	2.33	2.09
E. 1sp 27	37.08	15.89	20.57	18.59	36.07	7.75	8.81	2.52	1.37
E. 1sp 28	37.41	14.85	21.47	21.65	36.17	5.24	6.25	3.01	2.17
E. 1sp 29	35.71	15.13	17.6	17.46	32.88	3.24	5.81	2.99	1.87
E. 1sp 30	35.07	12.39	18.6	18.68	30.61	4.93	7.72	3.15	1.94
E. 1sp 31	35.09	13.67	20.45	20.2	34.32	5.46	8.06	3.47	1.91
E. 1sp 32	34.27	14.99	18.72	17.52	33.22	6.14	8.25	2.21	1.49
E. 1sp 33	31.62	12.95	18.67	17.04	31.7	6.98	8.23	2.63	1.81
E. 1sp 34	35.18	13.48	20.1	19.66	33.28	5.9	8.57	2.82	2.26
E. 1sp 35	27.7	9.71	17.55	15.22	26.85	6.77	7.92	3.71	2.24
E. 1sp 36	30	12.1	16.99	15.19	27.86	8.15	9.74	3.22	2.26
E. 1sp 37	26.99	11.54	15.44	13.19	25.98	7.25	9	2.91	1.54
E. 1sp 38	29.09	9.51	17.95	16.43	27.3	6.86	8.69	2.83	1.75
E. 1sp 39	33.16	14.11	18.46	17.95	32.12	5.25	5.59	2.75	1.13
E. 1sp 40	36.76	15.27	19.02	18.66	35.03	7.23	7.58	3.12	1.5
E. 1sp 41	36.57	15.83	18.83	18.45	34.78	5.78	6.74	3.53	2.3
E. 1sp 42	33.95	13.53	18.75	17.68	6.53	8.12	2.34	1.56	2.3
E. 1sp 43	36.05	15.95	18.88	18.56	34.69	5.59	6.8	3.57	2.04
E. 1sp 44	34.52	14.35	19.29	19.34	33.1	6.53	9.4	3.47	2.04
E. 1sp 45	34.38	14.08	20.2	16.87	34.14	5.59	7.91	2.43	1.13
E. 1sp 46	34.12	13.37	18.46	18.48	31.9	5.21	6.2	2.85	1.87
E. 1sp 47	35.66	15.09	20.47	18.71	35.32	6.03	8.96	3.56	1.78

E. 1sp 48	31.65	12.46	19.55	17.54	31.8	5.37	7.3	2.52	2.35
E. 1sp 49	31.5	10.88	15.9	16.39	27.03	6.15	9.94	2.86	1.87
E. alluaudi f nectophrynoides 1	27.33	11.94	13.12	12.62	24.79	6.98	9.36	3.93	2.1
E. alluaudi f nectophrynoides 2	17.05	8.24	8.27	7.33	16.16	3.69	6.02	2.2	1.29
E. alluaudi f nectophrynoides 3	26.04	12.6	10.91	10.48	23.6	5.48	8.68	3.69	2.03
E. alluaudi f nectophrynoides 4	27.85	14	13.63	12.28	26.7	7.62	11.17	3.89	2.17
E. alluaudi f nectophrynoides 5	24.37	11.22	12.82	10.52	23.78	6.92	10.92	3.62	2.74
E. alluaudi f nectophrynoides 6	27.62	12.41	12.59	11.74	24.83	5.85	8.88	3.81	2.19
E. alluaudi f nectophrynoides 7	65.03	35.49	28.35	24.35	61.46	20.7	24.42	9.12	7.31
E. alluaudi f nectophrynoides 8	25.95	11.97	12.6	10.97	24.36	5.88	10.37	3.26	2.14
E. alluaudi f nectophrynoides 9	18.84	7.37	11.47	9.77	18.62	6.29	7.16	2.88	1.69
E. alluaudi f nectophrynoides 10	22.62	10.32	11.87	11.22	21.45	4.02	4.16	2.28	1.67
E. alluaudi f nectophrynoides 11	27.62	12.41	12.59	11.74	24.83	5.85	8.88	3.81	2.19
E. alluaudi f nectophrynoides 12	24.37	11.22	12.82	10.52	23.78	6.92	10.92	3.62	2.74
E. alluaudi f nectophrynoides 13	27.85	14	13.63	12.28	26.7	7.62	11.17	3.89	2.17
E. alluaudi f nectophrynoides 14	26.04	12.6	10.91	10.48	23.6	5.48	8.68	3.69	2.03
E. alluaudi f nectophrynoides 15	17.05	8.24	8.27	7.33	16.16	3.69	6.02	2.2	1.29
E. alluaudi f nectophrynoides 16	17.05	8.24	8.27	7.33	16.16	3.69	6.02	2.2	1.29
E. alluaudi f nectophrynoides 17	27.33	11.94	13.12	12.62	24.79	6.98	9.36	3.93	2.1
E. alluaudi from maculatus 1	22.79	9.42	13.26	12.36	22.1	7.63	8.45	2.75	1.81
E. alluaudi from maculatus 2	14.63	4.95	8.57	7.41	12.92	5.97	7.73	2.73	1.46
E. alluaudi from maculatus 3	13.94	6.5	7.18	5.51	13.37	4.23	4.45	2.1	1.3
E. alluaudi from maculatus 4	16.94	6.76	10.85	8.31	17.01	7.57	9.1	3.34	2.47
E. alluaudi from maculatus 5	14.61	5.28	8.17	6.07	12.75	5.08	7.29	2.87	1.63
E. alluaudi from maculatus 6	14.25	5.52	8.67	6.76	13.04	5.61	8.43	2.75	1.97
E. alluaudi from maculatus 7	20.42	7.22	11.63	10.17	18.31	5.28	6.83	2.08	1.58
E. alluaudi from maculatus 8	17.41	7.33	8.23	8.11	15.12	3.41	4.07	1.74	1.48
E. alluaudi from maculatus 9	20.35	7.69	12.01	11.08	19.16	5.15	6.23	2.67	1.97
E. alluaudi from maculatus 10	14.37	6.38	7.95	5.53	13.82	5.94	7.68	2.47	1.83
E. alluaudi from maculatus 11	13.96	5.9	6.75	4.94	10.85	5.19	6.13	2.27	1.37
E. alluaudi from maculatus 12	18.44	6.83	11.37	9.08	18.04	4.98	6.32	2.45	1.87
E. alluaudi from maculatus 13	22.51	8.76	13.37	13	21.33	6.17	7.08	2.61	2.13

<i>E. alluaudi</i> from <i>maculatus</i> 14	23.2	9.5	13.58	12.88	22.63	5.77	6.3	2.88	1.69
<i>E. alluaudi</i> from <i>maculatus</i> 15	19.96	7.41	11.7	10.77	18.64	5.93	7.41	2.82	1.88
<i>E. alluaudi</i> from <i>maculatus</i> 16	14.67	4.41	9.87	8.16	13.82	5.94	7.65	2.19	1.58
<i>E. alluaudi</i> from <i>maculatus</i> 17	20.44	7.34	13.66	11.78	19.95	7.09	7.75	2.83	2.45
<i>E. alluaudi</i> from <i>maculatus</i> 18	18.71	7.71	11.46	9.21	5.83	8.03	2.5	1.69	6.72
<i>E. alluaudi</i> from <i>maculatus</i> 19	14.01	6.36	7.52	4.79	12.2	5.57	6.81	2.78	1.91
<i>E. alluaudi</i> from <i>maculatus</i> 20	20.44	7.56	12.79	10.79	20.36	6.05	6.47	2.31	1.59
<i>E. alluaudi</i> from <i>maculatus</i> 21	21.02	8.17	13.86	11.98	21.49	6.68	6.52	2.63	1.37
<i>E. alluaudi</i> from <i>maculatus</i> 22	19.16	7.43	10.33	9.36	17.11	5.51	5.91	2.67	1.98
<i>E. aluaudi</i> f <i>regularis</i> 1	16.74	6.69	7.79	6.9	13.8	3.31	4.5	2.45	1.48
<i>E. aluaudi</i> f <i>regularis</i> 2	24.89	7.51	12.57	12.06	19.66	5.6	6.42	2.63	1.87
<i>E. aluaudi</i> f <i>regularis</i> 3	20.51	7.79	12.56	10.65	20.25	7.67	8.39	3.26	1.83
<i>E. aluaudi</i> f <i>regularis</i> 4	22.8	7.13	15.26	14.58	22.33	7.9	9.1	2.69	2.03
<i>E. aluaudi</i> f <i>regularis</i> 5	12.72	4.37	10.42	7.9	13.47	6.62	7.34	2.25	1.65
<i>E. aluaudi</i> f <i>regularis</i> 6	20.79	8.12	11.85	10.66	19.41	6.01	6.77	2.72	1.48
<i>E. aluaudi</i> f <i>regularis</i> 7	20.7	8.01	12.75	10.91	19.93	5.7	6.35	2.59	2.04
<i>E. aluaudi</i> f <i>regularis</i> 8	24.59	8.81	15.01	14.65	23.54	5.84	7.66	2.36	1.82
<i>E. aluaudi</i> f <i>regularis</i> 9	22.39	8.4	12.44	10.63	19.91	6.18	7.91	2.65	1.63
<i>E. aluaudi</i> f <i>regularis</i> 10	17.01	7.02	9.52	6.82	15.89	4.45	6.9	2.85	2.47
<i>E. anterorchis</i> 1	24.98	8.27	17.98	16.07	26.15	5.97	6.85	3.62	2.53
<i>E. anterorchis</i> 2	23.74	12.11	10.61	10.36	22.34	5.01	6.33	2.83	1.84
<i>E. sp</i> 2 f <i>hoeschi</i> 1	27.73	13.3	13.93	13.25	26.96	5.83	7.29	2.37	1.36
<i>E. sp</i> 2 f <i>hoeschi</i> 2	33.39	13.65	18.8	18.75	32.56	5.47	8.44	2.58	1.92
<i>E. sp</i> 2 f <i>hoeschi</i> 3	31.17	13.63	16.4	15.33	30.45	5.09	8.31	1.98	1.7
<i>E. sp</i> 2 f <i>hoeschi</i> 4	31.15	14.02	16.23	16.08	30.25	5.46	7.57	2.88	1.76
<i>E. sp</i> 2 f <i>hoeschi</i> 5	33.26	14.63	17.51	17.12	31.9	5.59	8.33	2.69	1.66
<i>E. sp</i> 2 f <i>hoeschi</i> 6	28.21	14.45	14.94	12.59	28.49	6.33	8.22	2.28	1.61
<i>E. sp</i> 2 f <i>hoeschi</i> 7	31.35	13.71	16.97	16.3	30.14	5.81	8	2.96	1.67
<i>E. sp</i> 2 f <i>hoeschi</i> 8	33.66	15	17.54	16.41	32.11	6.76	8.72	2.7	1.93
<i>E. sp</i> 2 f <i>hoeschi</i> 9	29.65	16.09	13.46	12.65	28.6	5.69	6.26	2.38	1.95
<i>E. sp</i> 2 f <i>hoeschi</i> 10	32.84	13.31	17.38	16.99	30.54	5.24	7.19	2.76	1.9
<i>E. sp</i> 2 f <i>hoeschi</i> 11	30.12	13.58	16.26	14.9	29.65	6.16	8.75	3.13	1.32

<i>E. sp 2 f hoeschi 12</i>	27.57	11.1	13.37	12.38	24.15	5.56	7.14	2.22	1.84
<i>E. sp 2 f hoeschi 13</i>	33.54	15.95	16.63	15.37	32.05	7.22	9.08	2.79	1.61
<i>E. sp 2 f hoeschi 14</i>	33.33	14.71	17.49	16.38	32.23	6.35	8.24	2.76	2.12
<i>E. sp 2 f hoeschi 15</i>	30.79	14.67	15.21	13.07	29.64	7.51	9.46	2.79	2.19
<i>E. sp 2 f hoeschi 16</i>	33.07	14.56	18.24	16.57	32.8	6.31	9.04	3.23	1.52
<i>E. sp 2 f hoeschi 17</i>	34	15.28	17.92	17.46	33.07	7.03	9.93	2.82	2.08
<i>E. sp 2 f hoeschi 18</i>	33.78	17.27	15.94	14.75	33.49	6.48	8.48	3.34	2.06
<i>E. sp 2 f hoeschi 19</i>	35.2	15.75	19.38	18.34	34.39	6.98	9.3	2.53	1.99
<i>E. sp 2 f hoeschi 20</i>	35.16	14.75	20.16	19.58	34.43	6.29	8.83	2.47	2.13
<i>E. sp 2 f hoeschi 21</i>	29.76	14.75	15.06	14	28.92	7.69	9.71	2.98	1.81
<i>E. sp 2 f hoeschi 22</i>	29.61	13.71	16.45	13.91	29.17	7.74	9.5	3.2	2.25
<i>E. sp 2 f hoeschi 23</i>	32.9	16.03	17.22	15.61	32.56	6.53	8.52	2.44	1.84
<i>E. sp 2 f hoeschi 24</i>	33.87	16.76	16.89	15.81	33.23	6.24	9.32	2.94	1.81
<i>E. sp 2 f hoeschi 25</i>	35.41	17.09	17.89	17.2	34.41	6.44	8.97	2.48	2.11
<i>E. sp 2 f hoeschi 26</i>	34.1	16.74	16.37	15.9	32.73	6.16	9.11	3.22	2.08
<i>E. sp 2 f hoeschi 27</i>	34.22	15.76	17.49	16.58	32.82	7.08	9.63	2.17	1.81
<i>E. sp 2 f hoeschi 28</i>	34.22	15.76	17.49	16.58	32.82	7.08	9.63	2.17	1.81
<i>E. sp 2 f hoeschi 29</i>	34.3	15.06	18.35	18.37	33.12	7	8.65	2.71	1.59
<i>E. sp 2 f hoeschi 30</i>	33.02	13.84	18.63	17.85	32.17	6.04	8.51	2.32	1.76
<i>E. sp 2 f hoeschi 31</i>	31.13	15.2	15.7	14.23	30.57	6.16	7.94	2.4	1.76

Body Measurements of *Eupolystoma* spp.

<i>Eupolystoma alluaudi</i> from <i>Amietophrynus regularis</i>																					
	Body length	greatest width	haptor length	haptor width	oral sucker	pharynx length	pharynx x width	ovary length	ovary width	gen bulb width	n Gen spines	gen spine length	sucker 1A	sucker 2A	sucker 3A	sucker 1B	sucker 2B	sucker 3B	n Eggs in utero	egg length	egg width
1	3368.2	19.02	978.08	1160.5	235.8	173.2	128.4			40.94			238.73	224.08	187.4	208.4	177	160.3	120	141.49	70.9
2	2808.9	1460.43	616.82	1006.4									216.39	224.45		190.5	160		114	0	0
3	2767.6	1692.96	630.84	919.37			95.72			42.46	8		225.52	213.71	185.1	206.2	186.4	166	103	133.73	65.5
4	2367.4	1076	580.54	673	203.7	121.8	104.3			43.91		24.5	208.67	170.49	132.4	192.2	175.2	116.8	121	127.26	57

5	2381.2	1327.98		727.47	223.9	94.53	75.62	312.7	84.31	35.64			185.14	151.72		187.2	181.9		60	130.29	61.6
6			495.31	798.47									190.46	163.17		187	184.3		72	131.57	61.2
7	2372.9	1278.08		904.99	128.1								176.47	189.56	164.1	178.4	155.3	150.8	124	137.39	64.3
8					222.8	136.9	111			41.16									56		
9	2787.6	1520.44	560.82	908.37	244	134.3	108.3	305.4	78.21	40.09	5		194.82	163.65		213.2	231.4		106	128.51	61.1
10		1196.97	417.31	753.49				262.7	84.56	44.08	7		186.97	158.48		175.3	167.6		50	127.61	64.5
Average	2693.4	1196.483	611.389	872.46	209.7	132.1	103.9	293.6	82.36	41.18	6.67	24.5	202.57	184.37	167.2	193.2	179.9	148.5	92.6	117.5389	56.2
Minimum	2367.4	19.02	417.31	673	128.1	94.53	75.62	262.7	78.21	35.64	5	24.5	176.47	151.72	132.4	175.3	155.3	116.8	50	0	0
Maximum	3368.2	1692.96	978.08	1160.5	244	173.2	128.4	312.7	84.56	44.08	8	24.5	238.73	224.45	187.4	213.2	231.4	166	124	141.49	70.9
Number																					
STD	362.76	513.5294	177.726	152.44	42.26	28.42	17.54	26.98	3.596	2.871	1.53	####	20.909	29.323	25.46	13.34	22.08	22.03	29.684	44.32976	21.4
KV																					
<i>Eupolystoma krugerensis</i> from <i>Amietophrynus garmani</i>																					
	Body length	greatest width	haptor length	haptor width	oral sucker	pharynx length	pharynx width	ovary length	ovary width	gen bulb width	n Gen spines	gen spine length	sucker 1A	sucker 2A	sucker 3A	sucker 1B	sucker 2B	sucker 3B	n Eggs in utero	egg length	egg width
PL06 0103 B10	3498.6	1462.73	614.83	946.24	366.6	168.3	147.8	289.4	71.75	49.53			178.23	196.54	181	203.9	162.8	151.3	29	161.03	88.8
PL06 0103 B9	3198.7	1242.69	518.87	909.09	346.8	141.8	113.3	209	56.18	48.52			215.91	210.67		203.4	163.8		42	151.75	87.6
PL06 0103 B8	3678.1	1632.59	572.67	103.32	318.7	158.5	151.1	268.2	83.33	59.49	5		223.33		158.8	196.4		195.5	58	149.09	83.6
B7	4204.6	1311.41	574.18	1079.5	357.7	175.8	138.2	313.7	74.85	61.95			230.59	225.26	170.3	193.7	187.5	171.3	39	120.87	89.3
B6	3347.8	1676.87	503.56	1058.9	39.27	181.3	150	323	71.6	55.66			228.94		194.8	184.6		162.5	48	136.14	79
B5	3299.8	1340.39	557.94	885.07	259.5	149.7	136	315.4	66.91	52.98	5				178.4			174.2	35	132.22	86.4
B4	3678	1288.23	610.25	955.09	323.5	177.5	145.6	290.5	49.85	56.72	5		227.9	207.19	198	203.8	176.8	170.7	51	112.67	76.1
B3	3848.3	1411.33	534.95	975.6	374.8	169.1	149.6	292.3	81.71	60.08	5		218.08	208.83	159	173.3	173.3	181.4	36	146.38	66.7
B2	3282.9	1074.92	504.7	865.43	352.3	151.6	130.8	250.1	54.1	59.02			218.33	190.39	163.4	196.8	162	163.9	48	103.67	73
B1	3589.2	1322.99	501.36	958.21	371.3	154.4	131.9	212.1	79.94	41.68	5		189.41	174.87	171.9	218.2	197.9	150.6	56	109.22	64.8
B11	3321.9	1425.44	537.54	891.66	361.1	219.9	170	226.6	79.75	55.36			200.39	181.77	165.8	183.1	191.9	205.2	50	103.98	58.8
B12	3614.7	1335.31	579.98	985.83	350.6	167.4	148.4	303	78.13	53.56	5		203.76	187.94	182.8	189.8	199.5	172.9		116	62.9
B13	3014	1408.93	527.83	909.38	337.1	174.8	147.7	238.7	77.86	56.44			210.05	170.91	167.8	187.3	205.5	176	40	125.96	78.7

B14	3521.8	1292.52	567.24	910.4	350.2	179.5	139.8	222.7	80.96	55.45			205.43	183.94	174.9	204.4	190	176.4	44	132.29	69.9
B15	2980.2	1153.87	486	803.24	281.6	144.8	140.2			53.09	5		183.29	174.23	148.3	152.7	164.4	142.1	38	100.69	58.1
B16	2814.5	1322.04	520.13	826.54	355.8	165.4	143.4	233.3	85.88	56.04	5		183.94	170.41	169.7	174.1	167.6	157.4	39	154.9	86.8
Average	3430.8	1356.391	544.502	878.85	321.7	167.5	142.7	265.9	72.85	54.72	5	####	207.84	191	172.3	191	180.2	170.1	43.533	128.5538	75.6
Minimum	2814.5	1074.92	486	103.32	39.27	141.8	113.3	209	49.85	41.68	5	0	178.23	170.41	148.3	152.7	162	142.1	29	100.69	58.1
Maximum	4204.6	1676.87	614.83	1079.5	374.8	219.9	170	323	85.88	61.95	5	0	230.59	225.26	198	218.2	205.5	205.2	58	161.03	89.3
Number																					
STD	349.79	152.263	39.2532	219.48	81.65	18.8	12.15	40.38	11.26	5.001	0	####	17.644	17.524	13.33	16.18	15.78	16.65	8.1492	19.93368	11
KV																					

Eupolystoma namibiensis from *Poyntonophrynus hoeschi*

	Body length	greatest width	haptor length	haptor width	oral sucker	pharynx length	pharynx width	ovary length	ovary width	gen bulb width	n Gen spines	gen spine length	sucker 1A	sucker 2A	sucker 3A	sucker 1B	sucker 2B	sucker 3B	n Eggs in utero	egg length	egg width
1BH	3477	920.33	575.27	1013.2	326.6	178.8	129.7	277.9	93.72	55.36	5	35.9	233.32	271.84		233.3	223.8			128.23	70
2BH	585.52	244.5	196.68	243.86	115	49.95	52.56						100.47	91.99		96.49	78.21				
3BH						84.12	57.57														
4BH	4413.6	1293.68	927.63	1010.3	401.3	227.7	214	251.7	126.2		4	40.6	305.07	272.24	242.7	317.1	301.1	254.5	73	174.1	113
5BH	5212.9	1264.15		986.82	327.4					80.51	4	34.8								177.07	109
6BH	6522.6	2495.57	1266.88	1572.7	356.1								334.85	393.45	414.7	327.7	391.8	435.9		141.1	102
7BH	3582	826.32	760.57	872.27	206	157.7	120.6					30.7	268.19	280.5		283.6	290.4		3	183.09	102
8BH	4200.9	1832	851.17	1177.5	278.4	147	109.3	202	65.5		7		314.54	302.28	298.7	309	241.8	230.9	118	132.94	80.3
9BH	1875.7	431.38	409.48	578.94	203.9	125	93.18						172.82	137.27		174.7	161				
10BH	1488.9	418.77	445.82	562.78	167.9	108.1	81.82			39.52	7	37.4	167.42	157.99	125.5	170.2	155.5	121			
11BH	1104.5	380.96	287.34	447.54	173.7	92.74	72.65						141.2	110.39		132.8	113.3				
12BH	1372.8	364.19	381.51	497.77	177.4	112.3	86.4						152.4	109.75		136.6	108.5				
13BH	1342.7	383.3	395.38	549.25	205	117.9	93.65						171.07	136.62		170.3	125.6				
14BH	1355.7	391.8	332.91	547.77	159.2	109.7	91.09						159.38	130.57		143.6	109.7				

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KV	16.183	18.41863	17.8171	16.276	18.02	10.52	14.03	27.73	13.32	0	3.63	9.68	18.066									
Eupolystoma alluaudi from Amietophrynes maculatus																						
	Body length	greatest width	haptor length	haptor width	oral sucker	pharynx length	pharynx width	ovary length	ovary width	gen bulb width	n Gen spines	gen spine length	1A	1B	2A	2B	3A	3B	n eggs	egg length	egg width	
D1	8.8	4.3		2.8	2	1.6	1.25	3.3	0.7	22	7		1.85		1.8				35			
D2	10.4	5.1		3.3	2.2	1.6	1.1			23	7		2.15		2				52			
D3	11.2	5.9		4.5	2.2	1.7	1.4	3.2	0.6	27.5	8		2.75		2.1				49			
D4	10.1	5.75		2.7	2.1			3	0.7	25	7		2.25		2.1				49			
D5	8.3	5.6		2.9	2.1		1.1			23	7		2.05		2				48			
6	8.4	4.6		2.75	2.2	1.6	1.2	3.4	1	22	7		1.9		1.7				37			
7	12.7	6.3		4	2.4	1.6	1.1			27			2.4		2.2				73			
8	9	5.3			2.1	1.6	1			24	7								51			
9	10.4	5.8	2.3	3.4	2	1.6	1.2	3.3	0.8	26	6		2.25		2.2		1.8		64			
10	10.55	5.85		3.7	2.2					28			2.2		2.1				61			
11	12	7.4		3.8	2.1	1.6	1.3	6.7	1.6	22	7		2.45		2.4		2.2		67			
12	10.8	6.1	5.3	3.2	2.1		1.2			24	7		2.3		2.2		1.25		56			
13	10	5.6	5.5	2.8	2.1	1.6	1.2			24.5			2.25		2.1				57			
14	10.1	6.6		3.3	2	1.7	1.2			24	7		2.1		2.25				69			
15	9.8	5.7		3.55	2.2					26	6		2.25		1.95				58			
16	5.4	2.3		1.8	1.7	1.3	1	2.1	0.5				1.5		1.25							
17	8.7	4.9			1.9		1.2	2.6	0.7	23.5	6		2		1.8		1.45		41			
18	9.3	5.6			2.1	1.7	1.2			23	7		2.4		1.95		1.85		60			
19	6.7	3.8			2.1	1.4	1.2	2.8	0.8		7		1.85						37			
20	5.4	2.8			1.6	1.3	1	2.4	0.7	24	7		1.7		1.5				3			
21	9.3	5.1		3.5	2.1		1.2			22	8				2				40			
22	10.7	6.4	3.2	3.2	2.2	1.7	1.3		0.8	25	6		2.3		2.2		1.85		68			
23	10.4	5.2	1.8	3.3	2.4		1.3		0.7	25	7		2.2		2.05		1.9		54			
24	11.2	5.9		3.6	1.9					25	7		2.3	2.15	1.85				64			
25		4.4		2.6						11.5	7		2		1.85				41			
26	10.4	5.4			2.2		1.2		0.7	23.5	6		2		2		1.6		53			
27	10.9	6.2	5.4	3.2	2.1	1.7	1.3		0.8	22	7		2.3		2.1		1.85		76			
28	9.4	4.9	1.9	3.05	2		1.1	3.6	0.7			1.95	1.75						72			

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