Functional feeding groups of mayfly nymphs (Ephemeroptera) in South African rivers of the North West and Limpopo Provinces

Esi H. Bossman (Student no. 1688346)

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Supervisor: Dr Darragh J. Woodford

Co-supervisor: Dr James du G. Harrison

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General abbreviations and acronyms

Abbreviations for mouthpart microstructures measured (reproduced from Banegas *et al*., 2020):

Abbreviation	Description
gl	Glossae length
gpl	Combined glossa and paraglossae length
gpw	Combined glossa and paraglossae width
gw	Glossae width
hl	Head length
hsl	Hypopharynx superlingua length
hsw	Hypopharynx superlingua width
hw	Head width
lil	Lingua length
liw	Lingua width
ll	Labrum length
lmdl	Left mandible length
lmdw	Left mandible width
lpl	Labial palp length
lpw	Labial palp width
lw	Labrum width
mpl	Maxillary palp length
mpw	Maxillary palp width
pl	Paraglossae length
pw	Paraglossae width
rmdl	Right mandible length
rmdw	Right mandible width

Abbr./Acronyms	Definition
FPOM	Fine particulate organic matter
FFG	Functional feeding group
M	Magaliesberg
MMU	Microscopy and Microanalysis Unit
PAST	Paleontological Statistics Software
PCA	Principal Component Analysis
SASS	South African Scoring System
SD	Standard deviation
SE	Standard error
SEM	Scanning Electron Microscope
W	Waterberg
WRC	Water Research Commission

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And to my dearest Granny, Jeanette Cikie Watani, I think of you every day and I miss you. This one's for you and I hope you're proud of me.

Abstract

Researchers, primarily in the Northern hemisphere, have extensively studied mouthpart morphology and dietary variability in Ephemeroptera (colloquially called mayflies). Ephemeroptera are key primary consumers in rivers around the world and are used as aquatic biomonitoring indicators due to interspecific variation in ecological tolerances that make them good indicator taxa. Cummins (1973, 1974) created a functional feeding groups (FFG) classification system, based on northern hemisphere macroinvertebrates, as a universal tool to functionally categorise organisms based on their primary feeding mode within their habitat. The FFG classification system is useful in identifying interrelations between structural and functional components of an ecosystem.

The aim of this study was to contribute knowledge on FFGs for particular mayfly species, in the southern African savanna biome, through mouthpart morphology and gut content analysis. Four study sites were sampled from the Magaliesberg (M) and Waterberg (W) catchments. There were three species studied, namely *Afroptilum parvum* (Family Baetidae), *Afronurus barnardi* (Family Heptageniidae) and *Elassoneuria sp*. (Family Oligoneuriidae). These species' mouthparts were dissected and their macro- and microstructures were prepared for viewing with Scanning Electron Microscopy (SEM) and light microscopy. Micrographs and images showed variation in mouthpart morphology between all three species, with special attention to the labia and maxilla microstructures. The identification and description of microstructures that differentiate mayfly species (and the adaptations of these appendages to facilitate food uptake and ingestion) are important to address knowledge gaps regarding mayfly mouthpart morphology. A Principal Component Analysis (PCA) showed distinct grouping of the three species, with mandibles and maxillary palps showing the most variation between species. After analysis, the following feeding groups were assigned: *Afroptilum parvum* is a collector-gatherer feeder, *Afronurus barnardi* is a brusher-scraper feeder and *Elassoneuria sp.* is a filter feeder.

This study showed, in part, that the Cummins' (1973, 1974) classification categories were too broad for classifying organisms from a South African savanna stream. However, it is a good foundational starting point for region specific FFG classification systems. Thus, it is recommended to use the classification system Cummins created as a starting point for classifying organisms into FFGs with an initial definition that can be expanded upon to create individual classification systems for different localities.

Keywords: Ephemeroptera, Functional Feeding Groups, *Afroptilum parvum*, *Afronurus barnardi*, *Elassoneuria sp*., Magaliesberg, Waterberg

Chapter 1: General introduction

Freshwater resources in the world and South Africa

Freshwater ecosystems are regarded as some of the most impacted and endangered ecosystems in the world (Allan & Flecker, 1993; Malmqvist & Rundle, 2002; Dudgeon *et al*., 2006; van Deventer *et al*., 2018). The Earth has approximately 3% of freshwater resources which are spread through lakes (0.008%), soils (0.005%), atmosphere, rivers, and biota (0.001%), groundwater (0.61%) and ice (1.97%) (Carpenter *et al*., 1992; Malmqvist & Rundle, 2002; Dudgeon *et al*., 2006; NASA, 2020). The rate of biodiversity decline in freshwater systems is much greater than that experienced in terrestrial ecosystems (Dudgeon *et al*., 2006). Freshwater resources provide a large variety of high-demand ecosystem services mainly domestic use, industry and agriculture, drinking, sanitation, and transport (Carpenter *et al*., 1992; Malmqvist & Rundle, 2002; Dudgeon *et al*., 2006). Rivers are diverse, but information on taxonomic and functional diversity is lacking, thus making it difficult to fully encapsulate their value and how best to protect and conserve them (Allan & Flecker, 1993; Malmqvist & Rundle, 2002; van Wyk *et al.*, 2006).

South Africa is a semi-arid country that must balance conserving integrity and longevity of freshwater systems with, meeting the ecosystem needs of the people (van Wyk *et al*., 2006). Only 58% of the South African population has access to safe drinking water (Jackson *et al*., 2016). An ecosystem can only be maintained when the minimum flow is constantly supplemented to allow for instream and riparian biota to survive and regulate that ecosystem (Harding & Taylor, 2011). Headwater streams are responsible for providing ecosystem services, transport of organic material and for making strong links to terrestrial zones as they influence the productivity of their environment at various spatiotemporal scales (Naiman &

Turner, 2000; Benda *et al*., 2005; Dudgeon *et al*., 2006; Callisto *et al*., 2012). They are usually geographically isolated systems that contain genetically isolated species, thus making them important for aquatic biodiversity (Gomi *et al*., 2002; Wipfli *et al*., 2007). Anthropogenic activity impacts river health, water quantity and quality, channel morphology, concentration of pollutants deposited into systems, riparian vegetation and the presence of invertebrates and macroinvertebrates necessary for biomonitoring (Palmer *et al*., 1996; Dickens & Graham, 2002; Jackson *et al*., 2020).

Additionally, the effects of global warming directly impact freshwater ecosystems through changes in run-off patterns, increases in floods and long-term droughts, and change in the reliability and distribution of streams, lakes, and wetlands particularly in a water stressed country like South Africa (Carpenter *et al*., 1992; Schneider, 1993; Dallas & Rivers-Moore, 2014; Archer *et al*., 2018). Understanding the impacts that these stressors have directly and indirectly on the rivers and their inhabitants is important to conserve them for future use.

Initiatives that encourage countries to conserve their freshwater ecosystems together are known as transfrontier conservation areas (TFCAs) – Southern Africa has identified 20 TCFAs, with six currently in progress in South Africa (van Amerom & Büscher 2005; Büscher, 2005). These provide an opportunity for countries to meet and merge land management efforts to conserve large systems that transcend human borders, whilst creating jobs for local communities (Büscher, 2005). One example is the Maloti-Drakensberg Transfrontier Conservation and Development Project (MDTP) created to protect biological diversity and cultural heritage (Büscher, 2005).

To protect and conserve our freshwater systems, we must first understand their biology. There have been numerous bioassessment studies done on freshwater biota that provide

information at different spatiotemporal scales about what is happening biologically and chemically in a riverine system and the importance of these key role players.

Macroinvertebrates and food web dynamics

The importance of macroinvertebrates in a system is linked to complex food web dynamics. Food webs are useful in understanding the feeding relationships within an ecosystem (Kitching, 2001) (i.e., who eats who). Macroinvertebrates are short generation communities that provide information at a snapshot in time, meaning that they are useful in determining immediate effects of disturbance within a river system. The term “disturbance” in this thesis will follow the definition from White and Pickett (1985) as, “‘any relatively discrete event that disrupts the structure of an ecosystem, community, or population, and changes resource availability or the physical environment” (Yount & Niemi, 1990; Turner, 2010). How stable a river system is, is dependent on how resistant it is to disturbance and the rates at which it recovers from disturbance (Yount & Niemi, 1990). Rates of recovery can include levels of recolonisation of historically present species and measuring levels of primary productivity.

However, there is much debate about food webs and energy trophic cascades in the literature, so delving into ecosystem food webs and how they function at multiple levels with various feedback loops can quickly become complex and sometimes oversimplified to focus on specific role players (Power, 1992; O’Neill, 2001; Allen & Fulton, 2010).

Macroinvertebrates live among the substrate and sediment; they cycle deposited nutrients (e.g. decomposed matter, faecal pellets, etc.) that are distributed within the habitat which are essential for aquatic flora, thus indirectly contributing to primary productivity. The availability of other feeding material such as in stream vegetation and algae therefore remain

abundant for the consumption by other larger consumers, such as fish – interconnectivity between different trophic levels.

Order Ephemeroptera

A key group of macroinvertebrates regularly used in bioassessment are the insect Order Ephemeroptera, colloquially known as mayflies (Bauernfeind & Moog, 2000). Mayflies are an ancient monophyletic group of insects found in freshwater and brackish water, comprising of about 3500 species, 450 genera, and 42 described families (Brittain, 1982; Ogden & Whiting, 2005; Sartori & Brittain, 2015; Gattolliat *et al*., 2018; Salles *et al*., 2018). They are the oldest extant winged insect order, dating back to Carboniferous and Permian periods, with studies done on this order from as early as 1675 (Barnard, 1932; Brittain, 1982; Barber-James & Lugo-Ortiz, 2003; Barber-James *et al*., 2008; Sartori & Brittain, 2015). In South Africa, recent taxonomic work has documented 11 families, 47 genera and approximately 102 species of mayfly. Ephemeroptera undergo a hemimetabolous life cycle, meaning they hatch from eggs, grow, and mature as nymphs (by moulting several times – ranges from 10-50 moults have been recorded) and emerge as adults without a pupal stage (Barber-James & Lugo-Ortiz, 2003; Barber-James *et al*., 2008; Sartori & Brittain, 2015). There is large diversity in the external morphology in the order Ephemeroptera which is useful for taxonomy and for understanding how certain feeding adaptations facilitate efficient feeding in the various habitats in which they occur (Barnard, 1932; Brown, 1961; McShaffrey, 1992; Barber-James *et al*., 2008; Arimoro & Muller, 2010).

Ephemeroptera are key primary consumers in South African rivers. They are used as aquatic biomonitoring indicators due to their interspecific variation in ecological tolerance that make them good indicator taxa (i.e. some taxa can occur in degraded environments, others cannot,

hence the composition of the mayfly assemblage informs us on the quality of the habitat) (Barnard, 1932; Hubbard & Peters, 1978; Moog *et al.*, 1997; Lugo-Ortiz *et al.*, 2000; Dickens & Graham, 2002; Barber-James & Lugo-Ortiz, 2003; Menetrey *et al.*, 2008; Arimoro & Muller, 2010; Pereira-da-Conceicoa *et al.*, 2012).

The exclusive focus on late instar nymphs (nymphs with visible dark forewing pads folded over the mesothorax, are indicative that they are final instar (Salles *et al.,* 2018)) in this study is for standardisation, allowing for analysis and comparison at the same life stage. Mayfly nymphal life stages have digestive systems and mouthparts, whereas the winged life stages are only alive for hours up to a few days after emergence from their nymphal stages and contain neither mouthparts nor a digestive system as they specialise in dispersal and reproduction only (Hubbard & Peters, 1978; Barber-James *et al.*, 2007; Barber-James *et al*., 2008; Jacobus *et al.*, 2019).

Macroinvertebrate functional feeding groups

The classification of macroinvertebrates into functional feeding groups (FFGs) examines the behaviour and morphology of macroinvertebrates and how these behavioural and morphological traits are linked to the modes in which macroinvertebrates acquire their food (Cummins & Klug, 1979; Merritt *et al*., 1996; Wallace & Webster, 1996, Cummins *et al.*, 2005; Sartori & Brittain, 2015). It provides information about the available food organisms are feeding on in their habitat, how their mouthparts are suited for certain feeding material types and the condition of the ecosystem (King *et al.*, 1988; Faith, 1990; Cummins *et al.*, 2005; Tomanova *et al.*, 2006; Arimoro, 2007). Cummins (1973, 1974) created FFG classifications for macroinvertebrates, namely: scrapers, filterers, shredders, gatherers, and predators.

Lenat and Barbour (1993) describe the functional feeding group classification as an extension of the River Continuum Concept (RCC) (Vannote, 1980). The functional group dominance exists in a gradient within a freshwater system. The proportions of various feeding groups (e.g. scraper, grazer, filterer) often vary moving from smaller tributaries into larger mainstems. This makes them ideal for comparative studies between catchments and for long term water quality monitoring (Roback *et al*., 1969; Dale & Clifford, 1976; Green & Vascotto, 1978; King *et al*., 1981; Matthews *et al*., 1982; Furse *et al*., 1984; Wright *et al*., 1984; Marchant *et al*., 1985; Faith, 1990; Palmer *et al*., 1993a, b; Palmer *et al*., 1996; Schael, 2005; Kenney *et al*., 2009).

The relative dominance or change of a feeding group can be indicative of a habitat change or a change in stream order (e.g. a marker to indicate the moving from a first order stream to a fourth order stream) (Lenat & Barbour, 1993). When there is a change in feeding group type within a system or from one system to another it is useful to investigate other enviornmental factors that may be contributing to the shift such as changes in canopy cover, riparian vegetation and substrate (King *et al*., 1988; Cummins, 1973; Lenat & Barbour, 1993).

Additionally, the FFG classification is useful in identifying interrelations between structural components of an ecosystem (community structure, biomass distributions, distribution of nutrients and ranges of physico-chemical parameters) and functional components (energy flow and regulation through the trophic levels, nutrient cycling, biological and ecological regulation) (Cummins & Klug, 1979; Matthews *et al.*, 1982; Ramírez & Gutiérrez-Fonseca, 2014).

For example, there may be a system containing scrapers, grazers, and filter feeders with varying habitat sensitivities. A hydrological biotope assessment will indicate that in the river

there are various ideal habitats for these functional feeders to thrive in: there are cobbles and bedrock with deposited feeding material, diatoms, and algae – habitat for scrapers and grazers to thrive; and there are stones in rapid current that have lightly accreted feeding material that is constantly suspended into the water column for capture and consumption – habitat for filter feeders. Additionally, with measures of water quality parameters (also denoted as physicochemical variables) for each of these feeding groups to inhabit these waters their range of ideal physicochemical tolerances exist. These parameters in the right concentrations allow for primary producers to exist in the system as not only a food source for macroinvertebrates but for other secondary and tertiary consumers. This example can continue to expand, and new connections be made between organisms as one moves up the trophic ladder.

General mouthpart components of final instar mayfly nymphs

Nymph mouthparts consist of various structures. The labrum (upper lip) forms the upper border of the mouth – a flap-like structure; paired asymmetrical mandibles (jaws) are used for crushing, scraping, cutting, or piercing food; paired maxillae have a sensory function, often bearing a long, segmented palp; on the unpaired hypopharynx is a tongue-like structure; and labium (lower lip) with paired appendages called paraglossae covered in setae (stiff hairs). The maxillary and labial palps are used for touching, tasting, and manipulating food (Figures 1-4) (Barber-James & Lugo-Ortiz, 2003).

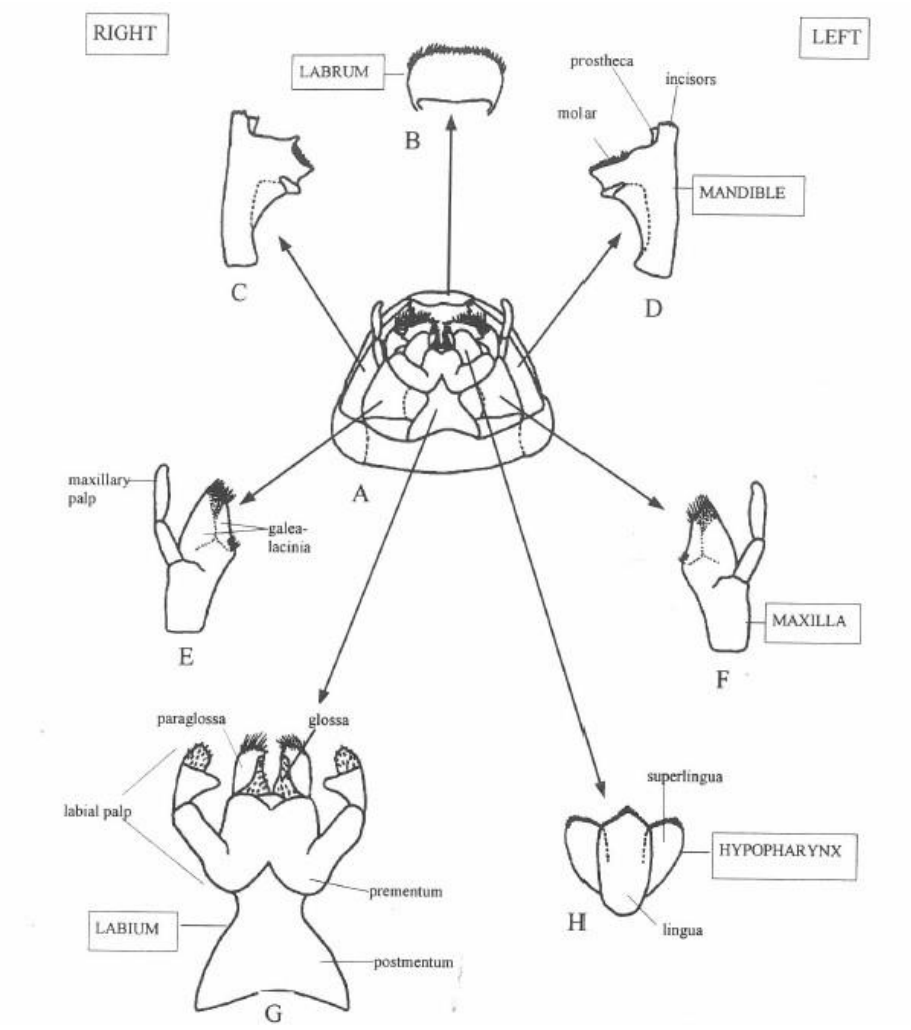


Figure 1. The head of an ephemeropteran nymph. A: head, ventral view, indicating position of various mandibulate mouthparts; B-H: ventral view of all mouthparts; B: labrum; C: right mandible; D: left mandible; E: right maxilla; F: left maxilla; G: labium; H: hypopharynx (found beneath the labium) (reproduced from Barber-James & Lugo-Ortiz, 2003).

Additionally, mayfly nymphs have distinguishing characteristics, namely: compound eyes that develop into turbinate eyes (a signature characteristic in males of some species of mayfly); ocelli are light-sensitive appendages on the head; slender antennae for sensory functions; well-developed legs; forewing pads with hidden hindwing pads; variously developed and orientated abdominal gills; two or three caudal filaments (tails or cerci) and one medial caudal filament; and chewing mouthparts (Barber-James & Lugo-Ortiz, 2003) (Figure 2).

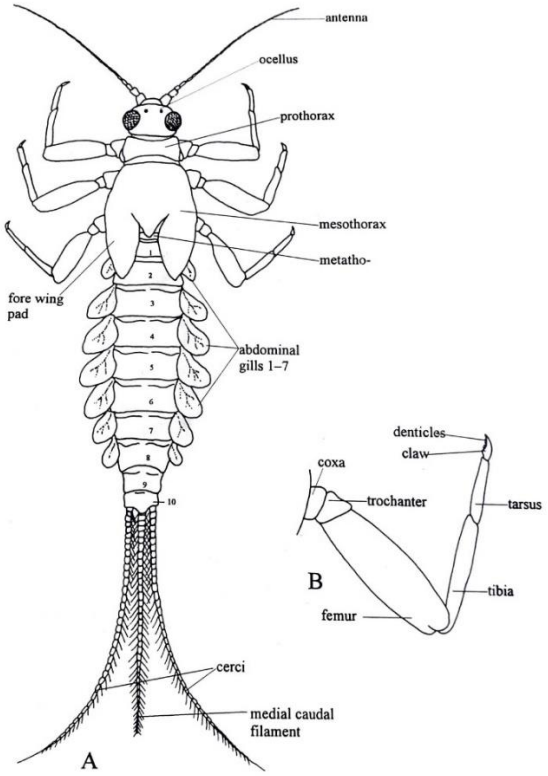


Figure 2. Features of a typical ephemeropteran baetid nymph. A: dorsal view of nymph; B: leg (reproduced from Barber-James & Lugo-Ortiz, 2003).

Information about the study families

Baetidae are a diverse group that follow the generalist morphologies described above for the family. Heptageniidae is an abundant and diverse flatheaded mayfly family with distributions throughout the world (Figure 3), consisting of about 59 genera, 1506 species and 66 subspecies, with six heptageniid species recognised in South Africa (Webb, 2007). They are often found in riffles, with females being generally larger than males – body length ranging from 10-11 cm (Barber-James & Lugo-Ortiz, 2003). The larvae are found to primarily feed on periphyton and detritus (McCafferty & Provonsha, 1986). This family is used in biomonitoring assessments as they are sensitive to anthropogenic activity (Webb, 2007). Heptageniidae consists of four subfamilies, namely *Aneporinae, Pseudironiae, Arthropleinae* and

Heptageniinae (Jensen & Edmunds, 1973). These families are generally widespread throughout sub-Saharan Africa (Jensen & Edmunds, 1973).

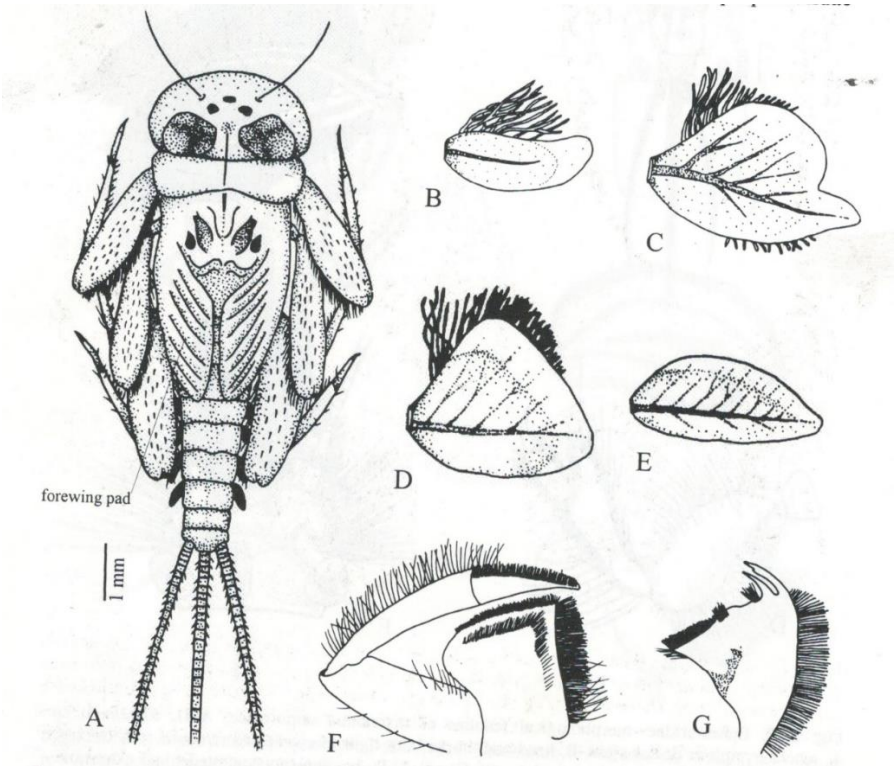


Figure 3. Heptageniid nymph morphological features. A: whole nymph; B – E: gill; F: maxilla; G: mandible (reproduced from Barber-James & Lugo-Ortiz, 2003).

Species from the family Oligoneuriidae are distinguished from other species by a carina on the head. They are found in fast-flowing streams at high elevations, with mature nymphs growing to 30 mm (3 cm) in size and are found in every continent around the world (except Australia) (Barber-James & Lugo-Ortiz, 2003). Oligoneuriids have two rows of setae on their forelegs (Figure 4), sometimes with a row of microtrichia grouped in bunches along the setae (Elpers & Tomka, 1995). They are found only in sections of rivers with highly oxygenated waters (Arimoro & Muller, 2010).

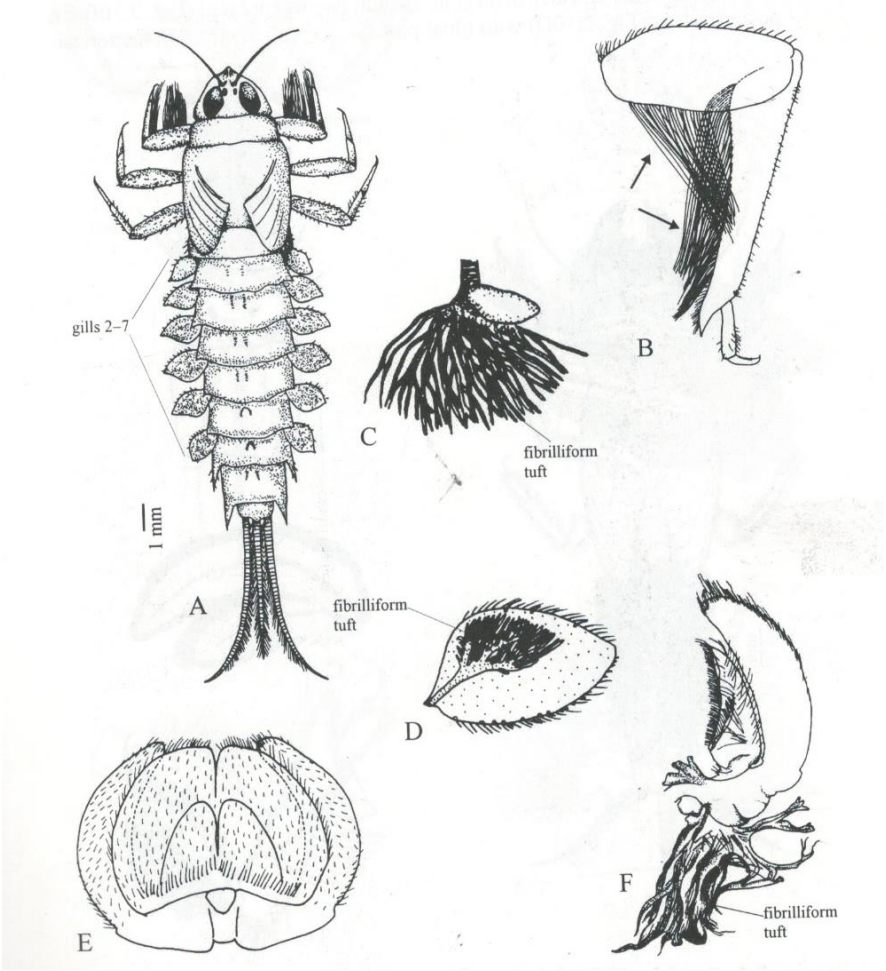


Figure 4. Oligoneuriid *Elassoneuria sp.* morphological features, A: whole nymph; B: details of foreleg; C – D: gill; E: labium, showing fusion of parts; F: maxilla, showing fibrilliform tuft (reproduced from Barber-James & Lugo-Ortiz, 2003).

Taxonomic diversity of study species

There have been numerous studies on families within the order Ephemeroptera to better understand the relationships between and within families through cladistical analyses (e.g., Spieth, 1993; Wang & McCafferty, 2004, Ogden & Whiting, 2005; Sun & McCafferty, 2008; Ogden *et al*., 2009; Sartori & Brittain, 2015; Gattolliat *et al*., 2018; Cai *et al*., 2018). Within the suborder Pisciforma comprising of a monophyletic group consisting of three families namely Baetidae, Heptageniidae and Oligoneuriidae (Figure 5). All three families have the same common ancestor and were chosen for this study as they all likely evolved from a common

ancestor with a common mouthpart plan that has since diverged through ecological pressures.

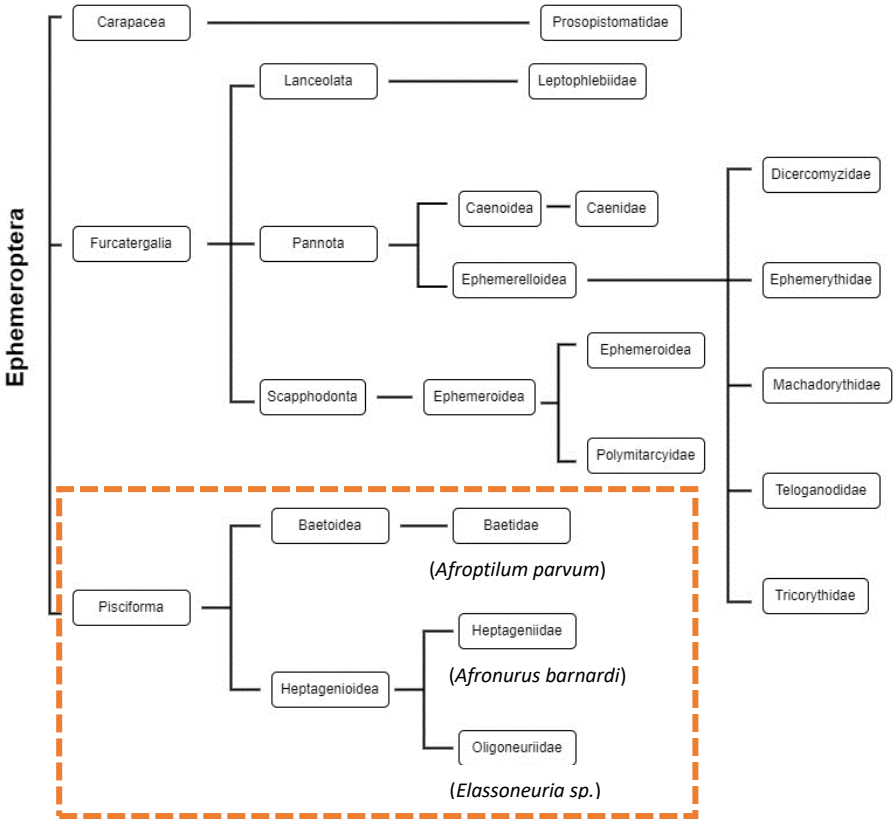


Figure 5. Ephemeroptera family tree with monophyletic clade containing descendants from the Pisciforma suborder (orange) (Sartori & Brittain, 2015) (figure adapted from writings by Fry (2021)).

The three study species of this thesis are *Afroptilum parvum* (baetid) (Gillies, 1990), *Afronurus barnardi* (heptageniid) (Schoonbee, 1968) and *Elassoneuria sp.* (W) (oligoneuriid) (Gillies, 1974; Agnew, 1980). They were collected from both the Magaliesberg (M) and Waterberg (W) catchments (see study sites and study species in Chapter 2). The species collected from each of the localities in this study were denoted with an 'M' or 'W' (Table 1). This study makes within-species comparisons (*A. parvum* compared between the Magaliesberg and the Waterberg) and between-species comparisons made with species of known FFG status studied by Palmer *et al*. (1993a, b), namely *Afroptilum excisum*, *Afronurus harrisoni* and *Neurocaenis reticulata* (Table 1). These taxa serve as comparative proxy taxa from the same

genus as the model study species, or as a representative taxon collected from the Buffalo River with similar mouthpart structures. A "proxy taxa" for this study is defined as a taxon that is different from the model species of this study and is in a comparable environment with a comparable ecology. Since it was unknown whether the model species chosen would have a comparable ecology or not, taxa were collected from the same genus where possible – *Afroptilum* and *Afronurus* – and from reading the literature Tricorythidae has some taxa with comparable ecology to taxa from Oligoneuriidae – therefore *Elassoneuria* is compared to *Neurocaenis*. This comparison will investigate whether the classifications will be the same between compared species collected from different localities and explore the implications should the compared taxa belong to different functional feeding groups.

Additionally, 21-22 mouthpart microstructure measurements were done for each species for comparison between model species and for FFG classification. These measurements were adapted from work done by Banegas *et al*. (2020) on the mouthpart morphology and food habits of a *Cloeon dipterum* population from Buenos Aries (Appendix C).

Table 1. Rivers sampled and species collected from the Magaliesberg and Waterberg catchments, with findings from the Buffalo River, Eastern Cape taxa for comparison and their respective FFG classifications (Palmer *et al*., 1993a, b).

Location	Magaliesberg	Waterberg	Eastern Cape
Rivers sampled	Sterkstroom/Maretlwane	Lephalala	Buffalo
Study sites	A-B	C-D	1-13
Study species and known FFGs	*Afroptilum parvum* (*M)*	*Afroptilum parvum* (W)	*Afroptilum excisum* (It was *Centroptilum excisum* at the time of publication by the author) **[FFG: gatherer]**
		Afronurus barnardi (W)	*Afronurus harrisoni* **[FFG: scraper]**
		Elassoneuria sp. (W)	*Neurocaenis reticulata* (family Tricorythidae) – different family to Oligoneuriidae but similar feeding apparatus **[FFG: passive filterer]**

The second chapter in this study examined the mouthpart morphology of *Afroptilum parvum*, *Afronurus barnardi* and *Elassoneuria sp*. The aim of this chapter was to classify nymphs of these mayfly species commonly found in the headwaters of the Magaliesberg and Waterberg tributary catchments (western Limpopo River basin) into FFGs based on their mouthpart morphology. This was achieved by the creation of four objectives dealing with, (1) nymph species identification through dissection; (2) the analyses of whether all species show distinct and divergent characteristics; (3) to determine whether *Afronurus barnardi* (W) and *Elassoneuria sp*. (W) show convergent mouthpart morphology when compared to same/representative taxa of known FFG status from Palmer *et al*. (1993a, b) and; (4) whether three congeneric taxa *Afroptilum excisum*, *A. parvum (M)* and *A. parvum (W)* which are similar morphologically and ecologically would still fall into the same FFG and what that infers about their feeding niches.

The third chapter investigates the degree of dietary variability between *A. parvum (M)* and *A. parvum (W),* respectively collected from headwaters of the Magaliesberg and Waterberg tributary catchments. The primary objective uses the mouthpart morphology results from the previous chapter determine whether there is significant difference in drivers causing morphological variation within a species, from different regions, and if this is linked to significant differences in diet.

Chapter 2: Mouthpart morphology

Introduction

Macroinvertebrates and functional feeding groups (FFGs)

Macroinvertebrates are abundant and diverse primary consumers that modify and regulate ecosystems through processes such as material transport, provision of ecosystem services and ecosystem engineering (Wallace & Webster, 1996; Covich *et al.*, 1999; Jackson *et al.*, 2020) – thus mediating essential river processes (Williams *et al.*, 2004; Dudgeon *et al.*, 2006; Ramírez & Gutiérrez-Fonseca, 2014). Understanding their feeding behaviours, habitat and food preferences help us infer their function within their ecosystems (Merritt *et al*., 1996; Baptista *et al*., 2006; Ramírez & Gutiérrez-Fonseca, 2014).

Macroinvertebrate community structure is dependent on the availability of ideal habitats in various biotopes and the feeding relationships between and within different species, in order to infer community structure and the roles played within an ecosystem (Vannote *et al*., 1980; Baptista *et al*., 2006; Wolmarans *et al.*, 2017). Macroinvertebrates are useful for detecting levels of disturbance in both lentic and lotic ecosystems (Wallace & Webster, 1996; Mandaville, 2002; Hauer & Resh, 2017). They are direct regulators of the nutrient cycle, as they ingest particles, process them metabolically and excrete nutrient-rich by-products that are essential for plants within their ecosystems (Anderson & Sedell, 1979; Hall Jr *et al.*, 2007; Ramírez & Gutiérrez-Fonseca, 2014; Fierro *et al.*, 2015). Nutrient cycling is a process facilitated by macroinvertebrates, and without this process plants and other animals would be unable to utilise or live in these water bodies (Cummins, 1973; Anderson & Sedell, 1979). So, when there is a loss of that species or an increase in the population size they can be indicative of a disturbance in their habitat (Johnson *et al.*, 1993; Mandaville, 2002) – this is

useful in infering river health based on the number of taxa, abundance and biotope they were collected from in each aquatic region (Dickens & Graham, 2002).

Criticism in the literature of FFG classification systems

There has been criticism in the literature from southern hemisphere ecologists of the North American FFG classification system (Cummins 1973, 1974; Vannote *et al*.,1980; Merritt & Cummins, 1984), as southern hemisphere ecologists have found that although these classification systems were stated to be useful universally they were not useful in a southern hemisphere river context as they yielded different results to local southern African classification systems (e.g. Winterbourn *et al*., 1981; King *et al*., 1988; Palmer & O'Keffee, 1992; Palmer *et al*., 1996; Ramírez & Gutiérrez-Fonseca, 2014). King *et al*. (1988) studying macroinvertebrate community structure and FFGs in Langrivier, South Africa, found that when using Merritt and Cummins' (1984) FFG classification method compared to their own classification method, the results differed substantially – the results of the proportional percentage of FFG groups in the Langrivier when using the local method (King et al., 1998) and the method created by Merritt and Cummins (1984) were so different that it questioned the validity of each of the classification methods. These same authors have questioned how different the northern and southern hemisphere rivers and their community structures must be to yield such different results or if rather there is an error with the classification system? (Winterbourn *et al*., 1981). Palmer and O'Keeffe (1992) successfully classified macroinvertebrates found in the Buffalo River, Eastern Cape into FFGs by investigating the feeding material consumed rather than the mechanism of food uptake, which is a slightly different angle to previously practised methods of FFG classification in the literature.

The largest controversy in the literature is the definition of the FFGs and which mouthparts or feeding behaviours and preferences are important to consider in this classification (Barmuta, 1988; Palmer & O'Keeffe, 1992). Baptista *et al.* (2006) and Palmer *et al.* (1993b) found that the labium and maxilla were the mouthpart structures with important information on feeding strategy, thus making them the most useful microstructures to observe when classifying macroinvertebrate nymphs into FFGs. The maxillary and labial palps have been described to be used for touching, tasting, and manipulating food across all species of insects (Barber-James & Lugo-Ortiz, 2003).

As a consequence of this methodological controversy an additional goal of this study will be to determine if the use of Cummins' (1973, 1974) and Merritt and Cummins (1984) methods of FFG classification are appropriate for classifying three common species of Ephemeroptera from South African Savanna streams into established FFGs. Investigation into whether the North American classification system is too broad and needs to be re-evaluated and modified per region to be useful in a southern African river context is, however, beyond the scope of this thesis.

Predominant FFG classification types in the literature

In the literature FFG classifications are derived from three sources, namely an investigation into mouthpart morphology (King *et al*., 1988; Palmer *et al*., 1993b; Baptista *et al*., 2006), gut content analysis (Gray & Ward, 1979; Sephton & Hynes, 1983; Feminella & Stewart, 1986; King *et al*., 1988; Walker *et al*., 1988; Palmer *et al*., 1993a; Wögerbauer & Kelly-Quinn, 2013) or feeding behaviour experiments (Palmer & O'Keeffe, 1992). However, there is difficulty in observing feeding behaviour (feeding behaviour trials were run for this study – see Appendix B) in macroinvertebrates because they are small, fast-moving organisms so analysis of gut

content has become the preferred method for assessing feeding material in conjunction with mouthpart morphology in determining FFGs (King *et al*., 1988; Palmer & O'Keeffe, 1992).

Aims, objectives and hypotheses

The aim of this chapter was to classify nymphs of mayfly species commonly found in the headwaters of the Magaliesberg and Waterberg tributary catchments (western Limpopo River basin) into functional feeding groups (FFGs) based on their mouthpart morphology. The project focused on baetid *Afroptilum parvum* (M), baetid *Afroptilum parvum* (W), heptageniid *Afronurus barnardi* (W) and oligoneuriid *Elassoneuria sp.* (W) which are recognised primary consumers of fine particulate organic matter (FPOM) and algae, in South Africa (Barber-James & Lugo-Ortiz, 2003). Observations of mouthpart morphology of these Savanna mayfly taxa were contrasted to those of same or similar proxy taxa collected from the Buffalo River (Palmer *et al*., 1993a, b), to determine whether the FFG assignment (modified by Palmer *et al*., 1993a, b) is consistent or different between the grassland/savanna ecoregions (Highveld, Western Bankenveld, Waterberg) and the Eastern Coastal Belt ecoregion.

The following four objectives were pursued to classify the mayflies into functional feeding groups:

Objective 1. Analysis under the ZEISS Stereo Discovery V8 light microscope of gill structure, overall body shape, size and microstructures were used to identify the species based on available taxonomic keys.

Objective 2. Comparison between species to determine distinct and divergent characteristics in their mouthpart morphology, through dissection and imaging. It was hypothesised that these mayfly taxa would show differences in mouthpart morphology because their feeding biomechanics differ.

Objective 3. Investigate whether *Afronurus barnardi* (W) and *Elassoneuria sp.* (W) differ in their feeding morphology to proxy taxa with confirmed FFG status in the Buffalo River, Eastern Cape. It was hypothesised that taxa occurring in the Waterberg would show convergent mouthpart morphology with the proxy taxa found in the Buffalo River.

Objective 4. Investigate whether three congeneric taxa *Afroptilum parvum* (M), *A. parvum* (W) and *A. excisum* Barnard 1932 (from the Buffalo River, Eastern Cape – Palmer *et al*., 1993b) – which are similar morphologically and ecologically – would fall into the same FFG and what that infers about their feeding niches. Information on FFG and mouthpart morphology for *A. excisum* was taken from Palmer *et al*. (1993b). It was hypothesised that species from the same genus that are morphologically and ecologically similar, from three different localities, will fall into the same feeding niche.

Materials and methods

Study catchment

The Waterberg catchment is located within the Waterberg Biosphere Reserve is in the Waterberg District of the Limpopo Province (Baber *et al.*, 2003). The Waterberg is a sandstone massif that is drained by the Matlabas, Mokolo, Mogalakwena and Lephalala perennial rivers and is characterised by Moist Mountain Bushveld vegetation (Kleynhans *et al.*, 2005; Pool-Stanvliet, 2013). These streams comprise the unique Waterberg aquatic ecoregion of South Africa (Driver *et al.*, 2011). The Waterberg Biosphere Reserve has been a site for tourism and environmental education for many years (Baber *et al.*, 2003). *Afronurus barnardi*, *Afroptilum parvum* and *Elassoneuria sp.* were all collected from this region.

The Magaliesberg mountain range is drained by the Gwathle River catchment in the Bojanala district of the North West Province. This catchment falls within the Western Bankenveld and

the Bushveld ecoregions and is fed by two major tributaries, the Sterkstroom and Maretlwane rivers, before it joins with the Crocodile (West) River catchment at Roodekoppies Dam.

The Magaliesberg range contains north-facing kloofs with perennial streams, the largest being the Sterkstroom River, which rises on the slopes between Breedtsnek and Olifantsnek (Carruthers, 2014). The Sterkstroom drains into the Buffelspoort Dam, which was completed in 1933 (Carruthers, 2014). The Buffelspoort Dam water is used to irrigate citrus, maize, wheat, and barley farms, and is a fishing and recreation attraction (Walmsley & Toerien, 1979). *A. parvum* was collected from this region.

Study sites

The study sites (A-D) were all pristine rivers upstream of the mines (Glencore, Tharisa and Lonmin Mines) with minimal agricultural impact, inside the Magaliesberg Biosphere Reserve (Figure 6) (ESRI ArcMap 10.5.1 was used to generate the study site map). All previous studies, including those conducted in South Africa (King *et al.*, 1988; Palmer *et al.*, 1993a, 1996), have used near-pristine headwater streams, not urban rivers for analysing freshwater species and classifying them into FFGs. In addition, the collection of heptageniid *Afronurus barnardi* (W) Lestage, 1924 and oligoneuriid *Elassoneuria sp.* (W) Eaton, 1881 were collected from the Lephalala River in the Waterberg catchment. *Afroptilum parvum* was collected from the Magaliesberg catchment – study rivers were the Sterkstroom and Maretlwane rivers (Figure 6).

Mayfly final instar larvae were collected in the middle to end of the wet season in April/May 2021 from the Sterkstroom (25°50’20.4” S, 27°22’17.4” E) and Maretlwane Rivers (25°44’29.04” S, 27°34’5.52” E) in the Magaliesberg catchment (Figure 6) the rivers sampled were at high summer base flow – this affected the ability to access the whole stream but did

not impede collection of specimens. Additionally, samples were collected from two sites on the Lephalala River *(*23°52’43.68” S, 28°20’9.59” E; 23°57’28.44” S, 28°23’0.24” E) in the Waterberg catchment in February 2022 just after the heavy rains.

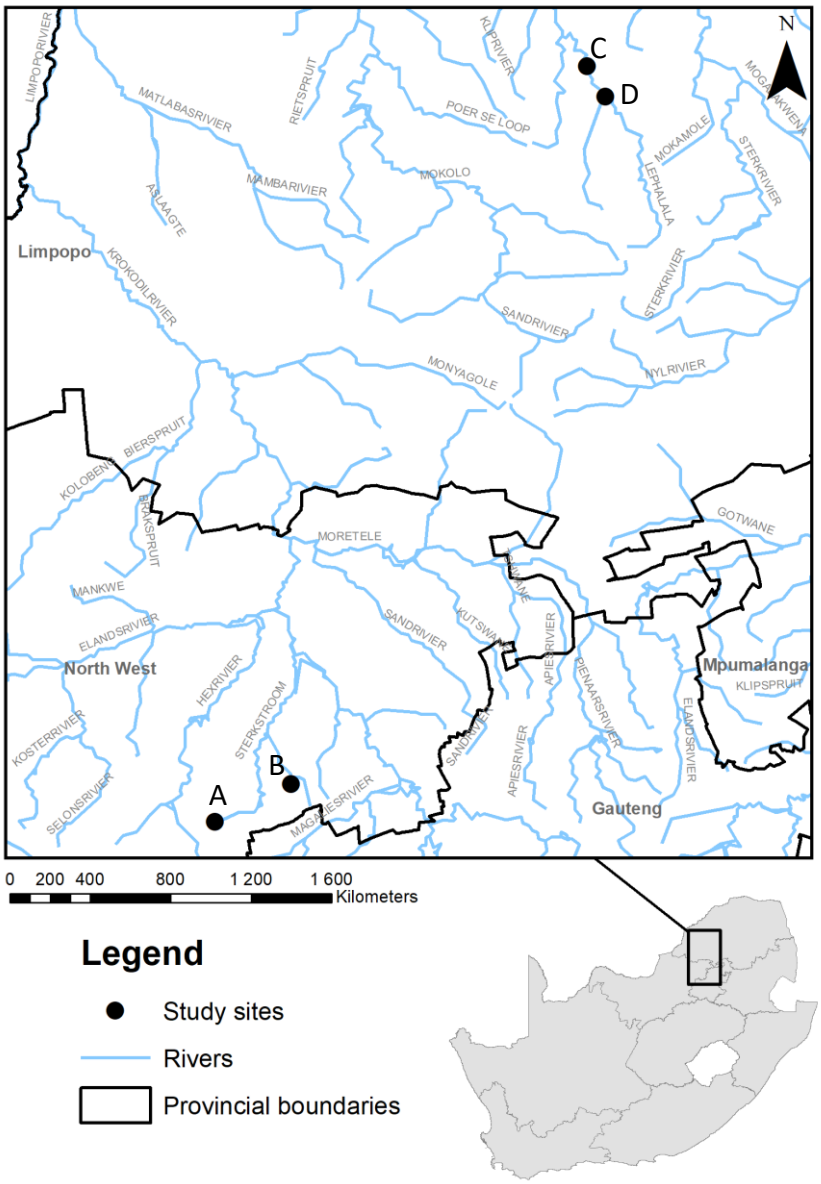


Figure 6. The location of the four sampling sites with two in the Magaliesberg catchment, one on the Sterkstroom river (A), one on the Maretlwane river (B), and two sampling sites on the Lephalala river (C and D) in the Waterberg catchment.

Study species

The baetid genus *Afroptilum* Gillies, 1990 (previously *Centroptilum* Lestage, 1924) has been studied in Northern hemisphere river networks (Geagan, 1963; Lowen & Flannagan, 1990; McCafferty & Waltz, 1990; Wiersema & Burian, 1999; Funk *et al*., 2006; Weaver *et al*., 2015), with some research done on this genus in parts of Africa (Soldán & Thomas, 1985; Gillies, 1990, 1991, 1992; Lugo-Ortiz & McCafferty, 1996) and South Africa (Palmer *et al*., 1993a, b).

Afroptilum parvum (order Ephemeroptera) was a focal species of this project as it is a common species found in river catchments that are congeneric to *A. excisum* which is a common local species in South African streams (Palmer *et al.*, 1991, 1993a, b; Palmer, 1994). In addition to *A. parvum* (W) and *A. parvum* (M) being studied, two additional species *Afronurus barnardi* (W) and *Elassoneuria sp.* (W) nymphs from the Waterberg were characterised and classed into functional feeding groups based on their mouthpart morphology.

Collection of final instar mayfly larvae and their preservation

Sampling was done using kick netting with the standard SASS5 net (1mm mesh on a 30cm frame) – this was done working upstream in fast flowing riffles, marginal vegetation, and stones in current to get maximum specimen numbers at each site (Dickens & Graham, 2002). All samples collected in the field were preserved in 70% ethanol and later replaced with fresh 70% ethanol in the lab upon return from the field. Final instar larvae were identified as larvae with dark overlapping forewing pads on the mesothorax (see Barber-James & Lugo-Ortiz, 2003; Salles *et al*., 2018). All measurements were corrected for allometry (by dividing each measurement by head width (hw2)) to account for individual variability within the species. All specimens were submerged in deionised water for approximately one minute for the sample

to rehydrate before dissection and identified using the Water Research Commission (WRC) Guide to the Freshwater Invertebrates of Southern Africa (Barber-James & Lugo-Ortiz, 2003).

Mouthpart measurements of final instar mayfly nymphs

To identify and measure the various mouthpart structures a total of five larvae of each species from both catchments were dissected under the ZEISS Stereo Discovery V8 light microscope using a dissecting needle and insect pin with the mouthparts individually placed on slides and fixed using DPX mounting media. Thereafter, microstructures were measured using the ZEISS Axio Imager M2 upright microscope at magnifications of 100X and 400X and imaged using the ZEISS six-megapixel Axiocam 506 colour microscope camera. Additionally, the final instar nymphs were dissected and imaged under the Phenom Pure Desktop Scanning Electron Microscope (SEM) to obtain images of finer details of the microstructures to compare between species (Figures 13-20). No dehydration or sputter coating preparation was needed for use of the desktop SEM, samples were dissected under the ZEISS Stereo Discovery V8 light microscope and placed onto aluminium stubs and viewed.

Following the methodology of Banegas *et al*. (2020) on the mouthparts of *Cloeon dipterum* (Appendix C), measurements from each species were taken of the: head (Figure 7), labium (Figure 8), hypopharynx (Figure 9), labrum (Figure 10), right mandible and left mandible (Figure 11), and maxilla (Figure 12).

The largest controversy in the literature is the definition of the FFGs and which mouthparts or feeding behaviours and preferences are important to consider in this definition (previously discussed in the literature review) (Barmuta, 1988; Palmer & O'Keeffe, 1992). Baptista *et al.* (2006) and Palmer *et al.* (1993b) found that the labium and maxilla were the mouthpart structures containing important indication on feeding strategy, thus making them the most

useful microstructures to observe when classifying macroinvertebrate nymphs into FFGs. The maxillary and labial palps have been described to be used for touching, tasting, and manipulating food (Barber-James & Lugo-Ortiz, 2003) and are further described in the results.

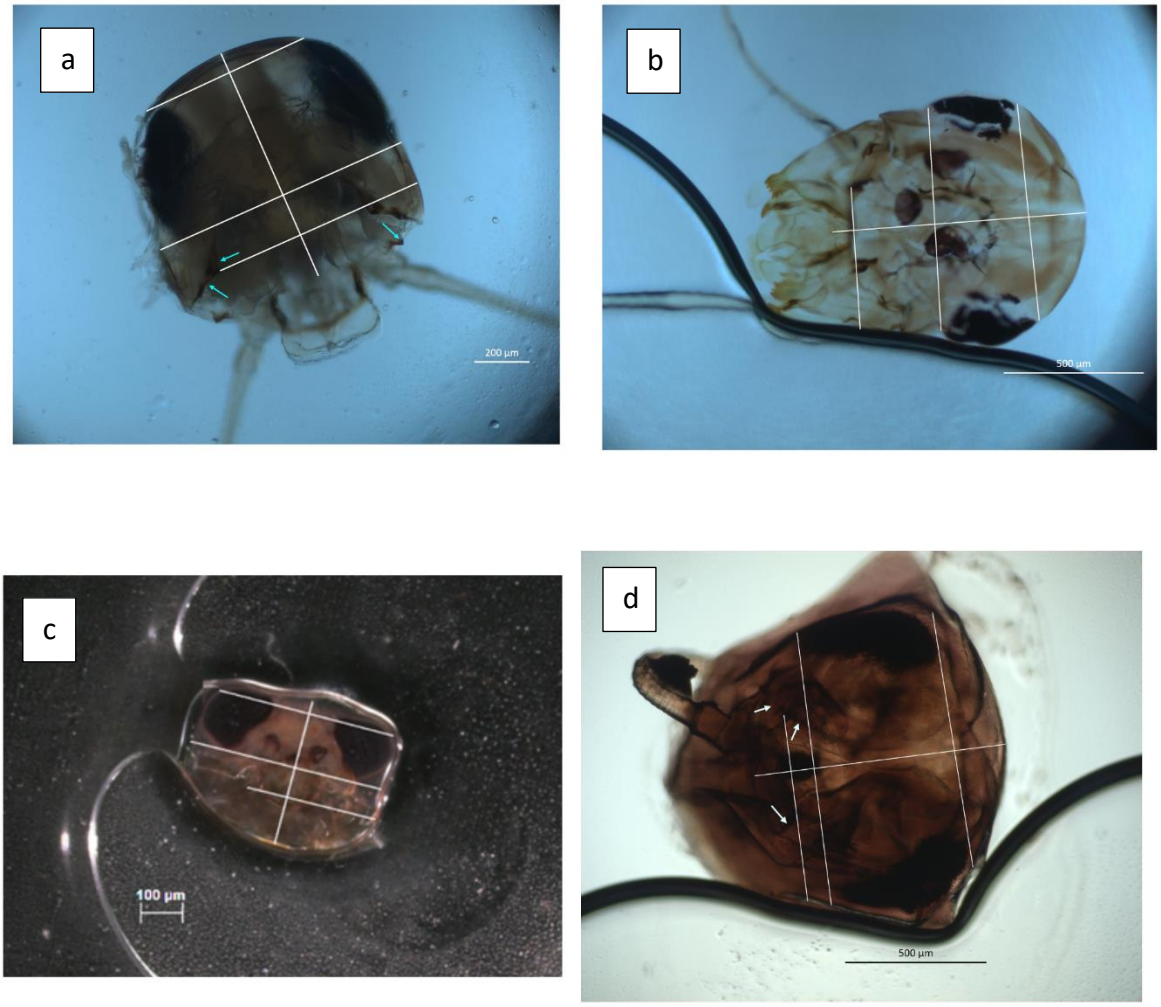
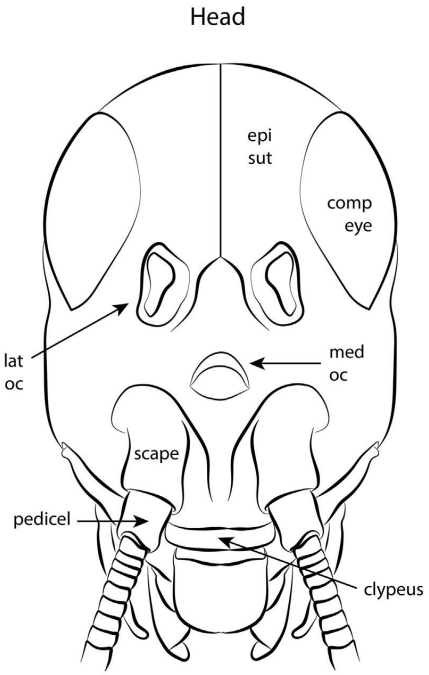


Figure 7. Images, from the light microscope, of the head measured for a) *Afroptilum parvum* (W), b) *Afroptilum parvum* (M), c) *Afronurus barnardi* (W) and d) *Elassoneuria sp.* (W). Baetid head diagram reproduced from Salles *et al*. (2018) to aid with the morphological interpretation. Scale bars: a = 200 µm; b-d = 500 µm, c = 100 µm. Measurement lines on images correlate to Appendix C (Banegas *et al.,* 2020).



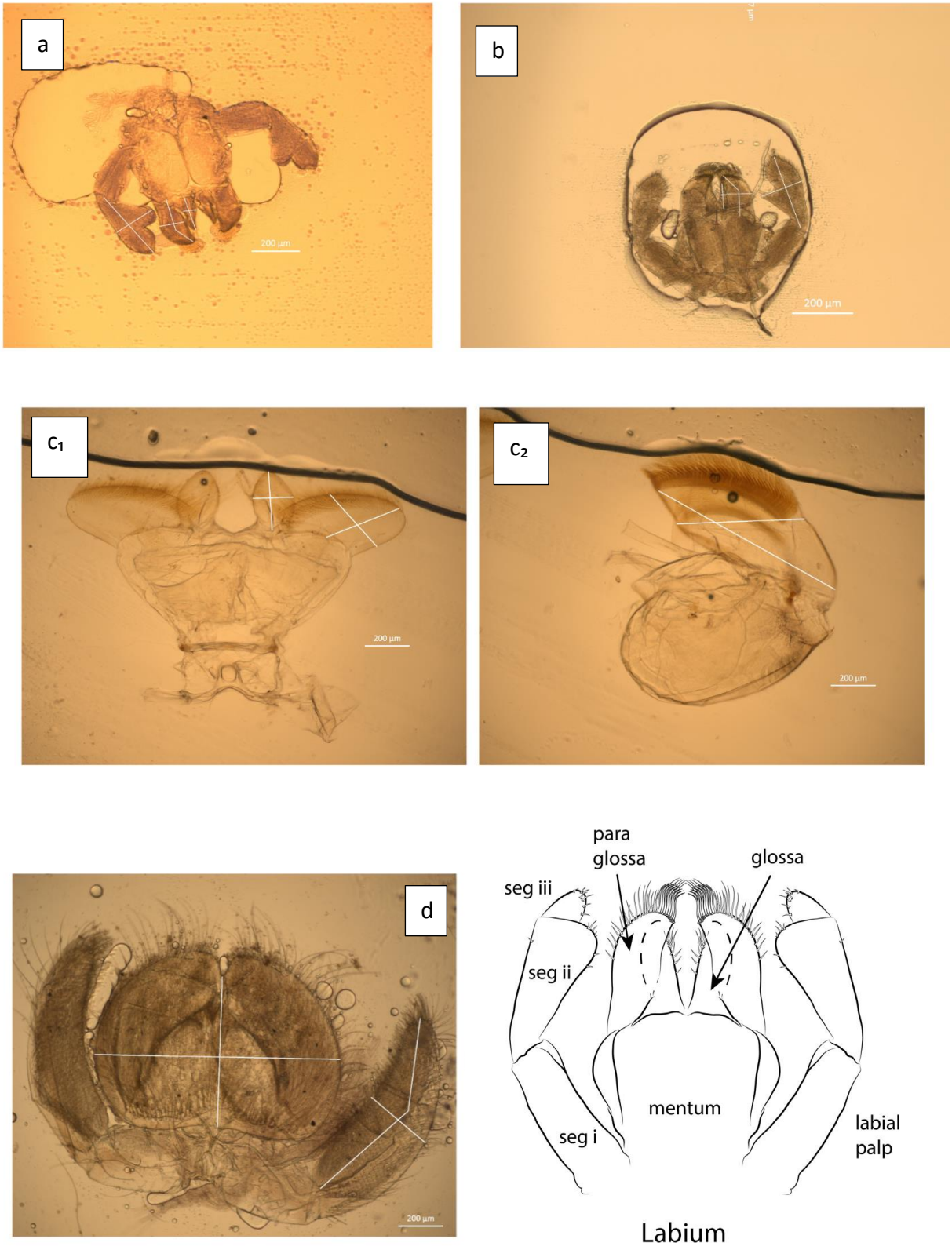


Figure 8. Images, from the light microscope, of labium measured for *Afroptilum parvum* (M) (a), *Afroptilum parvum* (W) (b), *Afronurus barnardi* (W) (c_1: fused labium and paraglossa; c_2: labial palp) and *Elassoneuria sp*. (W) (d). Baetid labium diagram reproduced from Salles *et al*. (2018) to aid with the morphological interpretation. Scale bars = 200 µm. Measurement lines on images correlate to Appendix C (Banegas *et al*., 2020).

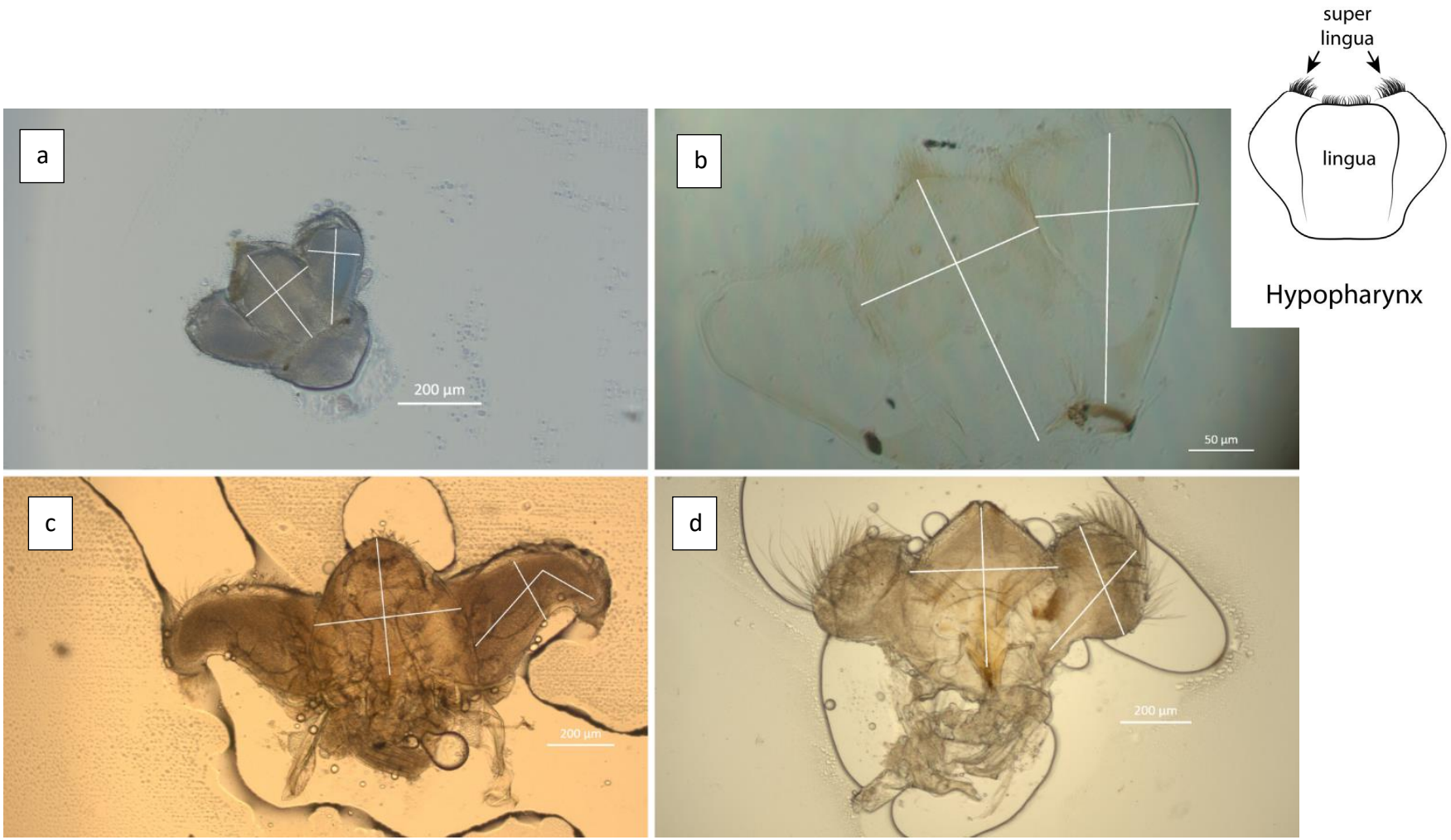


Figure 9. Images, from the light microscope, of hypopharynx measured for a) *Afroptilum parvum* (M), b) *Afroptilum parvum* (W), c) *Afronurus barnardi (W)* and d) *Elassoneuria sp.* (W). Baetid hypopharynx diagram reproduced from Salles *et al*. (2018) to aid with the morphological interpretation. Scale bars: a, c-d = 200 µm; b = 50 µm. Measurement lines on images correlate to Appendix C (Banegas *et al*., 2020).

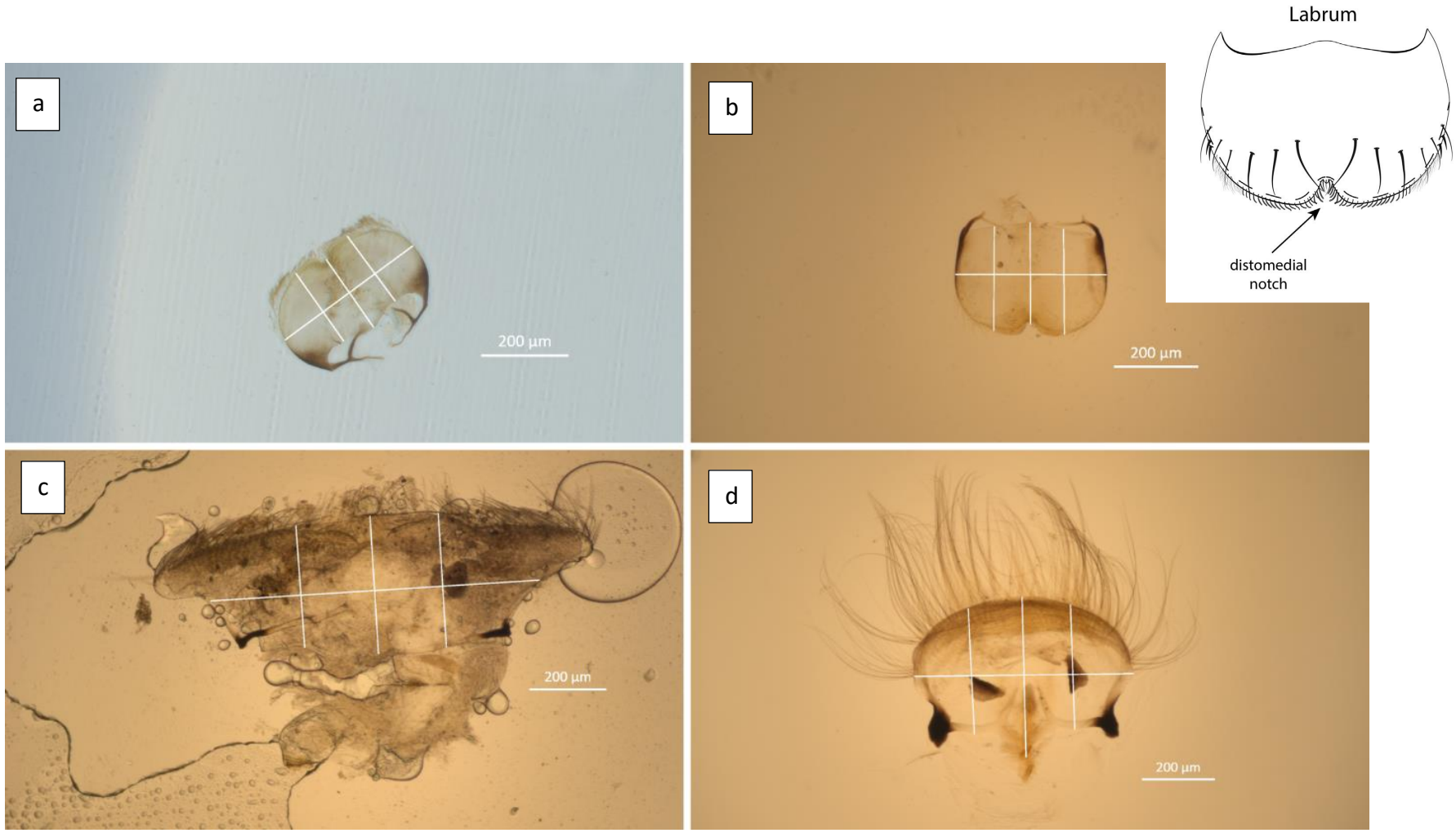


Figure 10. Images, from the light microscope, of labrum measured for a) *Afroptilum parvum* (W), b) *Afroptilum parvum* (M), c) *Afronurus barnardi* (W) and d) *Elassoneuria sp.* (W). Baetid labrum diagram reproduced from Salles *et al*. (2018) to aid with the morphological interpretation. Scale bars = 200 µm. Measurement lines on images correlate to Appendix C (Banegas *et al*., 2020).

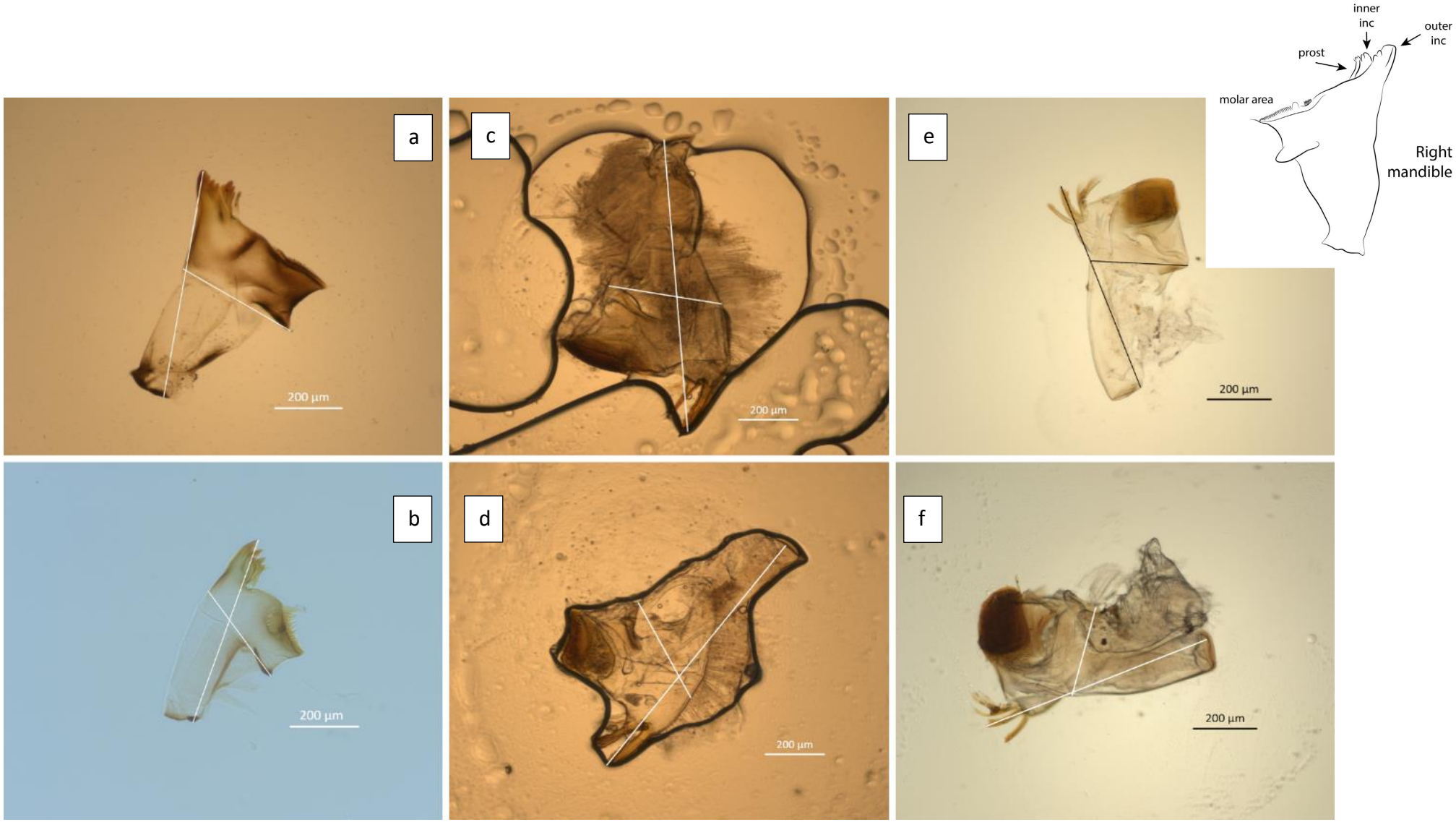


Figure 11. Images, from the light microscope, of left (top) and right (bottom) mandibles measured for *Afroptilum parvum* (W) and *Afroptilum parvum* (M) (*a-b), Afronurus barnardi* (W) (c-d) and *Elassoneuria sp*. (W) (e-f). Baetid mandible diagram reproduced from Salles *et al*. (2018) to aid with the morphological interpretation. Scale bars = 200 µm. Measurement lines on images correlate to Appendix C (Banegas *et al*., 2020).

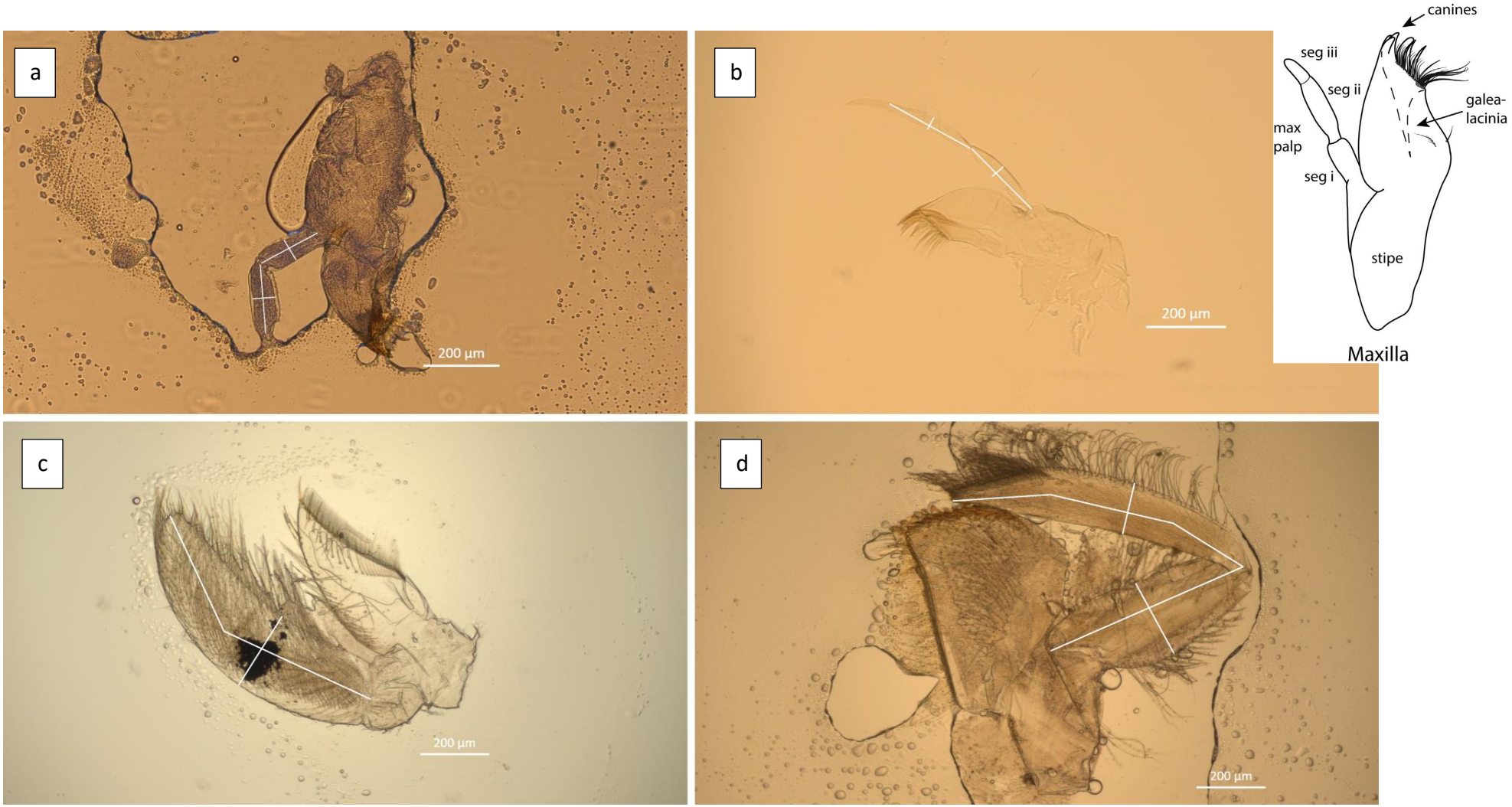


Figure 12. Images, from the light microscope, of maxilla measured for a) *Afroptilum parvum* (M), b) *Afroptilum parvum* (W), c) *Elassoneuria sp*. (W) and d). *Afronurus barnardi* (W). Baetid maxilla diagram reproduced from Salles *et al*. (2018) to aid with the morphological interpretation. Scale bars = 200 µm. Measurement lines on images correlate to Appendix C (Banegas *et al*., 2020).

Data analyses of nymph mouthpart measurements

Mouthpart morphometric data were collected from five final mayfly nymph instars for the four species studied. Means, standard error and minimum and maximum measurements were tabulated for comparison between species. All measurements were corrected for allometry to account for growth variability within the species (some final instars may be smaller than others due to environmental stressors during development) – by dividing each of the measurements by the head width (hw2) (adapted from Tocco *et al*., 2019). PAST (Hammer *et al*., 2001) was used to run a Principal Component Analysis (PCA) to determine links between mouthpart morphometrics for each species and their respective feeding groups (the FFGs were allocated through analysis of data obtained in this chapter and compared to classifications mentioned in the literature).

Thereafter, an ANCOVA was run in R Studio for average mandible length and width between *A. parvum* (W) and *A. parvum* (M) as they were the main drivers of each principal component in the PCA. The ANCOVA was run to investigate whether there was any significant difference between the species whilst controlling for the effect of each of the mandible measurements. Additionally, an F-test would show whether there was a significant interaction between mandible length and width.

Moreover, maxillary palp length was also shown to be a strong driver of the principal components. A comparison between the maxillary palp lengths were compared from both sites by use of a t-test.

Results

Micrographs of mayfly nymph mouthparts

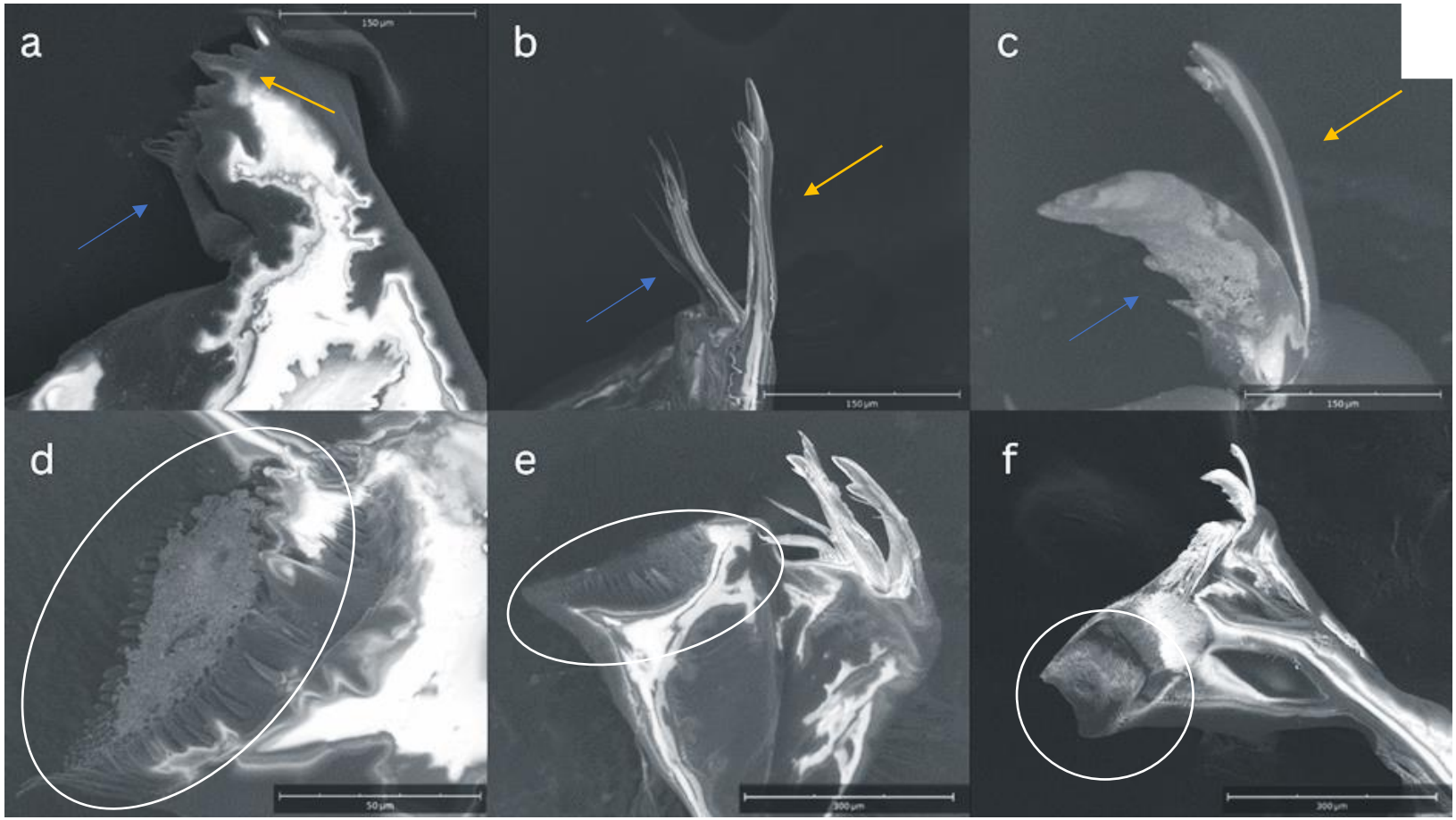
Micrographs of *Afroptilum parvum* (M) and *A. parvum* (W) showed unspecialised microstructures. Incisors are short and blunt with outer incisors (yellow arrow) and inner incisors (blue arrow) (Figure 13a). *Afroptilum parvum* (M) and *Afroptilum.* (W) has a "type V molar surface" sometimes referred to as the "thorn-carpet molar surface". This is a molar surface with numerous individual tubercules (Sroka, 2009) (Figures 13d and 17). The maxillary palps of *A. parvum* (M) and *A. parvum* (W) showed a simple thumb-like maxillary palp with no visible setae and are smaller in size when compared to *Elassoneuria sp.* (W) and *Afronurus barnardi* (W) (Figures 14a and 20,). Since *A. parvum* (M) and *A. parvum* (W) have unspecialised maxillary palps they have more complex labium with many differentiated parts. Although the mouthparts are unspecialised, they do possess scoop-like setae on the apices of their glossae and paraglossae (Figure 18) and bipectinate setae on the apices of the galea lacinia (Figure 15). In *A. parvum* (M) and *A. parvum* (W) the labium consists of three parts: labial palp, glossa and paraglossae with setae (Figure 14d).

The oligoneuriid *Elassoneuria sp.* (W) and heptageniid *Afronurus barnardi* (W) mouthparts were more specialised than those found in *Afroptilum parvum* (M) and *Afroptilum parvum* (W). Both species had more pronounced inner and outer incisors (Figure 13b-c) and different molar surfaces. *Elassoneuria sp.* (W) were found to have "type II molar surfaces". Where the ridges are marginally fused with non-articulated teeth and overlapping neighbouring grooves (Sroka, 2009) (Figure 13f). Ridges and setae assist in the removal of water from food particles before ingestion (Sroka, 2009). The maxillary palps of *Elassoneuria sp.* (W) are almost twice the relative size of those found in both *Afroptilum parvum* (M) and *Afroptilum parvum* (W),

covered in many fine setae (Figures 14b and 19, Tables 2-4). The difference in size and presence of setae on the palps reflects a change in the use and importance of the labium for processing food particles (McShaffrey, 1992; Thomson, 2013), specifically a change from brushing to filtering. The labium does not have differentiated labial palps and paraglossae as those seen in *Afroptilum parvum* instead they are fused forming a plate-like structure, with a slit down the centre (Figure 14e). An additionally interesting observation in the *Elassoneuria sp.* (W) was the presence of two rows of long setae on their fore-tibias.

Afronurus barnardi (W) were found to have "type I molar surfaces". This means that the spaces between individual ridges are filled by tiny hairs that are distributed evenly or in groups (Sroka, 2009) (Figure 13e). The maxillary palps are highly specialised with chitinous maxillary palps with setae on the apices of the palps (Figure 14c and 16). The labium shows fusion of the glossae and paraglossae structures with a dense brush of setae along the edge of the labial palp (Figure 14f).

The hypopharynx forms the dorsal and posterior wall of the preoral cavity, consisting of the lingua and superlingua (Banegas *et al.*, 2020). The linguae are on either side of the superlingua and are wider than they are long. However, the superlingua is longer than it is wide in all four species (Appendix G).



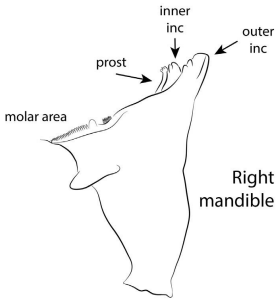


Figure 13. SEM Micrographs of *Afroptilum parvum* (W) (a, d), *Afronurus barnardi* (W) (b, e) and *Elassoneuria sp.* (W) (c, f) mandibles. Top row showing the inner incisors (blue arrow) and outer incisors (yellow arrow), with the bottom row showing the various molar regions (white circle) for each species. Mandibular setae can be seen between the inner and outer incisors. Scale bars: a-c = 150 µm; d = 50 µm; e-f = 300 µm.

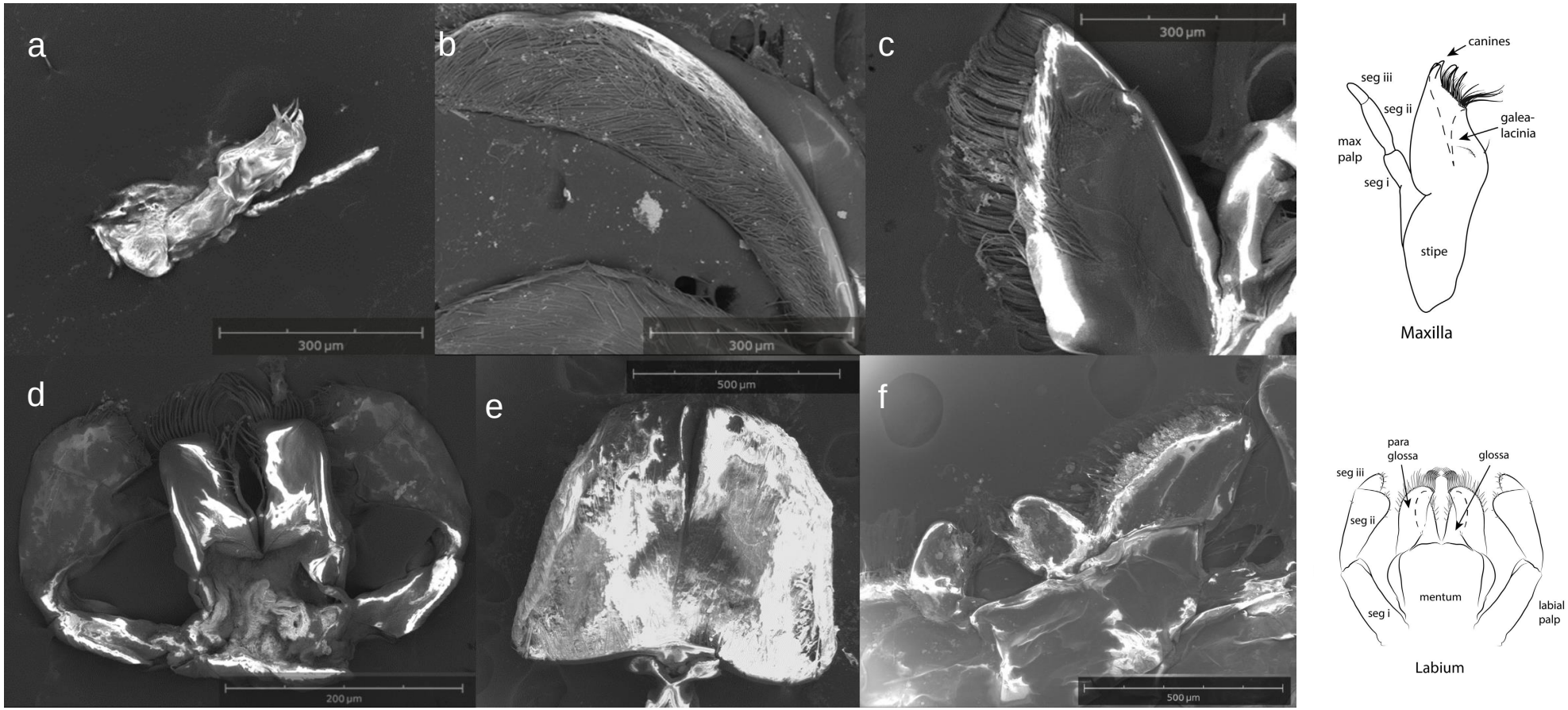


Figure 14. SEM Micrographs of labia and maxilla of *Afroptilum parvum* (W) (a, d), *Elassoneuria sp.* (W) (b, e) and *Afronurus barnardi* (W) (c, f). Top row showing the maxillary palps and the bottom row showing the variation in the labial structures. Micrographs show setae present on *Elassoneuria sp.* (W) maxillary palps (b) and *Afronurus barnardi* (W) maxillary palps with chitinous scrapers (c). The labial structures differ for all three species with a more complex labium consisting of multiple parts in *Afroptilum parvum* (W) (d); a fused tongue-like labium in the *Elassoneuria sp*. (W) (e); and a thickened labial palp with fusion of the glossae and paraglossae structures in *Afronurus barnardi* (W) (f). Scale bars: a-b-c = 300 µm; d- f = 200 µm; e = 500 µm.

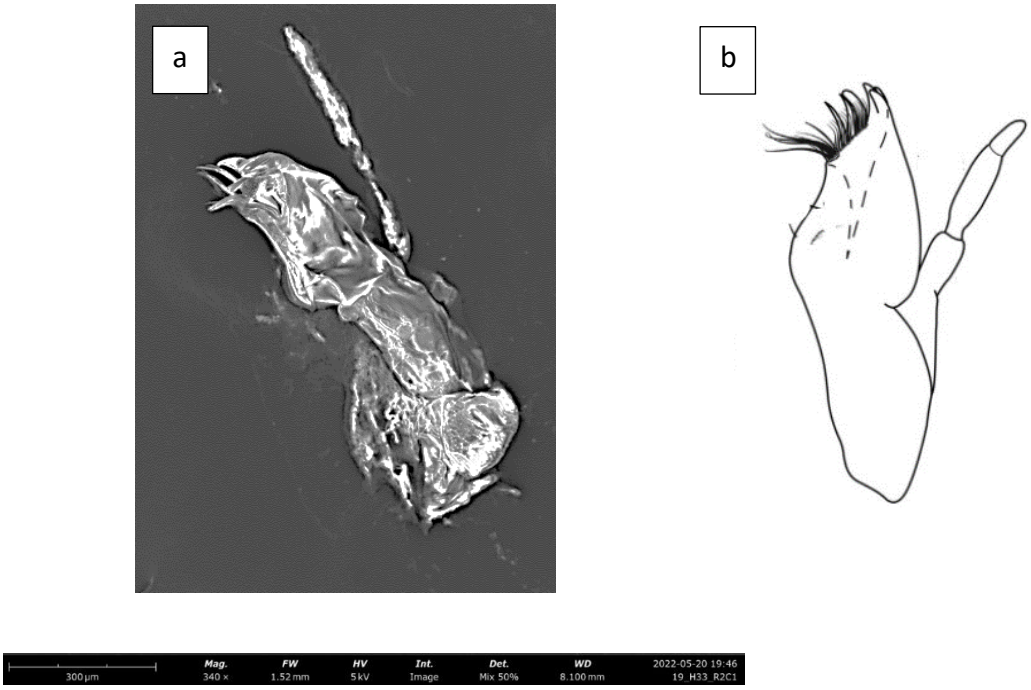


Figure 15. a) SEM Micrograph (340X) of *Afroptilum parvum* (W) maxillary palp with a tuft of bipectinate setae on the apace of the galea lacinia. Scale bar = 300 µm. b) Maxilla diagram reproduced from Salles *et al*. (2018) to aid with the morphological interpretation.

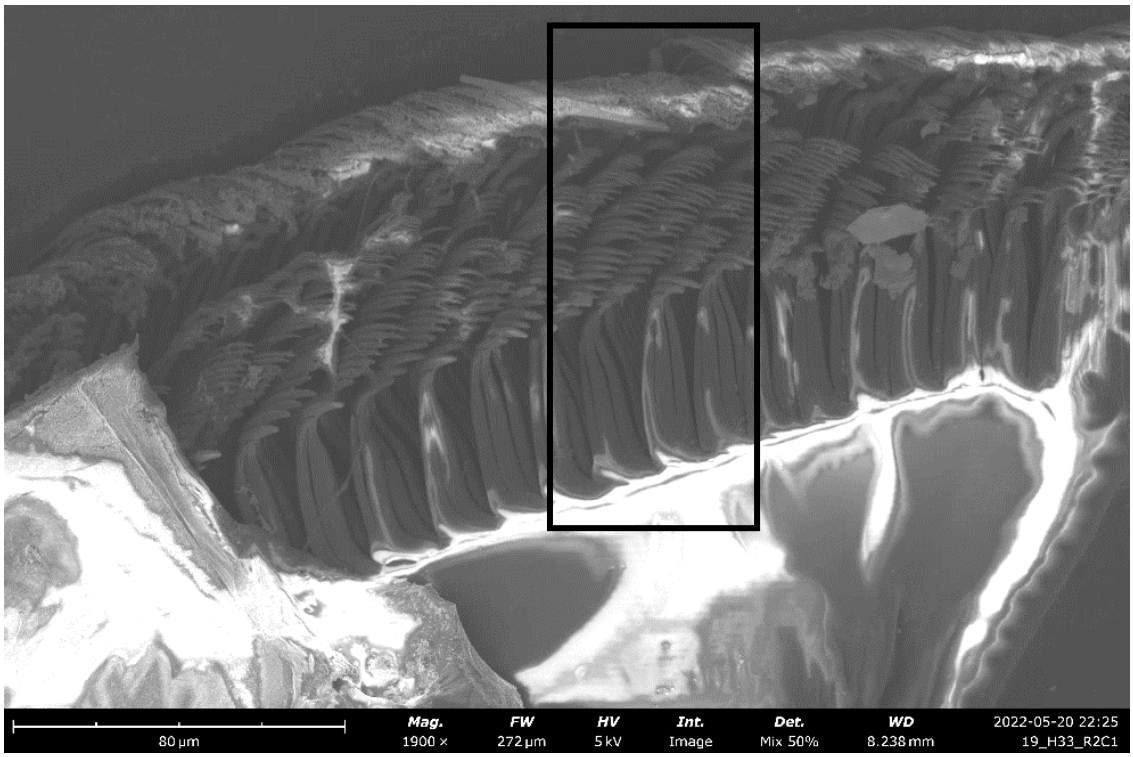


Figure 16. Micrograph (1900X) of brush of setae (black box) found on the ridge of the maxillary palp in *Afronurus barnardi* (W). Scale bar = 80 µm.

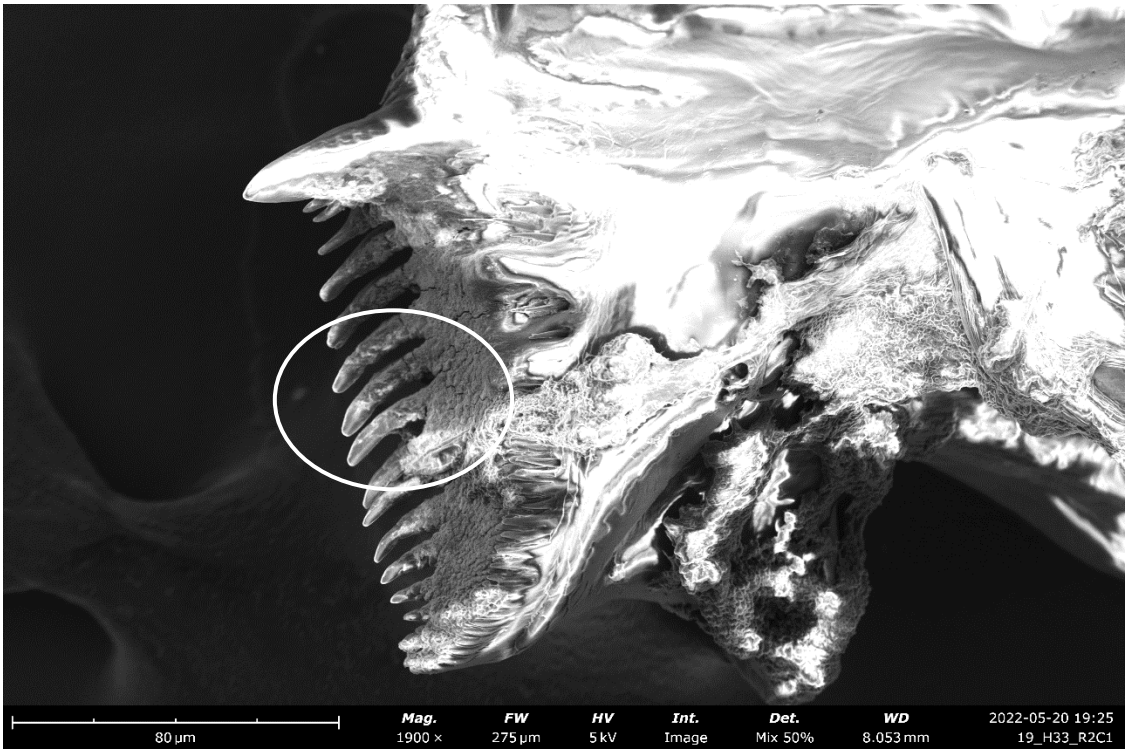


Figure 17. Micrograph (1900X) of tubercules (white circle) that make up *Afroptilum parvum* (W) molar region. Scale bar = 80 µm.

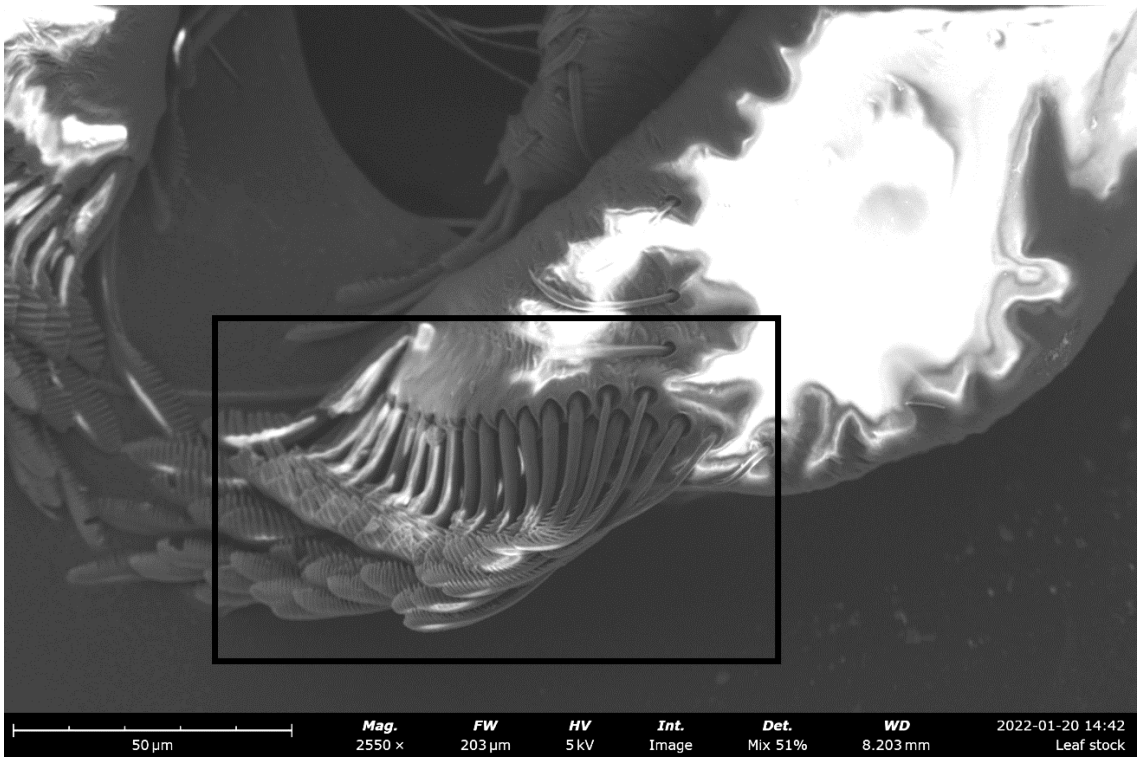


Figure 18. Micrograph (2550X) of the bipectinate setae (black box) on the apices of the glossae and paraglossae in *Afroptilum parvum* (M). Scale bar = 50 µm.

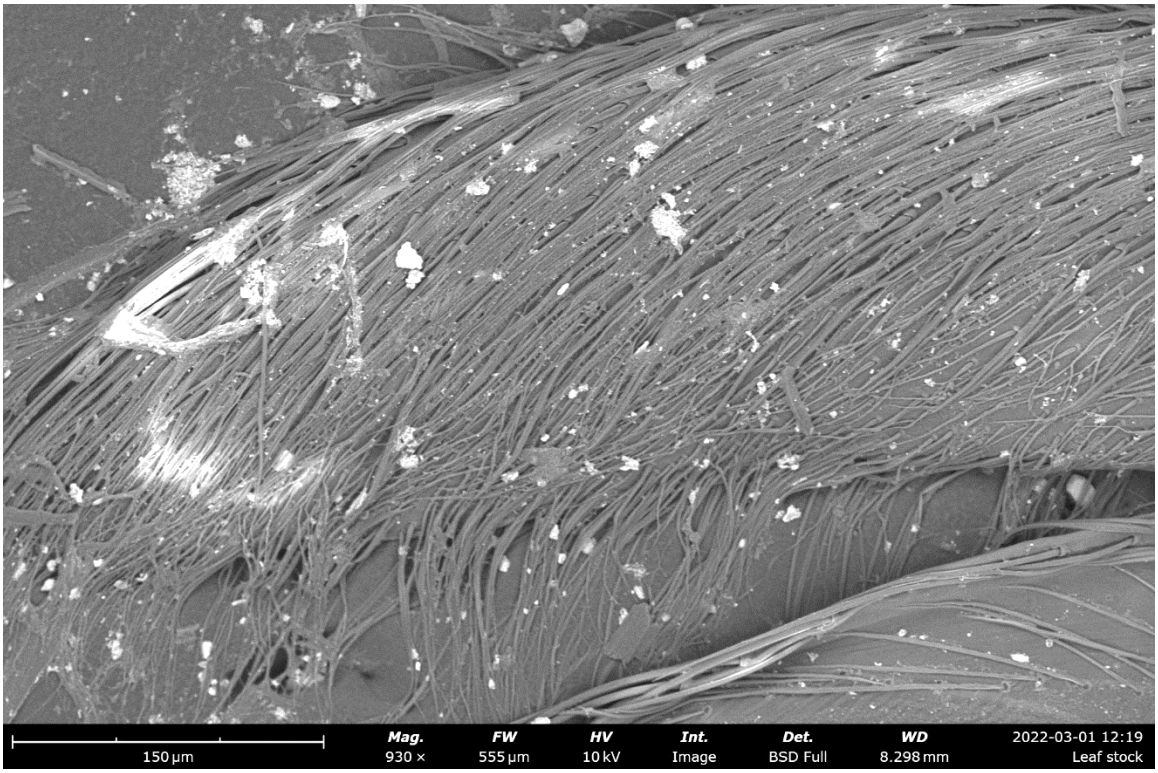


Figure 19. Micrograph (930X) showing thickened labial palp covered in setae of *Elassoneuria sp.* (W). Scale bar = 150 µm.

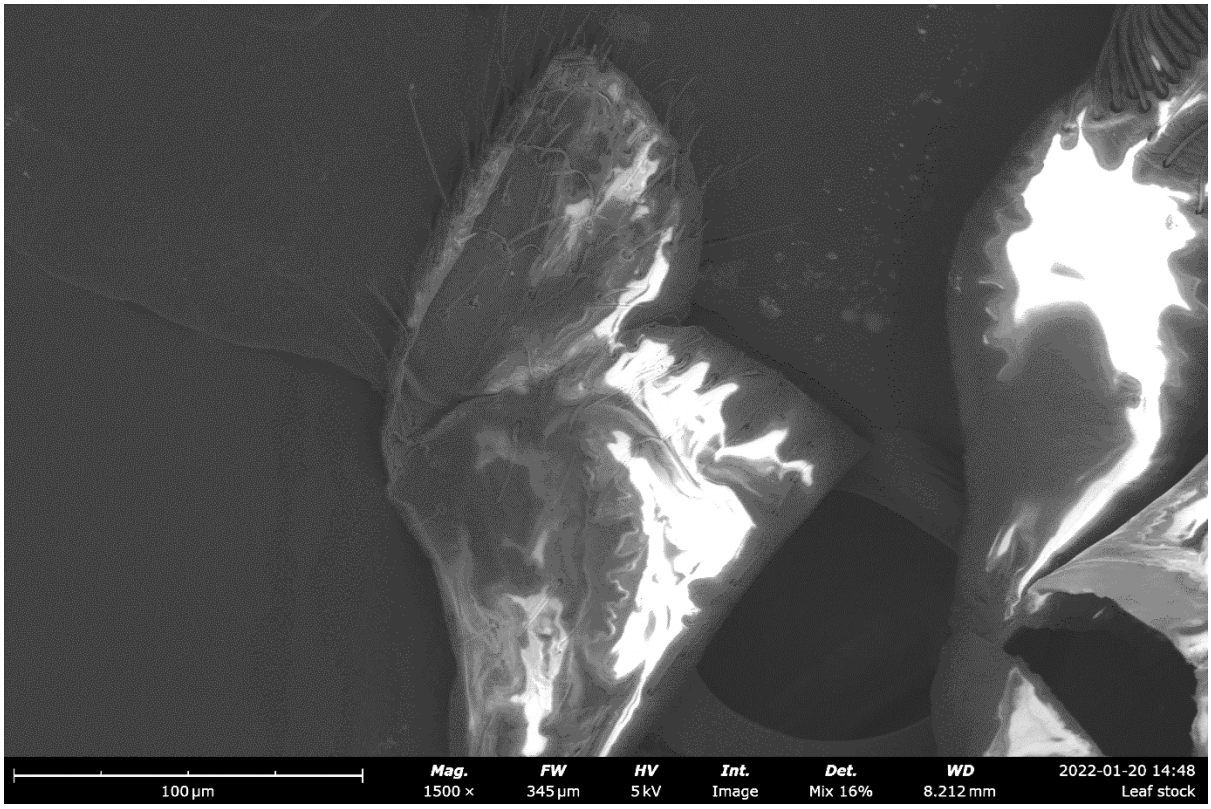


Figure 20. Micrograph (1500X) of blunt thumb-like labial palp of *Afroptilum parvum* (M), covered in sparse short setae. Scale bar = 100 µm.

Descriptions of head and mouthpart morphology

Afroptilum parvum (M) had an average head length (hl) of 974.0 µm (± 92.4 µm) which was relatively a third larger than that of *Afroptilum parvum* (W) averaging 680.5 µm (±202.8 µm) (Appendix D). Additionally, the relative head length of *Afronurus barnardi* (W) and *Elassoneuria sp.* (W) averaged 333.63 µm (±37.7 µm) and 1132.0 µm (± 142.6 µm) respectively (Appendix D).

The labrum for all species was wider (lw) than it was long (ll1, ll2 and ll3) with setae varying from short in *Afroptilum parvum* (M), *Afroptilum parvum* (W) and *Afronurus barnardi* (W) to long in *Elassoneuria sp.* (W) (Figures 7-9, Appendix E). The left and right mandibles for all species, like the labrum, were wider than they were long with the mandible widths (rmdw, lmdw) being between 2-2.5 times the mandible length (rmdl, lmdl) (Appendix F). Having explored the role of both labrum and mandible mouthparts in relative interspecies comparisons no further analyses were done on these structures.

The labium consists of the paraglossae, glossae and labial palps. The labium for all three species is longer than it is wide. The labial palps were relatively the longest in *Afronurus barnardi* (W) at 822.2 µm (± 157.9 µm) and relatively smallest in *Afroptilum parvum* (W) at 241.4 µm (± 27.1 µm) (Appendix H). Moreover, the relative paraglossae and glossae measurements vary substantially between species mainly due to the fusion of these structures in *Elassoneuria sp*. (W) (Figure 11e, Appendix I). On average they are longer than they are wide in all three species.

The relative maxillary palp measurements for all four species differed with *Elassoneuria sp.* (W) and *Afronurus barnardi* (W) having the largest maxillary palps compared to *Afroptilum parvum* (W). The cumulative relative length of the maxillary palps is the largest in *Afronurus*

barnardi (W) (Appendix J). *Afroptilum parvum* maxillary palps length show high variation between localities – the relative minimum and maximum maxillary palp lengths and widths had high variation (Appendix J). This variation was investigated by use of a Principal Component Analysis (PCA).

Principal Component Analysis (PCA) of nymph mouthpart microstructures measured

A Principal Component Analysis (PCA) was performed to determine whether any interspecific variations in mouthpart measurements reflected a species FFG classification or not. Twenty-two measurements were included into the PCA with all four species (Figure 21A) and twenty-one measurements were used in the PCA comparing *Afroptilum parvum* (W) to *A. parvum* (M) (Figure 21B). Distinct clusters can be seen of the four species with the cumulative percentage of PC 1 and PC 2 accounting for 93.50% of the variance in the data (Figure 21A, Table 2). *Afronurus barnardi* (W) split strongly from *Elassoneuria sp*. (W), *Afroptilum parvum* (W) and *Afroptilum parvum* (M) along PC 1 (Figure 21A). This was due to left (lmdw, lmdl) and right (rmdw, rmdl) mandibles, labial palp length (lpl) and labrum width (lw) being strong drivers on the first component (Table 2). There was some overlap observed between *Elassoneuria sp*. (W), *Afroptilum parvum* (W) and *A. parvum* (M) along PC 2.

Following the first PCA (Figure 21A) an additional PCA was run (Figures 21B) to better visualise and assess which mouthpart measurements were distinguishing the two regional samples of *A. parvum*. The PCA biplot showing the groupings of *A. parvum* (W) and *A. parvum* (M) (Figure 21B) had both PC 1 and PC 2 cumulatively account for 97.66% of the variation in the data (Table 3b). The *Afroptilum parvum* (M) is completely nested in *Afroptilum parvum* (W) along PC 2 with the maxilla palp length (mpl2) and left (lmdw, lmdl) and right (rmdw, rmdl)

mandibles having strong effects on the data (Figure 21B, Table 3). There is a greater variation in mouthpart measurements in *A. parvum* (W) when compared to the narrower variation in mouthpart measurements of *A. parvum* (M) indicating variation within the species from different localities but not enough to differentiate into separate FFGs.

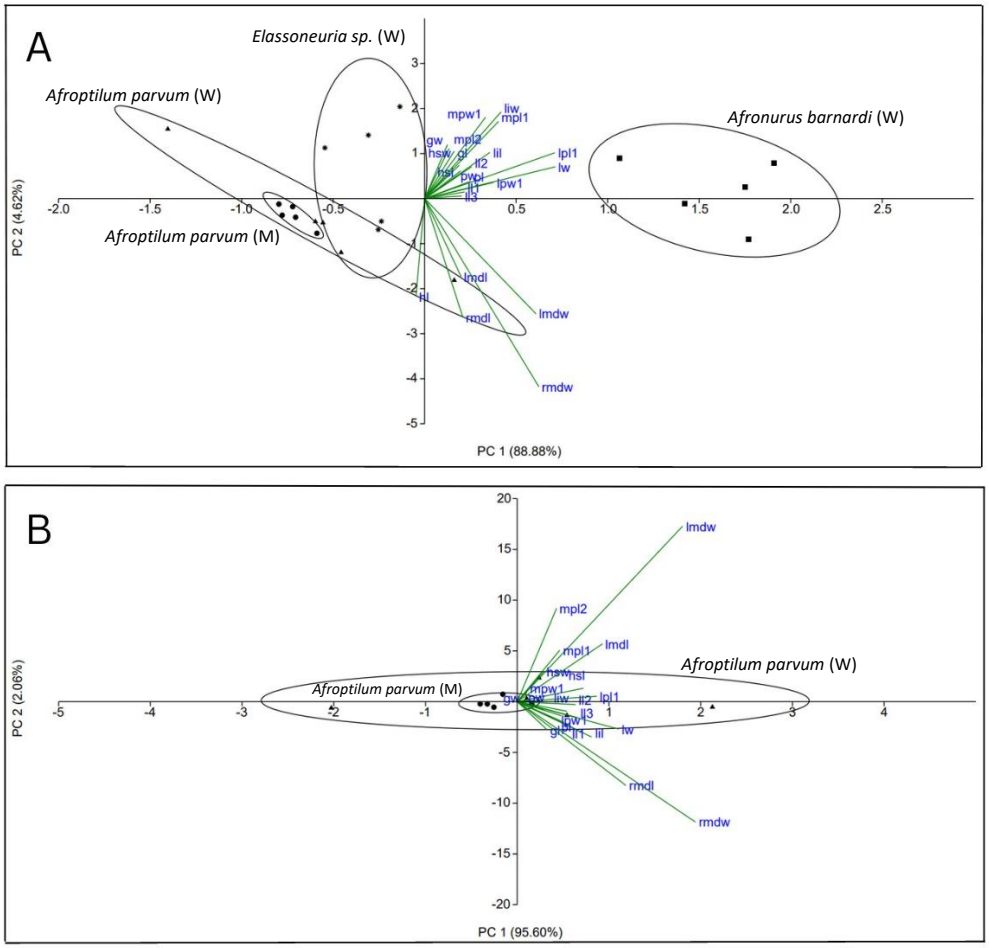


Figure 21. Clustering of (A) *Afroptilum parvum* (M) (dots), *Afroptilum parvum* (W) (triangles), *Afronurus barnardi* (W) (squares) and *Elassoneuria sp.* (W) (star), and (B) *Afroptilum parvum* (M), *Afroptilum parvum* (W) according to mouthpart morphology in a Principal Component Analysis biplot. Ellipses indicate significant ($p<0.05$) clustering of species from both the Magaliesberg and Waterberg sites, relative to the different mouthpart microstructures measured[1] within each of the two principal components.

[1] **gl**: glossae long; **gw**: glossae width; **hl**: head long; **hsl**: hypopharynx superlingua long; **hsw**: hypopharynx superlingua width; **lil**: lingua long; **liw**: lingua width; **ll**: labrum long; **lmdl**: left mandible long; **lmdw**: left mandible width; **lpl**: labial palp long; **lpw**: labial palp width; **lw**: labrum width; **mpw**: maxillary palp width; **mpl**: maxillary palp long; **pl**: paraglossae long; **pw**: paraglossae width; **rmdl**: right mandible long; **rmdw**: right mandible width.

Table 2. Five of the 22 mouthpart microstructures[2] measured from each species, being the main drivers of PC 1 and PC 2. Values shown in bold indicate strong drivers within each principal component (See figure 21A).

Abbr.	PC 1	PC 2
gl	0.1097	0.097895
gw	0.072382	0.15867
hsl	0.10238	0.064833
hsw	0.077811	0.14736
lil	0.20706	0.13578
liw	0.24291	0.25527
ll1	0.12703	0.019556
ll2	0.14944	0.093839
ll3	0.11777	0.007466
lmdl	0.11577	-0.22987
lmdw	**0.35365**	-0.33931
lpl1	**0.41345**	0.13485
lpw1	0.21869	0.047258
lw	**0.4151**	0.093509
mpl1	0.23449	0.22694
mpl2	0.093777	0.13947
mpw1	0.19323	0.23913
pl	0.14613	0.050949
pw	0.11382	0.081064
rmdl	0.12135	**-0.34955**
rmdw	0.36265	**-0.55381**
hl	-0.02759	-0.28835

[2] **gl**: glossae long; **gw**: glossae width; **hl**: head long; **hsl**: hypopharynx superlingua long; **hsw**: hypopharynx superlingua width; **lil**: lingua long; **liw**: lingua width; **ll**: labrum long; **lmdl**: left mandible long; **lmdw**: left mandible width; **lpl**: labial palp long; **lpw**: labial palp width; **lw**: labrum width; **mpw**: maxillary palp width; **mpl**: maxillary palp long; **pl**: paraglossae long; **pw**: paraglossae width; **rmdl**: right mandible long; **rmdw**: right mandible width.

Table 3. Four of 21 mouthpart microstructures[3] measured for *Afroptilum parvum* (M) and *Afroptilum parvum* (W), showing the main drivers of PC 1 and PC 2. Values shown in bold indicate strong drivers within each component. (See figure 21B)

Abbbr.	PC 1	PC 2
gl	0.083667	-0.10444
gw	0.018704	-0.00373
hsl	0.18508	0.04963
hsw	0.073477	0.11061
lil	0.20778	-0.13087
liw	0.13901	-0.03558
ll1	0.17234	-0.10692
ll2	0.16298	-0.01058
ll3	0.16757	-0.05849
lmdl	0.23842	0.21433
lmdw	**0.46243**	**0.64989**
lpl1	0.22238	0.020018
lpw1	0.12298	-0.05229
lw	0.28327	-0.10157
mpl2	0.11002	**0.34605**
mpw1	0.027203	0.034612
pl	0.13144	-0.07696
pw	0.075944	-0.03133
rmdl	**0.3038**	**-0.31023**
rmdw	**0.49833**	**-0.44594**

Mouthpart variation

An ANCOVA was run to test variation in mandible length and width between *Afroptilum parvum (W)* and *A. parvum (M)*. There was no significant difference in the mean mandible length between individuals from each site, however there was a significant interaction between mandible length and width (t-value = -1.52, p = 0.17); as mandible width increases length increases.

[3] **gl**: glossae long; **gw**: glossae width; **hl**: head long; **hsl**: hypopharynx superlingua long; **hsw**: hypopharynx superlingua width; **lil**: lingua long; **liw**: lingua width; **ll**: labrum long; **lmdl**: left mandible long; **lmdw**: left mandible width; **lpl**: labial palp long; **lpw**: labial palp width; **lw**: labrum width; **mpw**: maxillary palp width; **mpl**: maxillary palp long; **pl**: paraglossae long; **pw**: paraglossae width; **rmdl**: right mandible long; **rmdw**: right mandible width

An F-test revealed that there was a significant difference in variance between individuals from the Magaliesberg and the Waterberg in terms of mandible length (Sum sq. = 0.035, F-value = 75.42, df = 1, p-value = <0.0001), with Waterberg sites having higher variance among individuals.

In addition, the t-test comparing maxillary palp length between Magaliesberg and the Waterberg showed that they were not significantly different (t-value = 2.11, df = 4, p-value = 0.07).

Discussion

This chapter aimed to classify *Afroptilum parvum* (W), *Afroptilum parvum* (M), *Elassoneuria sp*. (W) and *Afronurus barnardi* (W*)* nymphs commonly found in the headwaters of the Magaliesberg and Waterberg tributary catchments respectively (western Limpopo River basin) into functional feeding groups (FFGs) based on mouthpart morphology. The identification and description of microstructures that differentiate species (and the adaptations of these appendages to facilitate food uptake and ingestion) are important to address knowledge gaps regarding macroinvertebrate mouthpart morphology.

The first objective focused on mayfly nymph identification. The mayfly nymphs from both the Magaliesberg and Waterberg catchments were classified to species except for *Elassoneuria sp.* (W). There have been potentially new species identified but not published (Agnew, 1980), so determining this species was a challenge and was left at genus level. The PCA does not give any evidence for taxonomic status of *Elassoneuria sp.* (W), as there were no congeneric species being compared to it.

The second objective investigated whether the different mayfly taxa examined showed any distinct and/or divergent characteristics in their mouthpart morphology. It was hypothesised that these mayfly taxa would show differences in mouthpart morphology because their feeding biomechanics differ – three different FFGs have been identified namely scraper, filter feeder and collector-gatherer. The observations of these variations in mouthpart morphology provide reasonable evidence to make inferences about their respective feeding groups.

Afroptilum parvum (M) and *A. parvum* (W) have simple maxillary palps with no visible setae that are relatively smaller in size when compared to the two other species. Maxillary palps are used to assist in the ingestion of food. Although the mouthparts are unspecialised, *A. parvum* (M) *and A. parvum* (W) have bipectinate setae on the apex of the paraglossae (Figure 18) and bipectinate setae on the apices of the galea lacinia (Figure 15) (Palmer *et al*., 1993b). These bipectinate setae are associated with trapping and retaining small food particles, such as fine particulate organic matter (FPOM) (McShaffrey & McCafferty, 1990; McShaffrey, 1992; Baptista *et al.*, 2006). It seems that *A. parvum* (M) and *A. parvum* (W) use the setae on the paraglossae to remove loose detritus from the substrate and the maxillae to manipulate the food into the alimentary tract (Palmer *et al.*, 1993b). *Afroptilum parvum* (W) and *A. parvum* (M) have an unfused labium, with maxillary palps without setae on the apicolateral margin of the palps, but bipectinate setae on the apices of the glossa and paraglossae and on the apex of the maxilla. Based on these traits *A. parvum* (W) and *A. parvum* (M) have therefore been classified as collector-gatherer functional feeders. Although other species of *Afroptilum,* from the Eastern Cape, have been found in depositional biotopes like gravel, sand, and mud (Palmer *et al.*, 1993b) and the *A. parvum* from this study were only found in the fast-flowing

riffles of all the study sites sampled, falling into the gatherer feeding group. This indicates that species from the *Afroptilum* genus are not restricted to a particular biotope.

An important feature to note is the position (marginally, dorsally, or apicolaterally) and type of setae present on the various mouthpart structures (Thomson, 2013), more specifically pectinate and bipectinate setae (McShaffrey, 1992). These two setae types are associated with trapping and retaining small food particles. If setae are present on outer mouthparts (those exposed to currents) or on forelegs, they are indicative of a filtering function – setae on forelegs are observed in various heptageniid and oligoneuriid species (for example, like those present on the forelegs of *Elassoneuria sp.* (W)). The presence of these setae on inner mouthparts (paraglossa, glossa, mandibles or maxilla) indicate where food is held during processing (McShaffrey, 1992).

When examining the maxillary palps of both *Elassoneuria sp.* (W) and *Afronurus barnardi* (W), in the study by McShaffrey (1992), he proposed that an increase in size and number of setae on the maxillary palp allows for efficient collection of various feeding materials and allows for feeding in faster flowing currents. It has been observed that an increase in maxillary palp size, shows a reduction in size and number of parts of the labium – as seen in *Afronurus barnardi* (W) and *Elassoneuria sp*. (W). As the maxillary palp increases in size and specialisation (e.g. development of chitinous scrapers seen in *Afronurus barnardi*) the labium begins to fuse together and is less complex (comparatively to *Afroptilum parvum* (W), for example). Therefore, the specialisation of the maxillary palps may be directly linked to the changes in the structure and overall use of the labium. The fused labium in *Elassoneuria sp*. (W) (due to larger more modified maxillary palps) has a reduced function when compared to the more differentiated labium of *Afroptilum parvum* (W) and *A. parvum* (M) by decreasing the amount

of food collected and filtered by the setae of the galea lacinia and the hypopharynx (McShaffrey, 1992). Additionally, the presence of filtering setae along the margin of the fibula and tibia of the forelegs are used to filter food from the water column, thus making it an important trait when classifying *Elassoneuria sp.* (W) as a filter feeder (McShaffrey, 1992). Whether or not *Elassoneuria sp.* (W) is an active or passive filter feeder is dependent on groups of microtrichia present on the filtering setae (Wallace & Merritt, 1980; Palmer *et al*., 1993b; Elpers & Tomka, 1995; Baptista *et al*., 2006). It was unclear how to determine if microtrichia are present or absent to determine passive or active filter feeding. There was no distinct evidence in the literature on how they look or what are clear signs of presence or absence. *Elassoneuria sp.* (W) is a filter feeder that removes suspended particulate matter from the current with its forelegs (Wallace & Merritt, 1980). An example of this filter feeding behaviour is exhibited by *Oligoneuriella rhenana* (Family Oligoneuriidae) and described by Elpers and Tomka (1995). *Oligoneuriella rhenana* use their meso- and metathoracic legs to hold onto substrate whilst filter feeding with their forearms. The head and front of the thorax are lifted above the substrate to allow adduction of the forearms to filter feed in the fast-flowing riffles (Appendix A) whilst the body remains parallel to the substrate (Elpers & Tomka, 1995).

Afronurus barnardi (W) has apicolateral setae on the maxillary palps, which are sclerotised. The sclerotization of *Afronurus barnardi (W)* maxillary palps increases the feeding possibilities as they are now able to scrape surfaces of rocks for feeding material. The mandibles can shear off food like algal filaments, whilst also being able to gather/filter fine food particles into the mouth from the substrate (Palmer *et al*., 1993b). The presence of chitinous scrapers is what classify *Afronurus barnardi* (W) as a scraper feeder. Additionally, because of the setae present

on the apicolateral margin of the maxillary palps they can collect loosely deposited organic matter on the substrate classifying them as brushers. Therefore, because of *Afronurus barnardi* (W)'s ability to both scrape and gather suspended material it is classified as a brusher/scraper feeder. The hypothesis in the second objective stated that these mayfly taxa would show differences in mouthpart morphology because their feeding biomechanics differ which has been shown in the descriptions of their mouthparts and the ways in which they use these microstructures to collect and filter food. Therefore, failed to reject the null hypothesis.

The third objective focused on whether *Afronurus barnardi* (W) and *Elassoneuria sp.* (W) differ in their feeding morphology to SEM images from taxa with confirmed FFG status in the Buffalo River, Eastern Cape. It was hypothesised that *Afronurus barnardi* (W) and *Elassoneuria sp.* (W) would show convergent mouthpart morphology with two taxa found in the Buffalo River as they are living in similar habitats with similar feeding materials. The proxy taxa used for comparison from the Palmer *et al*. (1993a, b) study were *Afronurus Harrisoni* (proxy for *Afronurus barnardi* (W)) and *Neurocaenis reticulata* (proxy for *Elassoneuria sp.* (W)). *Afronurus harrisoni* was characterised by Palmer *et al*. (1993b) as having bipectinate setae on the labial paraglossae and labial palps, and chitinous maxillary scrapers and sparse short setae on their maxillary palps. It was classified as a scraper feeder. *Neurocaenis reticulata* was characterised by Palmer *et al*. (1993b) as having long bipectinate setae on the labial paraglossae, long filtering setae on both the labial palps and maxilla, and small bipectinate setae on the maxillary palps. It was classified as an active filter feeder. From this study *Afronurus barnardi* (W) with its chitinous scrapers and bipectinate setae was classified as a brusher-scraper functional feeder. The presence of long setae on the tibia of *Elassoneuria sp.* (W) are indicative of a filtering function similarly to the proxy taxa *Neurocaenis reticulata*

(Tricorythidae), which has long setae on the outer mouthparts for filter-feeding (McShaffrey, 1992; Palmer *et al*., 1993b). Thus, it was classified as a filter feeder. These results are all in agreement with the findings from Palmer *et al*. (1993 a, b). Therefore, failed to reject the null hypothesis as feeding morphology does not differ to proxy taxa from Palmer *et al*. (1993)'s findings.

The fourth objective investigated whether three congeneric taxa *Afroptilum excisum* (taken from Palmer *et al.*, 1993b), *Afroptilum parvum* (M) and *Afroptilum parvum* (W) which are similar morphologically and ecologically would all fall into the same FFG and what that infers about their feeding niches and/or the classification system. Information on FFG and mouthpart morphology for *Afroptilum excisum* was taken from Palmer *et al*. (1993b). *Afroptilum parvum* (M) and *Afroptilum parvum* (W) were characterised as collector-gatherer feeders. Palmer *et al*. (1993b) classified *Afroptilum excisum* as a collector-gatherer due to its unspecialised mouthparts and the presence of bipectinate setae which are indicative of an organism that feeds on FPOM.

The definition of a niche has been confused, debated, and altered throughout the literature (e.g., Grinnell, 1917; Stevenson, 1982; Holt, 1987; Harmon *et al*., 2005; Whittaker, 1973; Winemiller *et al*., 2015), but the definition that will be used, relative to this study, is that a niche is the measure of resource utilisation by an organism (Holt, 1987). Hutchinson (1957) created a concept that details the necessary factors that must be present for a niche to be defined and distinguishable, and this is known as the "hypervolume concept". The hypervolume concept dictates that a niche exists within a range of specific environmental parameters necessary for an organism to optimally survive and thrive in. *Afroptilum excisum*, *Afroptilum parvum* (M) and *Afroptilum parvum* (W) are present in different catchments with

similar ecosystems (Buffalo River in the Eastern Cape, the Magaliesberg and the Waterberg) within a broad range of physicochemical parameters (i.e. rivers of varying condition) and they consume similar feeding material (gut contents were analysed for *Afroptilum excisum* in the Buffalo River, all species consuming FPOM, fungi, diatoms, and filamentous algae (see Chapter 3). *Afroptilum parvum* (W) and *A. parvum* (M) are two species with insignificant variation between them. Those collected from the Waterberg system come from a larger headwater stream with more variable habitat, whereas those collected from the Magaliesberg catchment are from smaller headwater streams. Observing the overlap between species in the PCA (after allometric correction) the variation in mandible length and width was found to be negligible between localities, with a strong interaction between length and width – as the width of mandible increases the length will increase proportionally. It was hypothesised that species from the same genus that are morphologically and ecologically similar, from three different localities, will fall into the same feeding niche. Based on these results, the hypothesis was not rejected as these congeneric taxa appeared to occupy the same feeding niche when there is high resource availability and ideal habitat for survival.

A study by Barmuta (1988) on organic matter and macroinvertebrates in an upland forested stream in Australia (Melbourne, Victoria), found that the FFG definition needs to be standardised and clearly defined to make the accurate and appropriate observations and inferences about functional community structure within streams and to be able to compare with other studies. The question of what to consider important when creating a definition of what encompasses a FFG for a genus or species is still complex. Although a clear definition was not made in this study, Cummins (1974) classification system was the foundation from which this study, Palmer *et al*., (1993a) and Schael (2005) used to make adapted FFG

classifications for South African rivers. The adapted FFG classification from Palmer *et al*., (1993a) is what was used for FFG classification in this study. The large focus around the FFG classification was adapted from Palmer *et al*. (1993a, b) and primarily focused on the mouthpart morphology and each of the functions of the available microstructures to provide information to classify each species into FFGs. This study found that the labia and maxilla possessed microstructures that provided the most useful information on food uptake and processing (Palmer *et al*., 1993b; Baptista *et al*., 2006).

There is, however, a need to address some of the criticism of the Northern hemisphere FFG classification system in the literature. It is true that there needs to be a Southern hemisphere FFG classification system that encompasses clear definitions of feeding behaviours and morphology that would clearly put an organism into a class (King *et al.*, 1988; Palmer & O'Keeffe, 1992) as the initial FFG classification by Cummins (1973) is too broad and not representative of the species found in the southern hemisphere, albeit from the same genera. King *et al.* (1988) discusses the core problem of the FFG classification concept being that the definitions of the functional groups are too vague. King *et al.* (1988) discusses the need to clearly differentiate whether FFGs are created with a focus on the feeding material or the feeders (independent of their feeding material). The example the authors used was with the FFG "shredder", trying to determine if shredder is inclusive of micro-shredders feeding on leaves and if it does, how it would be clearly distinguished from "collectors" that also similarly feed on leaves. If macroinvertebrates are useful in inferring the functionality of an ecosystem, it is important to determine what is more important to infer the levels of functionality – the mode of feeding, or the particle quality and size of what is being consumed? (King *et al.*, 1988).

From the findings of this study and through examination of the literature, the mode of feeding is seemingly more useful than the quality and size of the feeding material (Anderson & Sedell, 1979; Barmuta, 1988). In the examples from the literature of diet variability relative to FFGs, the diet shift from early instar larvae to late instar larvae (also referred to as ontogenic diet shifts) (Matthews *et al*., 1982; King *et al*., 1988; Lenat & Barbour, 1993; Masese *et al*., 2014; Ramírez and Gutiérrez-Fonseca, 2014, Banegas *et al*., 2020) are not indicative of a change in the habitat, but only of a change in feeding material preference at a different life stage. If an organism caught at the same instar stage (after a change to the habitat) exhibits changes in mouthpart morphology, it is likely that something in the environment has caused an adaptation (e.g., Šporka *et al*., 2006, Porst *et al*., 2012; Ganong *et al.,* 2021). It is possible that since aquatic organisms experience various disturbances (e.g. anthropogenic disturbance, floods, droughts, etc.) that observing organisms from the same species with variation in their mouthparts are exhibiting phenotypic plasticity in response to their external environment. Selection of advantageous features within the morphological variation present in a single generation of a taxon, lead to greater prevalence of that adaptation in the population of subsequent generations. A strong enough environmental change or event – potentially caused by a change in timing of regular seasonal events (e.g., seasonal rains) due to anthropogenic activity – could cause a behavioural adaptation to occur within a species for it to survive. At different instar stages macroinvertebrates will have a shift in diet (ontogenic diet shifts) – e.g., shift from herbivore-detritivores to carnivores (Feminella & Stewart, 1986; King *et al*., 1988). The stage that an organism is captured and analysed at is important and will influence what FFG class it falls into. This then means that for one species there can be at least two or more FFG classes, due to ontogenetic shifts in morphology.

In conclusion, the functional feeding groups of the four species from this study, based on the comparison to taxa collected from the Buffalo River (Palmer *et al*., 1993a), place *Elassoneuria sp.* (W), *Afronurus barnardi* (W), *Afroptilum parvum* (W) and *Afroptilum parvum* (M) into the feeding groups of filter feeder, brusher/scraper, and collector-gatherer respectively. The consistency in these three species' morphological features to those of Palmer *et al*. (1993a, b) suggest this classification system is valid. Cummins (1974) originally envisaged that FFGs could add to what we know about various macroinvertebrates and their feeding behaviour. This is true in part, but with a more explicit definition of FFGs they can tell us much more. Functional feeding groups can help to (1) add more information about the habitats the organisms are found in; (2) help infer community population dynamics through changes in relative proportions of available FFGs in a system; (3) determine levels of disturbance as known bioindicators; (4) evaluate organic material presence/absence and its available size classes through mouthpart specialisation and dietary analysis and; (5) asses for shifts in FFG type dominance, which can be indicative of changes in the physicochemical and physical river habitat (Barmuta, 1988; Palmer *et al*., 1996).

The findings of this chapter allow for one question: since there is no significant difference between *Afroptilum parvum* from both regions, do these findings correlate to differences in gut contents? The literature usually investigates mouthpart morphology in conjunction with gut content analyses, as discussed earlier in the chapter. Gut contents can add additional evidence for the placement of a species into its relative FFG or at a minimum provide information on the predominant feeding material ingested by a species. An investigation of feeding material ingested by this species collected will be covered in Chapter 3 of this study

Chapter 3: Dietary differences in *Afroptilum parvum*

Introduction

The diet of aquatic organisms is important in understanding the ecological state of an ecosystem, its processes and trophic energy cascades in a system (Koslucher & Minshall, 1973; Cummins, 1974). Cummins (1973) notes that there is variability in food habits of macroinvertebrates, due to their habitat and the difference in the ages of the organisms. Food preference has been described through investigation of particle size consumed and availability of microhabitats, not just from the available feeding material (Gray & Ward, 1979; Palmer *et al*., 1993b).

The analysis of gut contents can assist in assessing the range of feeding materials an organism feeds on, relative to what is available at different times of the year (Walker *et al*., 1988). For example fluorescence microscopy, utilising a fluorescent dye for illuminating chitinous cell walls in both fungi and algae (Maneval, 1936; Rodriguez-Tudela & Aviles,1991; Leck. 1999), has shown positive results in differentiating between feeding material types. There is an assumption that gut contents should help discern whether an organism is a specialist or generalist feeder, when the available feeding material is known in the habitat (Cummins, 1973; Walker *et al*., 1988).

The objective of this chapter was to investigate whether a lack of regional variation in mouthpart morphology is mirrored in a similar diet between the Waterberg and the Magaliesberg. When referring to Chapter 2's findings, *Afroptilum parvum* (W) and *A. parvum* (M) mouthparts are unspecialised and covered with bipectinate setae on the apices of the glossae and paraglossae which are indicative of feeding on FPOM.

However, Palmer *et al.* (1993a) noted that there are disadvantages with analysing gut content, such as: the disappearance of readily digestible material; predation without ingesting exoskeletons of the organisms; and difficulty in discerning the origin of any ingested leaf particles. Some solutions to resolve these disadvantages include analysing the foregut content; the use of additional techniques such as scanning electron microscopy (SEM) and fluorescent dye (Sephton & Hynes, 1983; Palmer *et al.*, 1993a; Wögerbauer & Kelly-Quinn, 2013). These techniques allow for the identification of different feeding material types (such as leaf matter, diatoms, filamentous algae, etc) and their relative proportions regardless of the mouthpart morphology of the animal.

The bipectinate setae found in *A. parvum* (M) *and A. parvum* (W) are associated with trapping and retaining small food particles, such as FPOM. *Afroptilum parvum* (M) *and A. parvum* (W) have an unfused labium, with maxillary palps without setae on the apicolateral margin of the palps, and bipectinate setae on the apices of the glossa and paraglossae and on the apex of the maxilla. These features are typical of this genus (Gillies, 1990). Based on these findings, they have been classified as collector-gatherer functional feeders. The aim of this chapter was to determine the degree of dietary difference between *A. parvum* (W) *and A. parvum* (M), collected from Waterberg tributary catchments (western Limpopo River basin) and headwaters of the Magaliesberg. The objective pursued to achieve this aim:

Objective 1. Determine whether differences in morphological variability observed between the two regions are mirrored in the variation in diet. It was hypothesised that Waterberg diets are more variable, representing a broader range of available food types than those found in Magaliesberg.

Materials and Methods

Field collections of final instar mayfly larvae

Afroptilum parvum were collected from the Sterkstroom and Maretlwane rivers in the Magaliesberg catchment and the Lephalala river in the Waterberg catchment, for cross catchment population comparisons of diet and feeding morphology. Study catchment and site specifications were described in Chapter 2.

Gut content preservation and dissection

All samples preserved for gut content analysis were preserved with a 10% formalin solution and after one week were placed in 50% ethanol. The collection and preservation of the samples were described in Chapter 2. Three to five individual replicate gut samples from the Magaliesberg and Waterberg catchments were observed under several complementary microscopes – each gut observed and its result are described, for each microscope, in Table 4.

The foregut (2–3 mm from the top of the thorax) was dissected out by removing the head from the last abdominal segment and pulling the gut out of the body cavity in one piece (Sephton & Hynes, 1983). The foregut contents were teased out and mounted onto a slide with DPX mounting medium. The slides were analysed under various microscopes using different techniques (Table 4) – microscopes that provided the most information were the SEM and the Zeiss Axio Imager light microscope. Various images were obtained using the following stains and microscopes: Lactophenol cotton blue stain method, ZEISS Axio Imager M2 upright microscope, ZEISS six-megapixel Axiocam 506 colour microscope camera and ZEISS Stereo Discovery V8 light microscope (described in Table 4).

Table 4. The various techniques used to analyse *Afroptilum parvum* (M) and *Afroptilum parvum* (W) gut contents.

Microscopes and methods	Techniques used
Lactophenol cotton blue stain method (n = 3-5 per locality)	Working with Lactophenol cotton blue is toxic, all staining and use of this substance were done under a fume-hood with appropriate lab ventilation and protective hand and eye gear. Samples were preserved in 70% ethanol and rehydrated before being stained. The samples were teased apart and stained with lactophenol cotton blue as this dye is known for illuminating chitinous cell walls in both fungi and algae (Maneval, 1936; Rodriguez-Tudela & Aviles,1991; Leck. 1999). The stain only appeared in one of all the samples analysed and was imaged under the ZEISS Axio Imager M2 upright microscope.
ZEISS Stereo Discovery V8 light microscope (n = ± 60-80 samples were made in total, of which not all were viable)	All gut contents were dissected under this microscope before being imaged with other microscopes.
ZEISS Axio Imager M2 upright microscope (n = 3-5 per locality)	Samples were preserved in Lugol's iodine solution in the field. Gut content samples were removed under the ZEISS Stereo Discovery V8 light microscope using a dissecting needle and insect pin. Thereafter, the samples were mounted onto glass slides using DPX mounting media and imaged for further analysis. The high magnification of the microscope and the high-quality camera made algae and diatoms in amongst FPOM easily discernible.
ZEISS six-megapixel Axiocam 506 colour microscope camera (n = 3-5 per locality)	
Brightfield microscopy (Olympus BX63 OFM) and Fluorescence microscopy (Olympus BX63 OFM) (n= 12, which is six samples per microscope)	Three samples were preserved in 70% ethanol in the field and three samples were preserved in Lugol's iodine solution in the field to prevent continued growth and colonisation of algal communities collected (Pomroy, 1984) for analysis under both the Brightfield and Fluorescence microscopes. The gut contents were removed under the ZEISS Stereo Discovery V8 light microscope using a dissecting needle and an insect pin. The samples were then mounted onto glass slides using DPX mounting media and sent to the Microscopy and Microanalysis Unit (MMU). The excitation wavelength was 525nm and the emission wavelength was 555nm. The Wilde & Fliermans (1979) method was used to determine the excitation and emission wavelengths for epifluorescence of algae.
Phenom Pure Desktop Scanning Electron Microscopy (SEM) (n = 3 per locality)	No preparation was needed for use of the desktop SEM. The gut contents were dissected out under the ZEISS Stereo Discovery V8 light microscope and placed onto aluminium stubs, dried, and viewed under the microscope.
Inverted microscopy (n = 10 – five samples per locality)	Samples were preserved in Lugol's Iodine Solution in the field. They were rehydrated using deionised water in petri dishes and observed under the inverted microscope. The amount of fine particulate organic matter (FPOM) in the gut contents obscured most view of algae and diatoms as it covered approximately >50-80% of the field of view (FOV) even after the gut contents were gently teased out for better viewing of gut samples.

Results

Gut content analyses

The gut content analysis revealed most of the algal and diatomaceous cells were obscured by large amounts of fine particulate organic matter (FPOM) making it difficult to do any proportional feeding material comparisons between the two species – only fragments of cell walls were visible (Figures 23 and 24). There were large amounts (60-90%) of FPOM in the guts of both species and presence of mineral material, filamentous algae, fungi, and diatoms (Figures 25-26). The similarity in the diet findings of the two species suggests that they are both gathering/grazing their feeding material from the substrate rather than filtering from the water column (e.g., *Elassoneuria sp*. (W)) or scraping from stones (e.g., *Afronurus barnardi* (W)). The results of all methods and techniques used to analyse gut contents were summarised, with remarks on what each of the results yielded (Table 5).

Table 5. Results for techniques used to analyse gut contents for *Afroptilum parvum* (M) and *Afroptilum parvum* (W).

Microscopes and methods	Results	Remarks
Lactophenol cotton blue stain method	The presence of fungi was minimal as it only appeared in one of the gut samples and is illuminated blue (Figure 24).	
ZEISS Axio Imager M2 upright microscope	Guts were imaged at 400X magnification and showed presence of FPOM, minerals, diatoms, and various filamentous algae (Figures 23-24).	Most images were obscured by large amounts of FPOM and some gut samples were almost completely empty.
ZEISS six-megapixel Axiocam 506 colour microscope camera		
Fluorescence microscopy (Olympus BX63 OFM)	At excitation wavelength = 525 nm and emission wavelength = 555 nm, images fluoresced green, but the gut contents were unclear and indistinguishable (Figure 25).	Unable to determine the correct wavelengths necessary for analysing freshwater gut algal samples, as information in the literature on freshwater fluorescence microscopy was limited.
Brightfield microscopy (Olympus BX63 OFM)	Images showed presence of FPOM, diatoms and algal cell walls (Figure 26).	Only cell walls and fragments of algal filaments were visible as the prolonged preservation of gut samples in formalin, 70% ethanol and 50% ethanol caused disintegration of the cell contents (Godhe *et al*., 2002; Sano *et al*., 2020). Cell contents are useful for identification and for better fluorescing with staining methods and fluorescence microscopy.
Phenom Pure Desktop Scanning Electron Microscopy (SEM)	Gut contents showed large amounts of FPOM and digested material that could not be further analysed. Fragments of diatoms were visible, but unable to see anything else in the images (Figure 27).	
Inverted microscopy	All algal samples analysed from the substrate (during the experimental trials) were visible under the microscope but all filamentous algae and diatomaceous gut contents that were analysed under the microscope were obscured by the large amounts of FPOM.	The magnification power and light intensity of the microscope were not high enough to be able to see through the FPOM for any presence of other feeding materials.

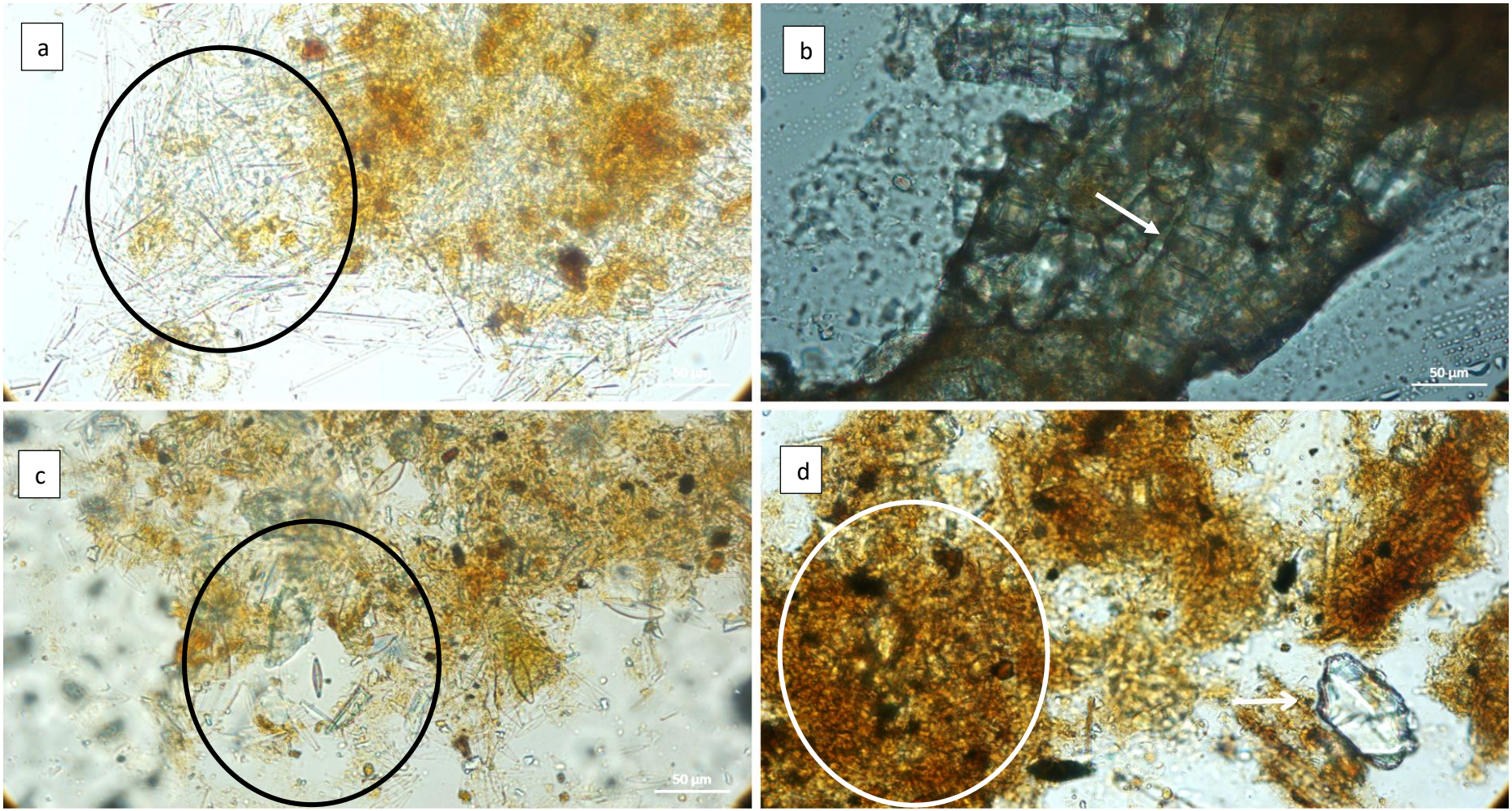


Figure 23. Zeiss Imager microscope images of food items found in the gut contents of *Afroptilum parvum* (M) showing (a) *Fragilaria* sp. a colonial diatom with cell filaments (black circle); (b) *Ulothrix* sp. a non-branching filamentous green alga (white arrow); (c) *Amphora* sp.*, Cymbella* sp., *Navicula* sp., *Nitzschia* sp. and *Achnanthes* sp. (black circle); (d) fine particulate organic matter (FPOM) (white circle) and mineral material (white arrow). Scale bars = 50 µm.

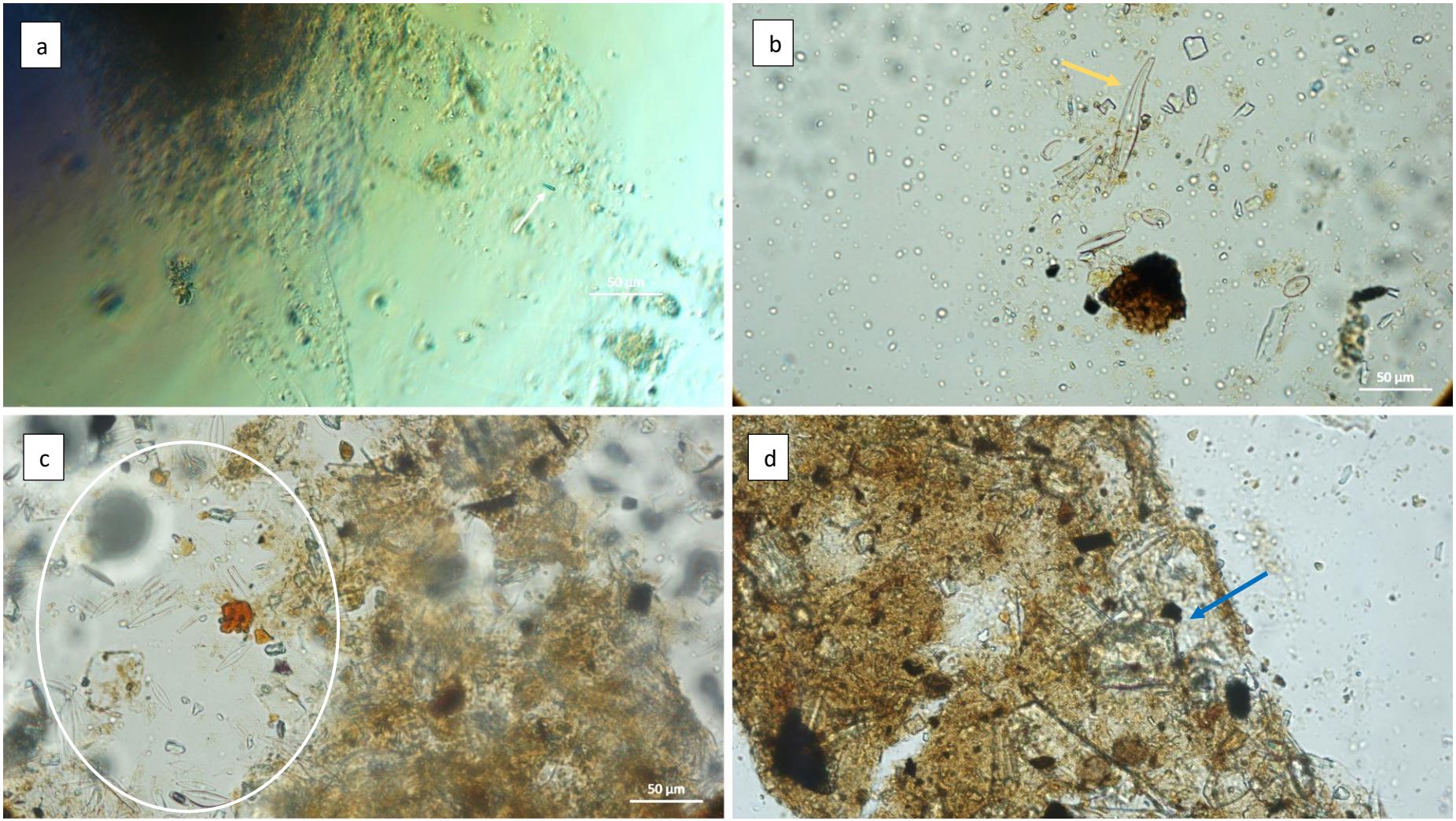


Figure 24. Zeiss Imager microscope images of food items found in the gut contents *Afroptilum parvum* (W) (a) fungus, dyed blue due to lactophenol cotton blue stain (white arrow); (b) *Diatomella* sp., *Acnanthes* sp. and *Gyrosigma* sp. (orange arrow); (c-d) FPOM, mineral material (blue arrow), alga and diatoms (white circle). Scale bars = 50 µm.

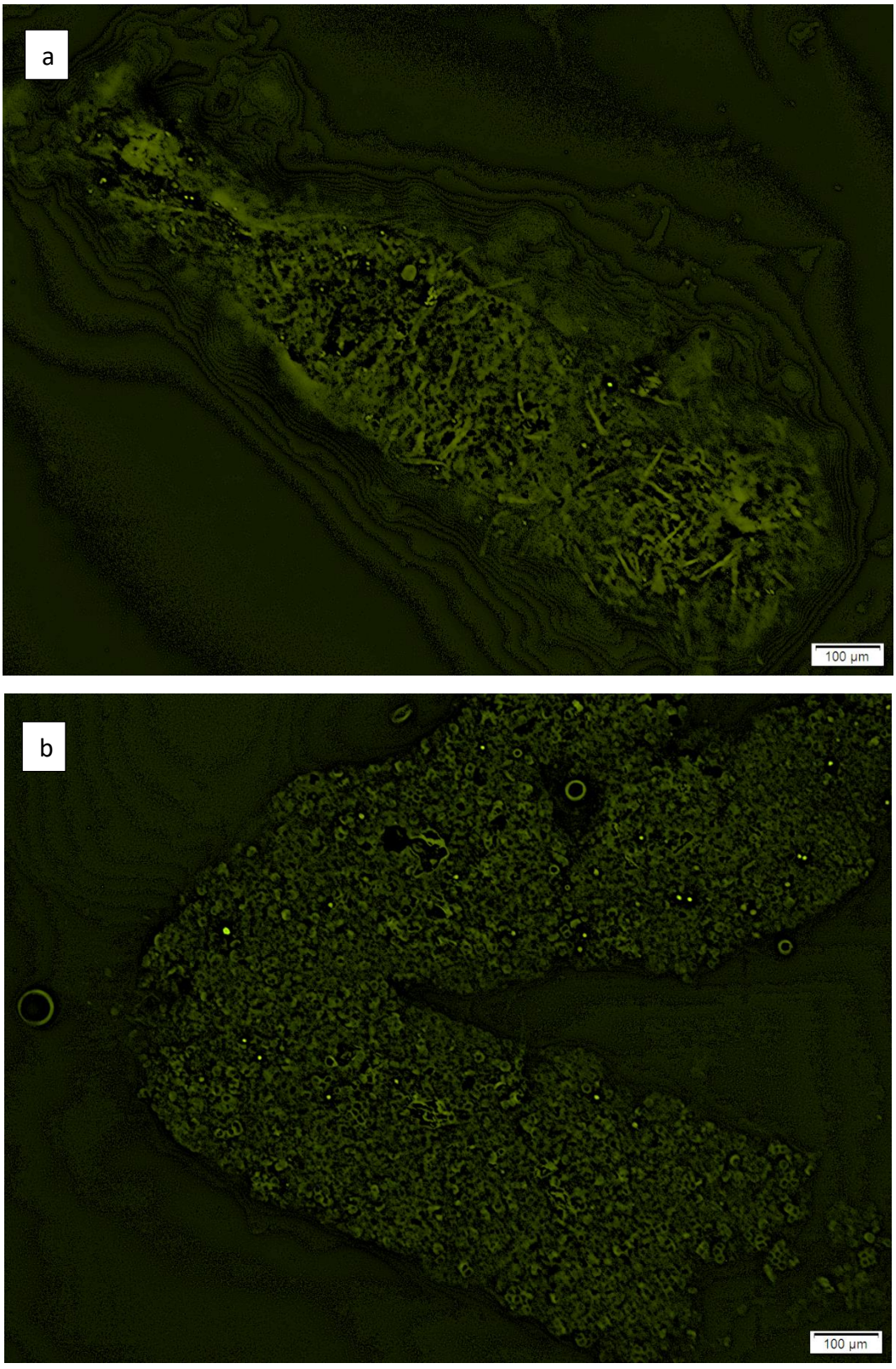


Figure 25. Fluorescence microscopy images (a-b) of gut contents of *Afroptilum parvum* (M), showing some gut contents illuminated bright green signifying some organic material, but few visible and discernible contents. Scale bars = 100 µm.

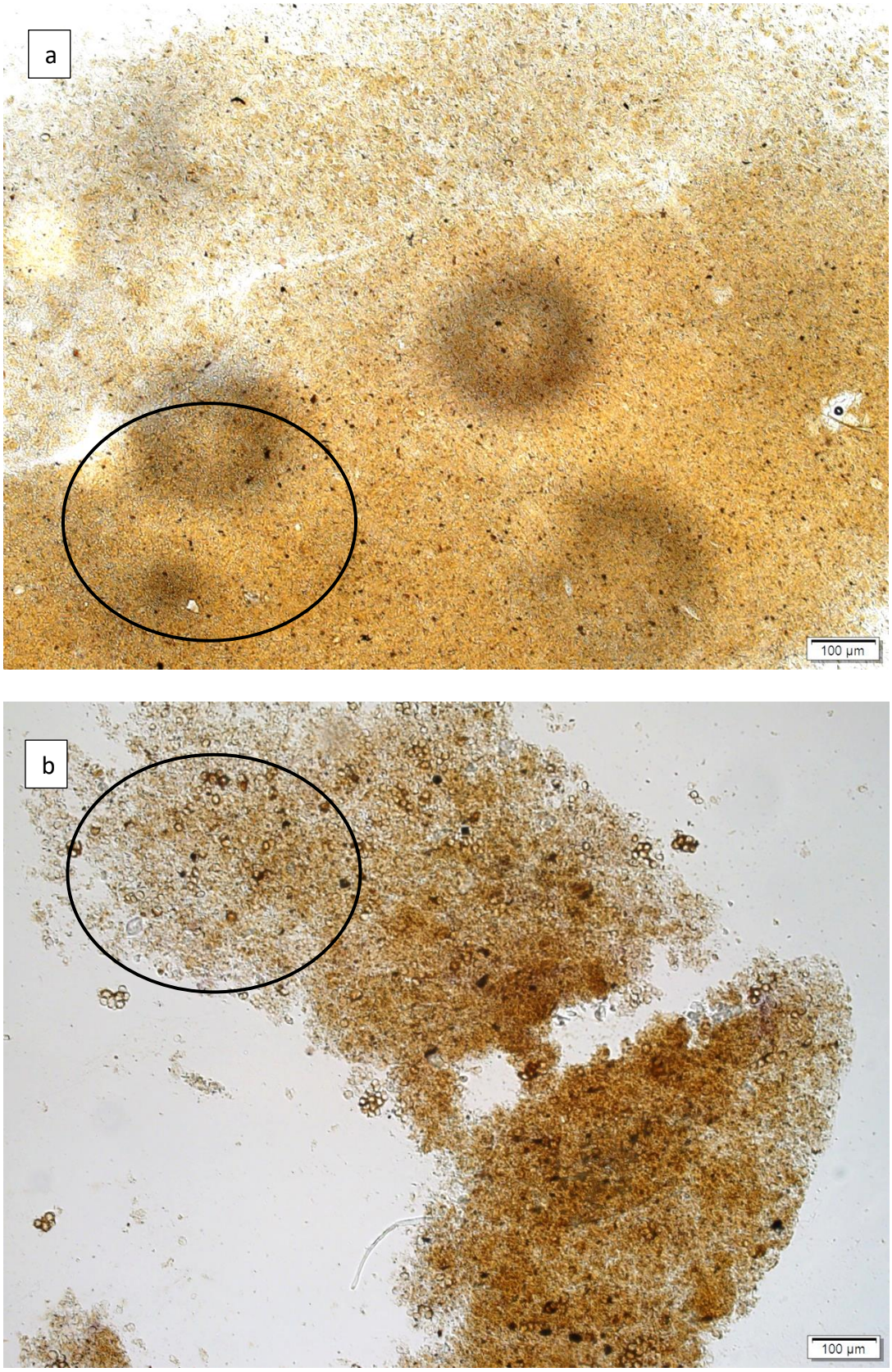


Figure 26. Brightfield microscopy images of gut contents of *Afroptilum parvum* (M) with a) FPOM (black circle) and b) some diatoms and small minerals present (black circle). Scale bars = 100 µm.

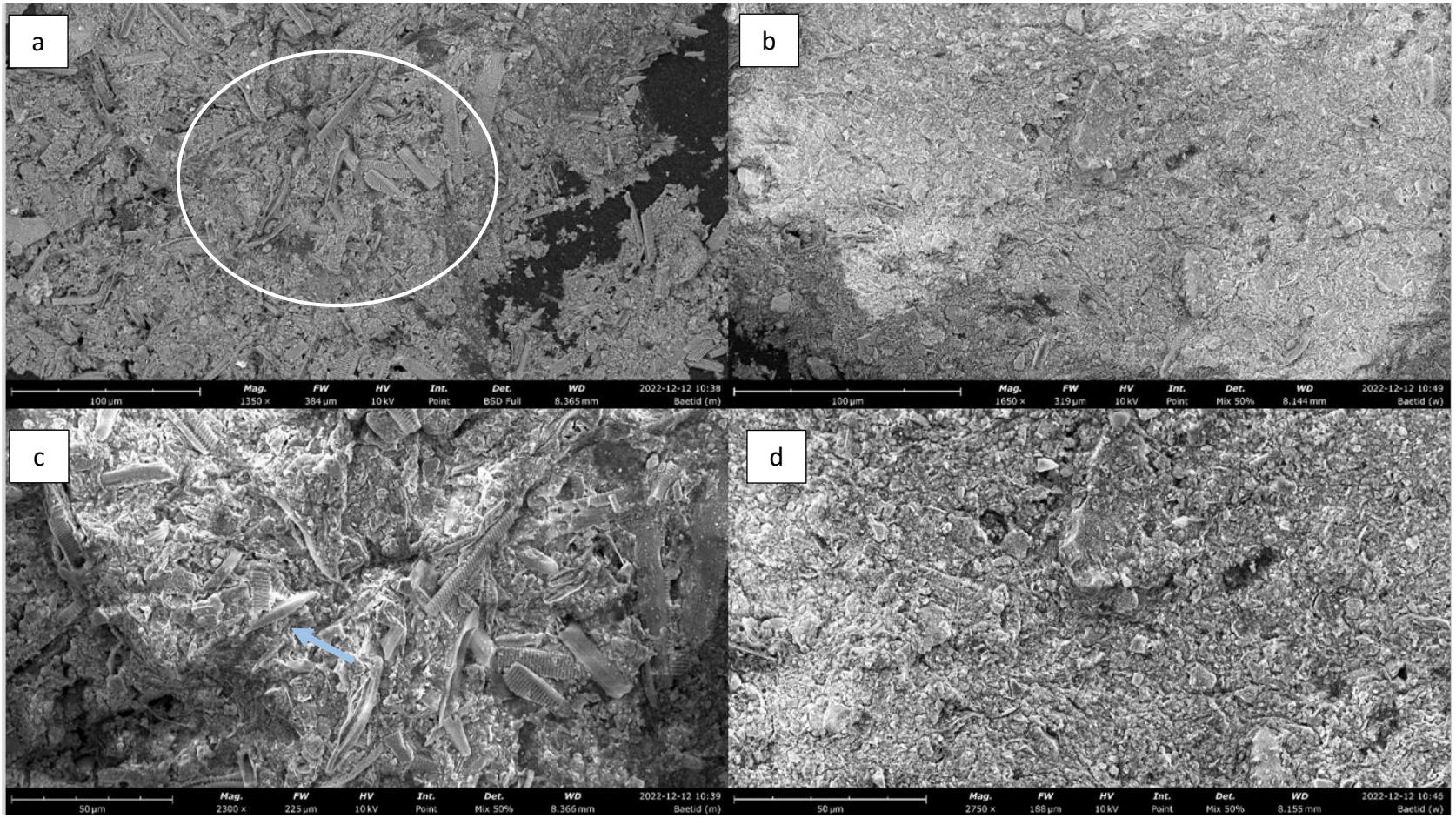


Figure 27. SEM Micrographs of the digested feeding material of *Afroptilum parvum* (M) with (a) fragments of filamentous algae (white circle) and (d) diatoms (blue arrow). Digested feeding material of *Afroptilum parvum* (W) (b and c) with indistinguishable fragments in the gut. Magnification (X) is 1350 for 29a, 1650 for 29b, 2300 for 29c and 2750 for 29d.Scale bars = 100 µm (a and b) and 50 µm (c and d).

Discussion

Afroptilum parvum are collector-gatherers with large amounts of FPOM, filamentous algae and diatoms in their guts according to what was found in the guts of the specimens sampled. In this study *A. parvum* (W) (1.99 – 2.96 cm) had a wider variation in physiological size of final instars when compared to *A. parvum* (M) (2.42 – 2.69 cm) which had a narrower variation in size of final instars. The difference in larval size, as found in the literature, to a certain degree can contribute to dietary differences (Matthews *et al*., 1982; King *et al*., 1988; Palmer *et al*., 1993a; Lenat & Barbour, 1993; Masese *et al*., 2014; Ramírez & Gutiérrez-Fonseca, 2014, Banegas *et al*., 2020), but that was not observed in this study.

The gut contents from both species showed presence of filamentous algae, diatoms, minerals, FPOM and fungi – both species were thus consuming the same feeding material. It was hypothesised that the physiological variation observed within a species collected from different regions could be linked to the abundance of feeding material available for consumption during earlier developmental instars. The results from the ANCOVA performed in Chapter 2 showed that within *A. parvum*, variance in mandible length differed significantly between from two different regions. This suggests that the mandibles have some phenotypic plasticity (a change in an organism's morphology to adapt to its environment). This plasticity allows them to feed on a variety of feeding material types. These findings could also be due to collection of larvae from a variety of habitats within the catchment. Thereafter a further investigation into the variety of feeding material was required. However, links to differences in diet could not be made.

The largest challenge faced in this study was the need to develop novel methods of dissection. In all the literature reviewed, no methods of how to dissect small aquatic organisms was

found (e.g. and Walker *et al*., 1988; Palmer *et al*., 1993a; Wögerbauer & Quinn, 2013). Their bodies were so fragile that any available tools in the laboratory were too big. After months of practice, trial and error, my co-supervisor Dr James du G. Harrison, created tools that I could use for dissection – a dissecting pin mounted on a wooden rod for holding and a needle for dissecting out mouthparts as scalpels were too big. It was not possible to clearly separate out the gut contents enough to avoid clumping of FPOM, as it obscured a clear view of any other feeding material. An additional problem arose with the preservation techniques used. Initially samples were preserved in 70% ethanol for mouthpart preservation, but almost all the guts were empty. The primary goal of the 70% ethanol preservation was for the conservation of the mouthparts. Thereafter, a second preservation technique was attempted where specimens were preserved in a 10% formalin solution and after two weeks specimens were transferred to a 50% ethanol solution that preserved both the specimens and the guts (adapted from Gray & Ward, 1979). However, the specimens initially preserved in formalin became rubbery and the gut contents had a film coating that (once dissected) erupted out of the body capsule in minute bubbles with a lipid membrane around them which was hard to collect and remove even after rehydrating in deionised water.

The preservation of specimens for months caused the algae chloroplasts to disintegrate which meant I could not use methods like those employed by Walker *et al.* (1988), or Wögerbauer & Quinn (2013), for example. Wögerbauer and Quinn (2013) and Walker *et al*. (1988) used a fluorescent dye 4’6 diamidino-2-phenylindole (DAPI) (nucleus dye) to differentiate algal types present in the guts as it binds to double-stranded DNA and illuminates various structures into a spectrum of colours under fluorescence microscopy. They managed to differentiate between different feeding materials in the guts of mayflies by shape and colour. They found

the predominant feeding materials ingested and their relative proportions with this method. This was not possible for this study, as by the time the samples were analysed the algal and diatomaceous cell contents had disintegrated due to the prolonged preservation in 70% ethanol, so all that was visible was predominantly cell walls. Fortunately, with the use of the Freshwater Algae Guide (Prescott, 1964) and the remaining cell walls visible in the microscope images, some of the feeding material was identified to genus level (see Figures 23-24).

Moreover, an additional task during this process was the differentiation of foreguts from hindguts – there is nothing in the observed literature that has clearly defined what differentiates a foregut from a hindgut morphologically (e.g., Palmer & O'Keeffe, 1992; Palmer *et al*., 1993 b) and determining already ingested matter from other matter still being processed in the gut was not easily discriminated.

Palmer *et al*. (1993a, b) and Palmer and O'Keeffe (1992) employed nymph mouthpart morphology and feeding experiments to supplement evidence from diet analyses. In the dietary analyses, they found that of all the gut contents analysed, 94.98% showed no significant difference in feeding material consumed between seasons, and the most significant difference (for all species studied) in feeding material ingested was observed when comparing juvenile (small) and large larvae gut contents of the same species (Palmer *et al*., 1993a). Proportion of gut content was directly linked to body size (i.e., larger organisms had more food in their gut when compared to smaller organisms). Significant difference in dietary composition can be due to change in season, and locality of sampling. Palmer *et al*., (1993a) found that dietary variability does not prevent a FFG classification of an organism, but it cannot be used alone to assign an organism to a FFG. Difference in diet will not necessarily denote a change in FFG but it is useful when noting common and rare food items ingested by

each species (Palmer *et al*., 1993a). Although gut content analysis would provide additional useful evidence for FFG classification it is not always the most informative in habitats where organisms have overlapping niches and feeding habits.

Therefore, the objective of assessing whether high variability in mouthpart morphology in Waterberg compared to Magaliesberg is reflected to the two populations' respective diets could not be achieved. In conclusion, in all the reviewed literature the definition of what comprises a FFG and what is important to consider (i.e., the feeder or the feeding material) changes based on the context in which FFG are being used to answer a bigger question (Anderson & Sedell, 1979; Barmuta, 1988). The criticism on the use of gut content analysis for assigning FFGs (that the FFG themselves are insufficiently defined; King *et al*., (1988)) is not completely unfounded but with the correct preservation and dissection of gut contents they still add useful information on feeding behaviour. Certain feeding material types can only be obtained through specific feeding behaviours and mouthpart adaptations (Palmer & O'Keeffe, 1992). *Afroptilum parvum* (W) inhabits a big river in the Waterberg compared to the smaller stream in the Magaliesberg *A. parvum* was sampled from. The floods during the rainy season in both these catchments may impose different environmental pressures on these species due to the difference in catchment size, creating intraspecific changes that drive morphological divergence.

Future research should focus on specific biomechanical and food processing tasks of the mandibles in this species, specifically using behavioural studies to better understand variance in specimens collected from different regions but of the same species. Additionally, the use of the correct preservation technique and storage time would allow for more data collection for analyses.

Chapter 4: General conclusion

This work was inspired by the doctoral theses and publications of Palmer and colleagues (Palmer & O'Keeffe, 1992; Palmer *et al*., 1993a, 1993b) and Schael (2005), as they were the most recent work done, in South Africa, on aquatic insect functional feeding group classification systems and their importance. This study has tackled the investigation and description of FFGs for three additional mayfly species and has outlined the major difficulties and complexities associated with creating FFG classification systems. The usefulness and effectiveness of FFGs in river ecology is of secondary importance if the definitions used in describing the functional feeding groups of organisms is inconsistent and not comparable between species within a population and between populations.

The first chapter found that identifying this study's mayfly nymphs to species level was possible for *Afronurus barnardi* and *Afroptilum parvum*, but *Elassoneuria sp*. could only be identified to genus level, as several oligoneuriid species have been identified from this genus, but not many have been published (Agnew, 1980).

In the second chapter, the three taxa studied showed distinct divergence in mouthpart morphology between each taxon. An increase in size and specialisation of the maxillary palps presumably influenced and altered the role of the labium in feeding behaviour of both *Afronurus barnardi* (W) and *Elassoneuria sp.* (W). The increased use of the maxillary palp has shown increased development of the distal part of the mandible used as a comb to remove food particles from the water column or the surface of the substrate (McShaffrey, 1992). Organisms with well-developed maxillary palps are likely to have been taken from stones-in-current microhabitats which means proportionally their mouthparts will process less detritus and more living material such as diatoms (McShaffrey, 1992). Between the three taxa studied,

the maxillary palps in *Afroptilum parvum* (from both localities) are unmodified with simple palps and setae absent; *Afronurus barnardi* (W) are the only mayfly with large chitinous palps and bipectinate setae; in *Elassoneuria sp.* (W) The palps are larger than *Afroptilum parvum (W)*, *Afroptilum parvum (M)* and *Afronurus barnardi* (W) and covered in many long setae. In this chapter there were three proxy species studied namely *Afronurus harrisoni*, *Afroptilum excisum* and *N. reticulata* (Palmer *et al*., 1993b). The paraphyletic *Elassoneuria sp.* (W) (family Oligoneuriidae) and *N. reticulata* (family Tricorythidae) are two completely unrelated species from two clades of the mayfly family tree (Fry, 2011; Sartori & Brittain, 2015). They were found to be convergent in their functional feeding niche as they are both filter feeders. *Afronurus harrisoni* and *Afronurus barnardi* are from the same genus and show slight divergence in their feeding groups but are both brusher-scraper feeders. Lastly *Afroptilum excisum* and *Afroptilum parvum* are from the same genus, but different species and are both collector-gatherer feeders.

Micrograph imaging showed that although the molar area has been traditionally considered for use in crushing food particles for mayflies living in habitats with detritus and live algae, the molar region for *Afronurus barnardi* (W) and *Elassoneuria sp.* (W) has been adapted for straining water from food, compressing and for grinding. Additionally, the multivariate analyses showed that many mouthpart measurements were influencing the clustering of species into their respective feeding groups – showing that although the maxilla and labia are important at differentiation feeding groups, they are not the only drivers. Between *A. parvum (W)* and *A. parvum (M)* there was variation in body size, left and right mandible length and width and maxillary palp length showing intraspecific variation within the species, which could be linked to differences dietary preferences between the two sampled regions. The

analysis of gut contents to determine dietary differences were unsuccessful but this study has found that *A. parvum* (W) and *A. parvum* (M) are two species with insignificant variation between them. Observing the overlap between species in the PCA the variation in mandible length and width was found to be negligible between localities, with a strong interaction between mandible length and width – as the width of mandible increases the length will increase proportionally. It was hypothesised that species from the same genus that are morphologically and ecologically similar, from three different localities, will fall into the same feeding niche.

Upon further review of the literature, a research question emerged, which asked what was more important when assigning FFGs: the traits of the feeder or the size and quantity of the feeding material (Barmuta, 1988). In this study, from the findings obtained, it was decided that the feeder was more important than the feeding material found in the gut because the best the gut content analyses was able to show was some presence and/or absence of available feeding material types. However, what is present in the gut at the time of sampling is influenced by several factors namely the stage at which the mayfly nymphs are collected and sampled, nymph body size (early versus late instar larvae – see Palmer *et al.*, 1993a) and how readily digestible the feeding material is (foregut and hindgut differentiation – see Sephton & Hynes, 1983). Gillies (1991, 1992) showed that *Afroptilum parvum* (previously *Afroptilum sudafricanum*) occurs throughout coastal South Africa and East Africa. It is found in many aquatic ecoregions, thus is likely a habitat generalist with a broad ecological niche. The analysis of feeding material in the gut isn't a stand-alone tool for the determination of a FFG classification, but in conjunction with mouthpart morphology or feeding behaviour experiments it can provide useful insights depending on the approach used. A review of

studies done by Palmer and O'Keeffe (1992), Palmer *et al*. (1993a), Baptista *et al*. (2006) and Wögerbauer and Quinn (2013) were all successful in classifying nymphs into FFGs as they were able to analyse either gut content, mouthparts, or both with the correct preservation techniques and after data collection – which was the biggest shortfall of this study, not necessarily the lack of data.

The experimental design and field collection presented severe challenges to successful hypothesis testing. Attempting various techniques and methods to extract some useable data proved to be almost as hard as the analyses themselves. There is not enough published literature that adequately describes the correct and most effective preservation techniques for preserving mayflies and gut contents. They must be preserved correctly and worked on as soon as possible after collection, as this study found that the formalin and ethanol caused degradation of the chloroplasts and the nuclei in the algae making it hard to adequately identify potential species (Wilde & Fliermans, 1979). To obtain useable data for this study, the use of the SEM, ZEISS Axio Imager M2 upright microscope, ZEISS six-megapixel Axiocam 506 colour microscope camera and ZEISS Stereo Discovery V8 light microscope were the best for imaging and analysing mouthpart and gut contents at high magnifications. The use of brightfield and fluorescence microscopy could be informative on living material such as the presence of blue-green algae or red alga but it is limited by preservation time and finding the correct wavelengths for viewing freshwater alga and diatoms (e.g. Wilde & Fliermans 1979; Walker *et al*., 1988; Wögerbauer & Quinn, 2013).

For the mouthpart preservation: use of 70% ethanol in the field for preservation is best for preserving the head and body for identification and dissection of mouthparts and to determine genus and species. Upon return to the lab, ethanol should be changed to fresh 50-

70% ethanol. Mouthparts can also be stored in a 10% formalin solution for longevity and changed to 50% ethanol upon return to the lab. For the preservation of gut contents, two key recommendations have emerged from this project's findings. The first recommendation is that the organism should not be preserved with ethanol for more than a few days (Godhe *et al*., 2002) before being placed in deionised water to rehydrate the body and gut contents, as it causes degradation of cell walls and chloroplasts in the gut of the organism. In the study by Godhe *et al*. (2002) they found that preserving microalga in 75% ethanol for two weeks, resulted in no cells left intact for DNA extraction and amplification. However, samples preserved in 95% ethanol lost 10% of cells after two weeks. They further went on to recommend preserving samples for 10-30 minutes in a formalin solution and then transferring the sample to 100% methanol. This method of preservation was most successful for DNA extraction and amplification. Preservation techniques like these allow for use of fluorescent dyes (see chapter three) as the chloroplasts and nuclei of the cells are still intact. The second recommendation is that it may also be useful to attempt preservation of the entire organism with Lugol's iodine solution in the field as this may preserve the entire organism and its guts long enough for analysis but not for longer than a week as it causes dark staining of the gut contents (the iodine solution stains the gut contents darker the longer it remains in the solution). Substrate from where the organisms were collected should also be collected and stained with Lugol's iodine solution for analysis. The collection and identification of the available feeding material present to the organism can provide useful information (coupled with physicochemical data) as to what may be comprising material ingested (also allows for selective versus generalist feeding theories), predominant feeding material ingested comparatively for an organism and environmental parameters influencing food availability and abundances.

An increasing number of studies from around the world are finding the FFG classification system (from the northern hemisphere) cannot be used in a southern hemisphere context (King *et al*., 1988; Palmer *et al*., 1993a, b; Schael, 2005). Cummins' (1973, 1974) broad definition of what a FFG is, make comparisons between northern and southern communities difficult. This study concluded that it is possible to use the classification system of Cummins as a starting point for classifying organisms into FFGs and expanding on that initial definition to create region specific classification systems, with the same foundation, but for different regions around the world.

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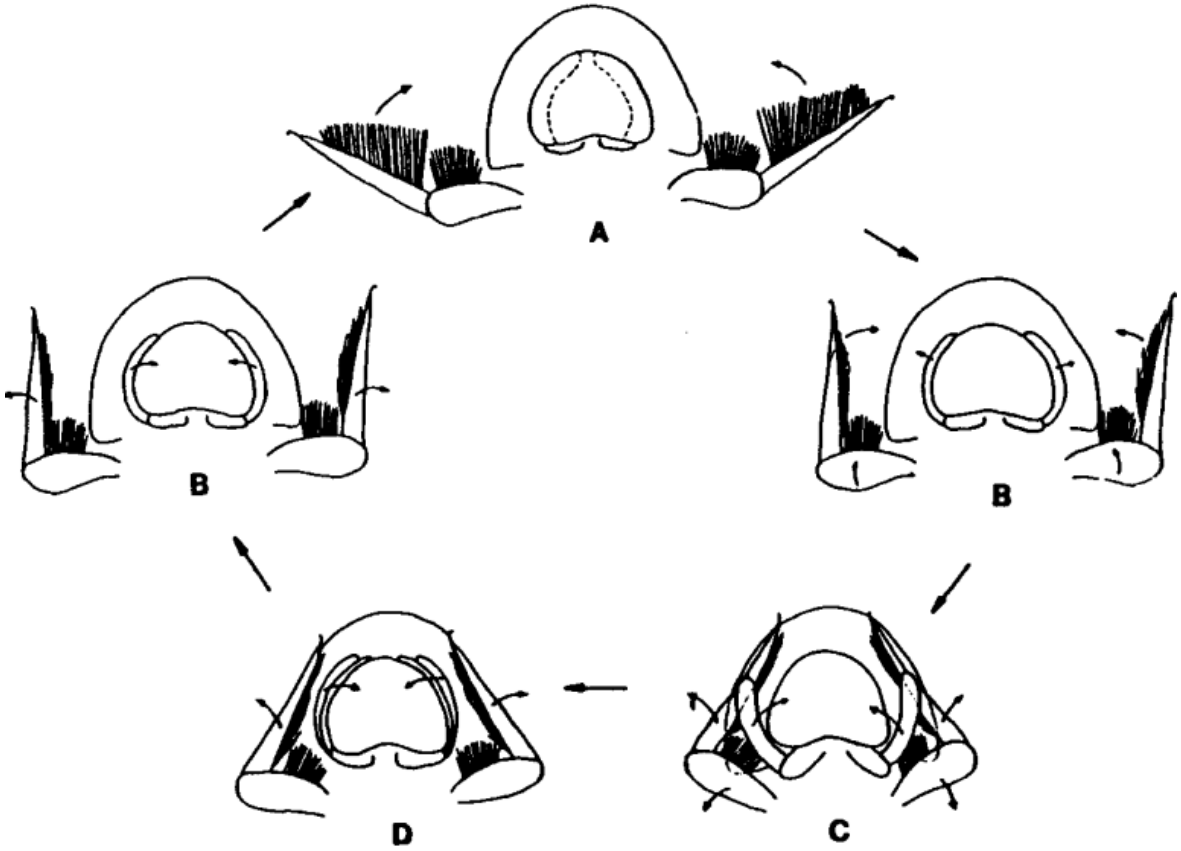
Appendices

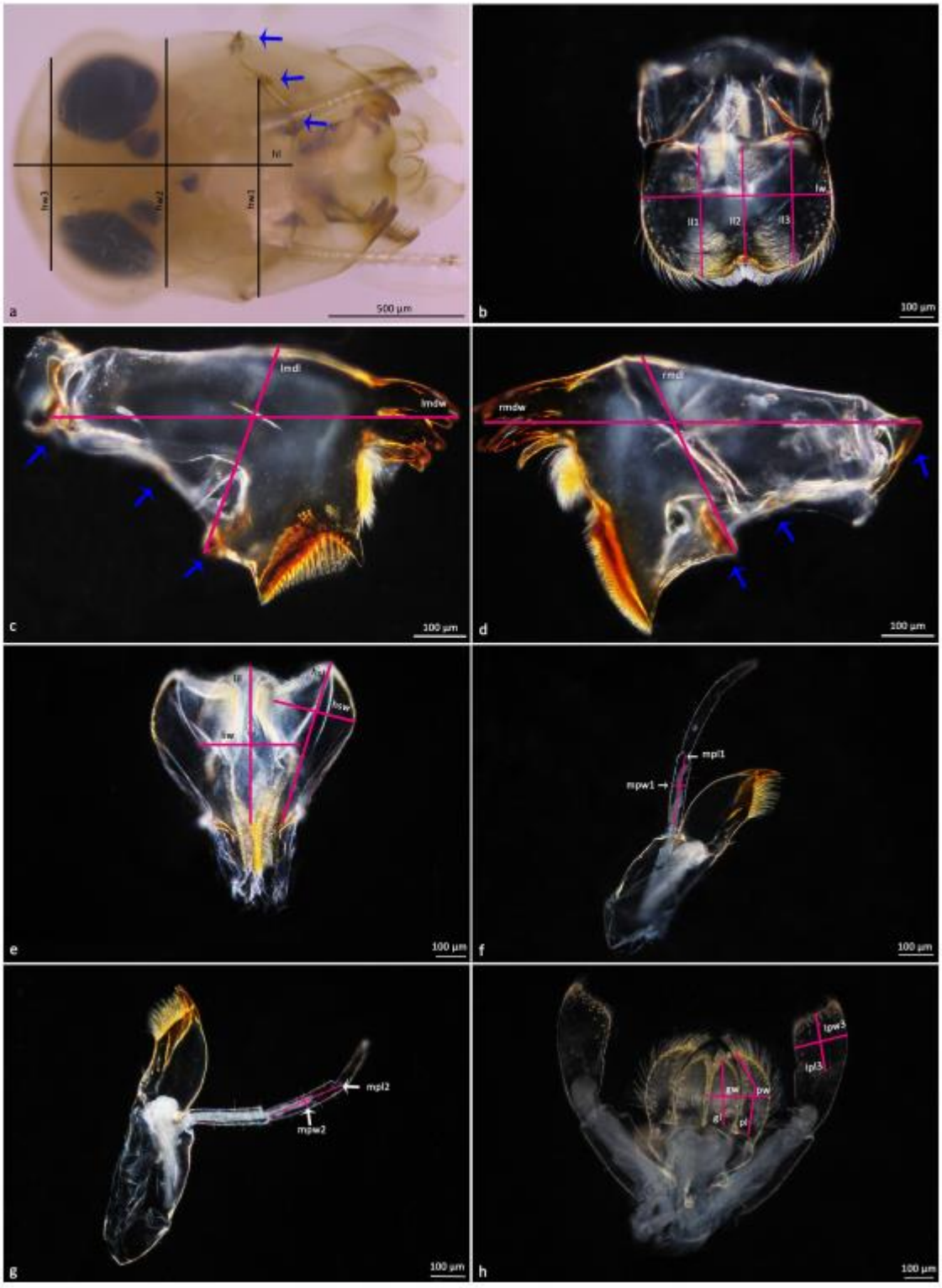


Appendix A. Filtering cycle of the *Oligoneuriella rhenana*, ventral view. A: filtering position, B: inward movement of the prothoracic legs, C: labial and maxillary palps in outward position (maxillary palps not visible), protohracic legs in maximum inward position: setae of prothoracic legs are situated between dorsal side of labial palps and ventral side of the maxillary palps, D: prothoracic legs in outward movement, palps in inward movement:

particles, which are caught in the setae pf prothoracic legs during the filtering cycle remain in setae of palps (Elpers & Tomka, 1995).

Appendix B. Trial experiments to observe feeding behaviour for *Centroptilum sp. 1*. Methodology was adapted from Palmer and O'Keeffe (1992).

Method	Outcomes and remarks
Feeding behaviour trial 1: Dissecting Microscope	1. Algal feeding material collected from the riffles was grown on rectangular stone tiles (10.3 cm x 5.2 cm), by placing these tiles amongst boulders/rocks from the river so that the feeding material could gradually transfer and reproduce on this new surface. 2. The initial experiment was to place these feeding tiles in a feeding tank and observe feeding behaviour with a dissecting microscope. 3. Tiles were to be placed on a grid in the microscope FOV and feeding behaviour observed. 4. This was unsuccessful as insects were small, fast and did not remain stationary long enough to observe any feeding behaviour. 5. No feeding behaviour could be observed with the dissecting microscope, as the mouthparts were not visible at any magnification.
Feeding behaviour trial 2: Binocular microscope	1. A Binocular microscope was used to observe feeding behaviour but could not observe any behaviour from above the insect. 2. Was unable to observe the selection of the feeding material or how it was being consumed. 3. Insects were fast and did not remain stationary long enough to observe feeding behaviour.
Feeding behaviour trial 3: Inverted Microscope	1. Insects were placed in petri dishes with river water that they had acclimatised to for 24 hours in the lab. 2. Five insects were placed in the petri dish with filamentous algae placed into the petri dishes, 3. However, no feeding behaviour was observed because the insects were too opaque and the light filtering from beneath the insect did not illuminate the head or mouthparts enough to observe any kind of feeding behaviour. 4. Insects were fast and did not remain stationary enough to observe feeding behaviour.



Appendix C. Mouthparts of *Cloeon dipterum* (Linnaeus, 1761) measured with their acronyms. a Head, dorsal view. b Labrum. c Right mandible, d Left mandible. e Hypopharynx. f Right maxilla. g Left maxilla. h Labium. hw: head width; hl: head long; lw: labrum width; ll: labrum long; rmdw: right mandible width; rmdl: right mandible long; lmdw: left mandible width; lmdl: left mandible long; hsw: hypopharynx superlingua width; hsl: hypopharynx superlingua long; liw: lingua width; lil: lingua long; mpw: maxillary palp width; mpl: maxillary palp long; gw: glossae width; gl: glossae long; pw: paraglossae width; pl: paraglossae long; lpw: labial palp width; lpl: labial palp long. The blue arrows indicate the articulatory surfaces (Banegas *et al*., 2020).

Appendix D. Head mouthpart measurements of *Afronurus barnardi*, *Afroptilum parvum* and *Elassoneuria sp*. Abbreviations used for the mouthpart structures are defined in Appendix C; (SD = Standard deviation).

Variable	Location	Species	Abbr.	Mean	±SD	Minimum	Maximum
Head	Waterberg	*Afronurus barnardi*	hl	333.6	37.7	270.2	362.9
			hw1	295.5	33.4	244.5	327.5
			hw2	463.2	48.2	392.4	526.1
			hw3	395.9	36.2	335.8	430.1
	Waterberg	*Elassoneuria sp.*	hl	1132.0	142.6	893.0	1246.0
			hw1	745.8	59.3	660.0	822.3
			hw2	1165.2	110.8	969.1	1229.7
			hw3	1137.9	151.9	908.7	1828.2
	Waterberg	*Afroptilum parvum*	hl	680.5	202.8	340.0	881.8
			hw1	536.4	246.1	202.3	790.1
			hw2	720.7	208.8	406.9	958.1
			hw3	525.4	113.3	365.7	648.6
	Magaliesberg	*Afroptilum parvum*	hl	974.0	92.4	909.9	1133.8
			hw1	704.7	137.6	502.3	827.1
			hw2	957.1	105.2	800.1	1046.0
			hw3	761.1	69.8	658.5	841.9

Appendix E. Labrum mouthpart measurements of *Afronurus barnardi*, *Afroptilum parvum* and *Elassoneuria sp*. Abbreviations used for the mouthpart structures are defined in Appendix C; (SD = Standard deviation).

Structure	Location	Species	Abbr.	Mean	±SD	Minimum	Maximum
Labrum	Waterberg	*Afronurus barnardi*	ll1	299.4	47.5	243.8	351.4
			ll2	349.7	40.8	294.4	394.5
			ll3	287.6	42.8	239.5	328.0
			lw	851.7	118.8	668.1	964.2
	Waterberg	*Elassoneuria sp.*	ll1	371.9	121.9	283.1	586.4
			ll2	488.6	132.0	379.5	718.2
			ll3	377.3	100.4	279.8	546.4
			lw	644.1	129.8	558.1	874.2
	Waterberg	*Afroptilum parvum*	ll1	175.4	15.1	162.1	196.5
			ll2	172.5	17.0	151.6	190.2
			ll3	174.0	16.3	154.4	191.0
			lw	302.0	41.2	267.1	363.8
	Magaliesberg	*Afroptilum parvum*	ll1	213.8	16.1	190.4	235.9
			ll2	200.9	16.0	175.7	218.4
			ll3	213.0	15.7	321.0	379.6
			lw	346.8	22.4	196.4	238.5

Appendix F. Left and right mandible measurements of *Afronurus barnardi*, *Afroptilum parvum* and *Elassoneuria sp*. Abbreviations used for the mouthpart structures are defined in Appendix C; (SD = Standard deviation).

Structure	Location	Species	Abbr.	Mean	±SD	Minimum	Maximum
Right mandible	Waterberg	*Afronurus barnardi*	rmdl	321.5	55.8	255.3	376.4
			rmdw	846.2	137.2	673.7	995.2
	Waterberg	*Elassoneuria sp.*	rmdl	320.8	192.6	177.4	539.2
			rmdw	732.9	438.4	393.6	1245.3
	Waterberg	*Afroptilum parvum*	rmdl	286.9	46.2	230.0	340.7
			rmdw	541.2	81.2	421.1	639.9
	Magaliesberg	*Afroptilum parvum*	rmdl	294.3	15.7	277.4	312.0
			rmdw	554.7	24.8	587.0	520.3
Left mandible	Waterberg	*Afronurus barnardi*	lmdl	316.4	72.2	223.3	380.1
			lmdw	838.6	165.8	608.0	1008.5
	Waterberg	*Elassoneuria sp.*	lmdl	378.6	110.8	295.0	509.4
			lmdw	902.3	265.8	690.8	1215.1
	Waterberg	*Afroptilum parvum*	lmdl	269.9	36.3	229.9	326.1
			lmdw	539.8	96.4	432.1	666.8
	Magaliesberg	*Afroptilum parvum*	lmdl	294.8	30.9	263.2	334.3
			lmdw	556.4	40.4	496.2	594.4

Appendix G. Hypopharynx mouthpart measurements of *Afronurus barnardi*, *Afroptilum parvum* and *Elassoneuria sp*. Abbreviations used for the mouthpart structures are defined in Appendix C; (SD = Standard deviation).

Structure	Location	Species	Abbr.	Mean	±SD	Minimum	Maximum
Hypopharynx	Waterberg	*Afronurus barnardi*	hsl1	175.1	35.7	124.9	212.6
			hsl2	360.7	49.7	301.3	418.4
			hsw	191.6	34.4	152.7	244.7
			lil	452.5	35.7	406.8	492.4
			liw	483.8	26.4	440.6	511.5
	Waterberg	*Elassoneuria sp.*	hsl	394.5	113.4	321.9	593.6
			hsw	357.8	54.6	303.6	447.6
			lil	444.0	135.1	349.6	676.1
			liw	447.3	163.6	314.5	728.6
	Waterberg	*Afroptilum parvum*	hsl	210.4	16.4	188.1	231.8
			hsw	102.6	17.4	83.3	126.9
			lil	224.1	16.7	194.8	233.9
			liw	136.0	24.1	108.4	160.3
	Magaliesberg	*Afroptilum parvum*	hsl	212.9	24.3	194.6	254.0
			hsw	94.9	17.5	77.8	119.9
			lil	220.8	35.3	189.6	279.3
			liw	127.3	21.1	96.0	147.3

Appendix H. Labial palps mouthpart measurements of *Afronurus barnardi*, *Afroptilum parvum* and *Elassoneuria sp*. Abbreviations used for the mouthpart structures are defined in Appendix C; (SD = Standard deviation).

Structure	Location	Species	Abbr.	Mean	±SD	Minimum	Maximum
Labial palps	Waterberg	*Afronurus barnardi*	lpl	822.2	157.9	603.5	990.8
			lpw	435.3	127.9	306.9	574.7
	Waterberg	*Elassoneuria sp.*	lpl1	580.5	122.6	483.3	793.5
			lpl2	578.8	202.2	410.4	904.1
			lpw	324.4	94.0	239.4	485.3
	Waterberg	*Afroptilum parvum*	lpl1	241.4	27.1	208.7	277.0
			lpw1	136.5	19.1	114.5	165.8
	Magaliesberg	*Afroptilum parvum*	lpl1	255.1	14.7	242.8	278.5
			lpw1	147.3	12.6	126.2	159.6

Appendix I. Glossae and paraglossae mouthpart measurements of *Afronurus barnardi*, *Afroptilum parvum* and *Elassoneuria sp*. Abbreviations used for the mouthpart structures are defined in Appendix C; (SD = Standard deviation).

Structure	Location	Species	Abbr.	Mean	±SD	Minimum	Maximum
Glossae	Waterberg	*Afronurus barnardi*	gl	251.5	49.7	183.8	306.2
			gw	158.5	30.7	115.7	182.8
	Waterberg	*Afroptilum parvum*	gl	100.5	7.9	90.8	109.5
			gw	25.7	5.1	20.2	32.8
	Magaliesberg	*Afroptilum parvum*	gl	129.8	29.5	102.4	179.2
			gw	45.2	4.6	39.3	49.4
Glossae and paraglossae	Waterberg	*Elassoneuria sp.*	gpl	744.5	202.7	600.2	1101.6
			gpw	655.3	155.2	548.1	929.5
Paraglossae	Waterberg	*Afronurus barnardi*	pl	334.7	39.3	288.6	372.9
			pw	242.1	53.3	176.4	313.7
	Waterberg	*Afroptilum parvum*	pl	150.8	8.1	145.7	163.7
			pw	83.6	7.2	74.0	92.5
	Magaliesberg	*Afroptilum parvum*	pl	193.5	25.3	166.6	227.5
			pw	81.8	26.7	44.4	107.3

Appendix J. Maxillary palps mouthpart measurements of *Afronurus barnardi*, *Afroptilum parvum* and *Elassoneuria sp*. Abbreviations used for the mouthpart structures are defined in Appendix C; (SD = Standard deviation).

Structure	Location	Species	Abbr.	Mean	±SD	Minimum	Maximum
Maxillary palps	Waterberg	*Afronurus barnardi*	mpl1	479.3	76.2	411.8	606.0
			mpl2	240.5	50.1	186.4	319.5
			mpl3	335.0	40.7	288.1	387.5
			mpl4	219.9	65.7	142.1	282.9
			mpw1	196.8	35.7	147.1	231.6
			mpw2	153.1	12.5	138.2	172.3
	Waterberg	*Elassoneuria sp.*	mpl1	481.6	74.4	419.5	610.4
			mpl2	460.9	80.4	369.3	583.8
			mpw	276.8	53.0	236.4	367.7
	Waterberg	*Afroptilum parvum*	mpl1	154.2	36.8	125.5	212.9
			mpl2	151.7	45.7	121.9	230.3
			mpw1	35.8	8.7	28.9	50.5
			mpw2	27.4	11.8	14.9	43.5
	Magaliesberg	*Afroptilum parvum*	mpl1	151.4	32.5	118.8	195.3
			mpl2	130.7	42.9	78.7	182.3
			mpw1	41.2	28.0	16.4	79.3
			mpw2	35.2	24.8	14.4	64.9