

Mopane worms (*Imbrasia belina*) as indicators of elemental concentrations in a trophic system

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Declaration

I, the undersigned, declare that this dissertation is my own unaided work. This dissertation is being submitted for the degree of Master of Science in the field of Ecology, Environment and Conservation at the University of the Witwatersrand, Johannesburg, South Africa. This dissertation has not previously been submitted for any degree or examination to any other institution.



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Disclaimer:

This dissertation consists of a series of chapters that have been prepared for submission to a range of scientific journals for publication. As a result the styles may vary between chapters in the dissertation and content overlap may occur between chapters.

This thesis uses the *Imbrasia* genus nomenclature in reference to the mopane moth, but we do acknowledge that the *Gonimbrasia* genus nomenclature is also used in reference to this species.

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Abstract

The impacts of mining on the environment continue to pose a risk in many regions. Palabora Mining Company (PMC) situated in Limpopo Province, South Africa is of environmental interest because of its shared border with South Africa's Kruger National Park. PMC generates both gaseous (emitted from the reverberator (Rev; also known as a smokestack)) and solid (stored in the tailings storage facility (TSF)) waste products. To assess possible pollution to the environment from PMC's activities, two study species were chosen, the Anomalous Emperor Moth (*Imbrasia belina* [Westwood 1849] (Lepidoptera: Saturniidae)) and its primary host the mopane tree (*Colophospermum mopane* [Kirk ex Benth.]). In addition to these two species being abundant on and around PMC, the mopane moth and all stages of its lifecycle are a source of food to many animals, and mopane caterpillars are a valuable source of food and income to many people in this region. Mopane caterpillars and mopane leaves were collected from on and around PMC at varying distances from the reverberator and TSF, as well as from several control sites. The elemental contents of these samples were analysed for 25 elements, focusing on copper, iron, aluminium, silicon, arsenic, zinc, nickel, lead, mercury, chromium and cadmium. In addition to these elemental analyses, the carbon and nitrogen isotopic compositions of these samples as well as caterpillar headwidth, body mass and mandible wear were also measured to assess the impacts of elemental concentrations on caterpillar growth. No mopane caterpillars were found at the two reverberator sites closest to the reverberator itself. Overall these two reverberator sites appear to be most contaminated, as concentrations of 14 of the 25 elements measured were highest in the mopane leaves from these two sites. The copper concentrations at these two sites, 42 ppm and 29 ppm respectively, were at levels that are considered toxic to plants. Assuming that 10 mopane caterpillars (each weighing 5g) are consumed a day, copper concentrations in the mopane caterpillar bodies across sites (between 8 ppm and 12 ppm) would equate to a maximum of 0.56 mg.day⁻¹, which is less than the recommended maximum intake level of 10 mg.day⁻¹ suggested for humans. As there was little difference between sites in terms of leaf carbon and nitrogen composition, elemental concentrations do not appear to be affecting the nutritional content of the leaves. This would suggest that caterpillar growth is not affected by elemental concentrations at sites where they

can survive. Similarly, caterpillar headwidths did not vary greatly between sites for each instar, suggesting that elemental concentrations are not affecting mopane caterpillar growth. Headwidths and mandible wear were found to be useful tools to age mopane caterpillars within an instar, and can be used in future to ascertain if mopane caterpillars are bioaccumulating elements or eliminating excess elements when they moult.

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Chapter 1

Mopane caterpillars and mopane trees

Mopane caterpillars, commonly referred to as mopane worms, are the larval stage of the Anomalous Emperor Moth (*Imbrasia belina* [Westwood 1849] (Lepidoptera: Saturniidae)) (Banjo *et al.* 2006; Hrabar 2006; Akpalu *et al.* 2007). The species is widespread throughout central and southern Africa and occurs in abundance in Namibia, Zimbabwe, Botswana, Zambia, Angola and South Africa (Gaston *et al.* 1997; Frears *et al.* 1999; Picker *et al.* 2004; Banjo *et al.* 2006; Akpalu *et al.* 2007; Gondo *et al.* 2010). Although the name mopane worm is derived from the tendency of the caterpillars to defoliate mopane trees (*Colophospermum mopane* [Kirk ex Benth.]) (Gaston *et al.* 1997; Mpuchane *et al.* 2000), the caterpillars also forage on other plant species such as *Carissa macrocarpa*, *Diospyros* spp., *Ficus* spp., *Searsia* spp., *Sclerocarya* spp., *Commiphora glandulosa*, *Acacia tortilis*, *Cassia abbreviata*, *Terminalia* spp. and *Trema orientalis* (Pinhey 1972; Dithlogo 1996; Illgner and Nel 2000; Picker *et al.* 2004).

Although the contribution of various plant species to the diet of mopane caterpillars is well known, examining the diet at a finer scale can provide us with information on the quality of the diet. A tool that can be used to investigate the caterpillars' diet at a finer scale is stable isotope analysis. Stable isotopes can be very useful to ecologists as they provide a method of tracking the movement of elements through the biosphere. Two of the elements that ecologists often use in isotopic studies are carbon and nitrogen.

Carbon is most often used to broadly identify the diet of individuals, and is especially useful for distinguishing the diet of herbivores, as plants using different photosynthetic pathways produce different carbon isotope signatures (Cerling *et al.* 2003; Cerling *et al.* 2006; Fry 2006). Nitrogen isotopes on the other hand are often used to work out where organisms lie within a food web (Minagawa and Wada 1984; Post 2002; Fry 2006). Both carbon and nitrogen isotopes may provide us with a means to investigate the relationship between mopane caterpillars and their food sources, particularly the mopane tree. These isotopes can provide some insight into the trophic links between

the caterpillars and mopane leaves, as well as providing a means of assessing relative food quality of mopane leaves at each of the sites.

The mopane tree, the primary host of mopane caterpillars, is a deciduous xeric savanna woodland species occurring in many regions of southern Africa including Namibia, Botswana, Zimbabwe, Mozambique and South Africa (Fears *et al.* 1997; van Wyk and van Wyk 1997). South Africa's mopaneveld, located in Limpopo Province and Mpumalanga Province, is dominated by mopane trees and is an arid environment characterised by highly seasonal and variable rainfall and high summer temperatures (Fears *et al.* 1997; Rutherford *et al.* 2006a). Mopane trees tend to form monospecific stands, and range in height from shrubs of 1-2m to trees of 20m, termed cathedral mopanes, with most individuals growing to about 10m (van Wyk 1993; van Wyk and van Wyk 1997). These plants can easily be identified by their pinnate leaves with two large leaflets, together thought to resemble butterfly wings (van Wyk and van Wyk 1997; Hrabar 2006).

Mopane leaves have a high total phenol and tannin content, the latter of which has been found to make leaves both unpalatable and indigestible to mammalian herbivores (Kumar and Vaithianathan 1990; Macala *et al.* 1992; Styles and Skinner 1997; Hooimeijer *et al.* 2005). Apart from a few species such as African elephants (*Loxodonta africana*), eland (*Taurotragus oryx*) and greater kudu (*Tragelaphus strepsiceros*) which supplement their diet with mopane leaves throughout the year, most mammalian browsers only feed on mopane leaves in the late dry season when high quality forage is not widely available (Styles and Skinner 1996, 1997; Smallie and O'Connor 2000; Hooimeijer *et al.* 2005). This is also when young green leaves with low tannin content are available (Styles and Skinner 1997).

As there are trade-offs between the various plant functions because of a plants' limited resources (Bazzaz *et al.* 1987), the reduced tannin content in the young leaves is most likely because growth has been prioritised in order to generate new leaves, with defence possibly being prioritised at a later time. This is in line with the nutrient stress hypothesis which suggests that during times of higher nutrient availability, resources will be directed towards plant growth, and during times of lower nutrient availability, excess carbon will be focused toward anti-herbivore defences (Bryant *et al.*

1983; Tuomi *et al.* 1984). Styles and Skinner (1996) found this same temporal pattern in plant function and stated that younger mopane leaves utilise the products of photosynthesis for growth and not protection. Such a temporal pattern in plant function prioritisation was also shown by Ferwerda *et al.* (2005) who found that the concentration of chemical defences in mopane leaves was positively correlated with time since manual defoliation of a mopane tree.

Unlike many other herbivores however, mopane caterpillars are able to survive solely on mopane leaves despite the high levels of phenols and tannins in the leaves. Young green mopane leaves, which emerge in spring, have the highest crude protein content and lowest total phenolic and tannin contents in spring as compared to other seasons (Styles and Skinner 1997; Smit 2001). Mopane caterpillars however appear to prefer mature mopane leaves over young green mopane leaves (Styles and Skinner 1996; personal observation). These mature green mopane leaves contain their highest crude protein content and their lowest tannin content during the summer season, which is when the majority of mopane caterpillars are abundant (Frears *et al.* 1997; Gaston *et al.* 1997; Styles and Skinner 1997; Hrabar 2006).

Despite the high phenolic content of mopane leaves, mopane trees are sometimes completely defoliated by mopane caterpillars in some regions of the mopaneveld where large and unpredictable outbreaks of mopane caterpillars occasionally occur (Frears *et al.* 1997; Gaston *et al.* 1997; Frears *et al.* 1999; Picker *et al.* 2004; Hrabar 2006). After these defoliation events, leaf growth often occurs, commonly referred to as compensatory growth or compensatory regrowth (McNaughton 1983; Gold and Caldwell 1989; Haile *et al.* 1998; McIntire and Hik 2002; Khan and Lone 2005). In mopane trees, compensatory leaf growth is smaller in size than mature leaves, but has also been found to be nutritious for herbivores, most likely because compensatory growth has a lower total phenolic and tannin content than the older leaves (Styles and Skinner 1997; Hrabar 2006). Tuomi *et al.* (1984) have however suggested that the nitrogen content of leaves decreases following a defoliation event. These defoliation events typically occur in late spring or early summer after the larval stage of the first generation of the mopane moth has been completed, as this generation of larvae usually produce the largest caterpillar populations, and so the caterpillars' need for mopane leaves would be highest at that time (Dithlogo 1996; Gaston *et al.* 1997).

Adult mopane moths live for just a few days and do not eat, having only rudimentary mouthparts, but rely instead on fat and water stores attained during the larval stage (Styles and Skinner 1996; Hrabar 2006; Gondo *et al.* 2010). Male mopane moths locate females by detecting female pheromones (Gondo *et al.* 2010). After mating has occurred, female mopane moths lay a single cluster of eggs around twigs or on leaves, with a cluster typically containing 30 to 335 eggs (Hrabar 2006; Akpalu *et al.* 2007; Gondo *et al.* 2010). Approximately 10 days after the eggs have been laid, small black larvae hatch and begin their 4-6 week long larval stage, during which time they pass through five growth stages, known as instars, with each instar lasting 5-7 days (Klok and Chown 1999; Akpalu *et al.* 2007; Gondo *et al.* 2010). The first two instars are light brown in colour while the last three instars are black with aposematically coloured scales which are yellow, white and red in colour (Gaston *et al.* 1997). The last three instars are also covered in white hairs, which may act as irritants as has been found in other caterpillars (Allen 2010; Schabel 2010), and sharp black spines to deter predators (Ditlhogo 1996; Dube and Dube 2010; Gahukar 2011). As with many insects, mopane caterpillars undergo a large variation in body size over the course of their life. The caterpillars experience an estimated 4 000 fold increase in body size over the larval period (Gaston *et al.* 1997, Akpalu *et al.* 2007; Gondo *et al.* 2010), with fifth instar caterpillars usually weighing about 12-13g and measuring approximately 80 mm in length (Frears *et al.* 1999; Hrabar 2006). This increase in body size results in caterpillars moulting, because chitin is rigid and cannot stretch to accommodate the increased body size (Gondo *et al.* 2010).

During the first three instar stages, the mopane caterpillars are gregarious and forage in groups of 20 to 200 caterpillars (Akpalu *et al.* 2007; Gondo *et al.* 2010), while caterpillars of the remaining two instars tend to be solitary (Akpalu *et al.* 2007; Gondo *et al.* 2010). At the end of the larval stage, the fifth instar caterpillars descend from the tree and burrow 10-15 cm into the ground where they undergo metamorphosis (Ghazoul 2006; Hrabar 2006; Akpalu *et al.* 2007; Gondo *et al.* 2010). The mopane moth is bivoltine throughout most of its distribution, meaning there are usually two generations each year (Hrabar 2006; Akpalu *et al.* 2007). Both generations of the moth emerge during the summer months when temperatures are typically high, rainfall is most likely to occur and

mopane trees are in leaf (Frears *et al.* 1997). Depending on the generation, pupae remain underground for six to seven weeks (first generation) or experience a period of diapause and emerge six to seven months later (Hrabar 2006; Gondo *et al.* 2010). The life cycle of the first generation of the species for the season begins when adult moths from the previous season mate and lay eggs, usually between October and January, and the first generation of adults then gives rise to the second generation of eggs (Figure 1.1) between February and May (Frears *et al.* 1997; Gaston *et al.* 1997; Hrabar 2006; Akpalu *et al.* 2007; Gondo *et al.* 2010). In the case of bivoltine populations, the first generation tends to be more abundant than the second, but the size of the population for both generations is greatly variable from year to year and is dependent on many factors such as food availability and rainfall (Dithlogo 1996; Gaston *et al.* 1997; Wiggins 1997). In some more arid areas, the species is univoltine (Hrabar 2006; Akpalu *et al.* 2007), so only one emergence of moths occurs each year in summer (Wiggins 1997).

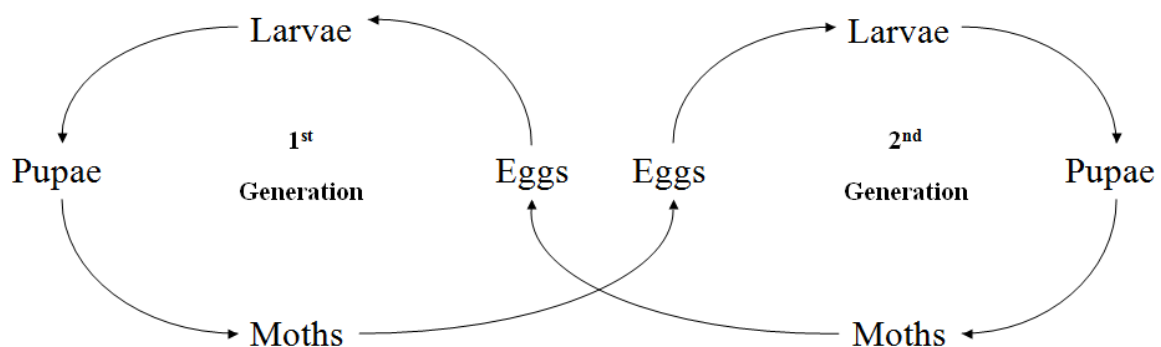


Figure 1.1. Life cycle of the Anomalous Emperor Moth (*Imbrasia belina*) in regions where the species is bivoltine.

Mopane worms as a food source

This life cycle of the mopane moth as well as the population size of each generation is very important for ecologists to understand, as all stages of the species' life cycle are a source of food, with Gaston *et al.* (1997) estimating the species to form part of as many as 70 trophic links during periods of outbreak. Mopane larvae are eaten by mammals such as baboons (*Papio ursinus*) and vervet monkeys (*Chlorocebus pygerythrus*), as well as by at least 34 bird species, and invertebrates such as

spiders, ants and mantids (Styles 1995; Styles and Skinner 1996; Gaston *et al.* 1997; Hrabar 2006).

The pupae are eaten by black-backed jackals (*Canis mesomelas*), bat-eared foxes (*Otocyon megalotis*), warthogs (*Phacochoerus africanus*) and aardvarks (*Orycteropus afer*) while the moths of the species fall prey to spiders, birds and bats (Hrabar 2006).

In addition to forming an important part of the natural ecosystem, mopane caterpillars are also one of the most important edible insects for humans in southern Africa for both dietary and economic reasons (Frears *et al.* 1997; Barany *et al.* 2001; Akpalu *et al.* 2007; Ghaly 2009). Processed mopane worms (caterpillars that have had their gut content squeezed out, and are dried and ready for consumption) contain approximately 60% crude protein (three times the protein content of beef per unit weight), 17% crude fat and 11% minerals including calcium, iron and phosphorus (Akpalu *et al.* 2007; Dube and Dube 2010). As a result of the high levels of iron that most caterpillars contain, they are often given to pregnant and nursing women as well as people suffering from anaemia to increase the amount of iron, calcium and protein in their diet (Akpalu *et al.* 2007). Mopane caterpillars are also rich in vitamins and contain high levels of essential amino acids such as lysine, tryptophan and methionine (Dreyer 1968; Dube and Dube 2010). As caterpillars have numerous nutritional properties, dried caterpillars are even made into flour to feed children in some African countries in an attempt to suppress malnutrition where access to good quality food is limited (Akpalu *et al.* 2007).

Mopane worms are in high demand in poor, rural areas of southern Africa because of their great nutritional value, but they are also a highly sought-after delicacy in many regions, such as Zambia and Malawi (Dreyer 1968; Frears *et al.* 1997; Mbata *et al.* 2002; Banjo *et al.* 2006; Gondo *et al.* 2010). The caterpillars also contribute to peoples' livelihoods through trade (Ditlhogo 1996; Stack *et al.* 2003; Hope *et al.* 2009; Dube and Dube 2010; Thomas 2013). Tonnes of caterpillars were recorded to be collected per annum in the early 1980s, with Dreyer and Wehmeyer (1982) indicating that approximately 1 600 000 kg of mopane caterpillars were being sold annually according to an estimate by the South African Bureau of Standards. The sales value of mopane caterpillars (dry mass) increased from less than US \$0.5 / kg in the early 1980s to US \$10 / kg by 1996 (Ditlhogo 1996; Dube and Dube 2010).

As a result of the value placed on mopane worms, they are being unsustainably harvested in many regions (Barany *et al.* 2001; Banjo *et al.* 2006; Akpalu *et al.* 2007; Ghaly 2009; Gondo *et al.* 2010) and this non-selective harvesting has led to the disappearance of many mopane worm populations in some parts of Botswana and South Africa (Banjo *et al.* 2006; Hrabar 2006; Akpalu *et al.* 2007). In South Africa, the demand for and overexploitation of mopane worms is so great that mopane worms are even being imported from other countries such as Zimbabwe (Gondo *et al.* 2010).

Mines and impacts of mining

Most South African mopane caterpillars are harvested for food in Limpopo Province, where the majority of South Africa's mopaneveld is located, but this region is also the location of numerous mining operations (Rutherford *et al.* 2006b; Council for Geoscience 2011). Mining can adversely affect the environment through land clearing and pollution. Land clearing has the potential to lead to habitat fragmentation or even complete habitat loss. This can result in the extirpation of many species of plants and animals in those regions, particularly of species that are highly specialised, and that can only survive under specific conditions or in specific areas (Brown 1971; Owens and Bennett 2000). Pollution from mining activities however can affect both generalist and specialist species, and this includes adverse effects on the human population.

The most well-known form of mine pollution is Acid Mine Drainage (AMD), which occurs when sulphide-bearing rocks come into contact with oxygen and water, and through a number of chemical reactions, can be transformed into compounds such as sulphuric acid (Johnson and Hallberg 2005; Akcil and Koldas 2006). This polluted water can contaminate surface water and groundwater, as well as soil (Akcil and Koldas 2006). Contaminants may then be taken up from any of these sources by plants which can themselves be adversely affected and later affect other organisms at higher trophic levels. Organisms at higher trophic levels are more likely to be exposed to greater amounts of pollutants through biomagnification, particularly if large quantities of the pollutants are bioaccumulated in organisms that form part of that trophic link (Woodwell 1967; Connolly and Pedersen 1988; Gobas *et al.* 1993). Mining pollutants thus have the potential to cause a reduction in

biodiversity as a result of the potential toxicity of those pollutants (Akcil and Koldas 2006). Types of mining most commonly associated with AMD include copper, gold and nickel mining (Akcil and Koldas 2006).

One group of mining pollutants that is of great interest to scientists ecologically are heavy metals. The term “heavy metals” is misleading as the elements to which the term refers are not all “heavy” in terms of atomic number, density or atomic weight, and some are not truly metallic at all in terms of their properties (Volesky 1990). The grouping of elements as heavy metals has instead been made because these elements are all identified as having the ability to be toxic. Heavy metals tend to have a disproportionate effect relative to their low concentration in the environment (Volesky 1990). Although some trace elements required by plants and other organisms are heavy metals, high concentrations of these substances can be toxic (Kumar *et al.* 1995; Hall 2002). Metal toxicity could then pose lethal or sub-lethal threats to organisms exposed to heavy metal pollution, even in small doses (Boyd *et al.* 2006; Lürling and Scheffer 2007; Boyd 2010).

Effects of heavy metals include inhibition of seed germination in plants, and inhibition of growth in both plants and animals (Scott *et al.* 2003; Sharma and Agrawal 2005; Lürling and Scheffer 2007; Pavel *et al.* 2013). The sub-lethal effects of heavy metals are possibly of greatest concern because they can negatively impact important ecological relationships within and between species (Klaschka 2008; Boyd 2010). This disruption can for example affect species interactions by altering normal predator avoidance behaviours (Lürling and Scheffer 2007; Klaschka 2008; Boyd 2010). Scott *et al.* (2003) showed that when exposed to high levels of waterborne cadmium (Cd) ($2\mu\text{g Cd l}^{-1}$), juvenile rainbow trout (*Oncorhynchus mykiss*) did not respond to chemical alarm substances warning of nearby predators.

In insects, heavy metals have been found to have a number of detrimental effects including reductions in survival, growth, fecundity, weight and eclosion success, and increases in development time (Haney and Lipsey 1973; Kazimírová and Ortel 2000; Scheirs *et al.* 2006; Butler and Trumble 2008). In addition to the negative effects that heavy metals can have on organisms, heavy metals can also remain in the environment for long periods, increasing the likelihood that they may cause harm

(Kumar *et al.* 1995). The group of heavy metals includes elements such as mercury, arsenic, cadmium, chromium, lead, nickel and copper.

Copper, like some other elements classed as a heavy metal, is an essential element for optimum plant and animal function, but at high concentrations can be toxic (Gaetke and Chow 2003; Dučić and Polle 2005). Copper functions as a cofactor in many enzymes, and also plays a key role in processes such as oxidative phosphorylation, transcription and iron mobilization (Fernandes and Henriques 1991; Gaetke and Chow 2003; Yruela 2005; Turnlund 2006). High concentrations of copper can however be toxic, with toxic effects including chlorosis, inhibition or stunting of growth, discolouration of leaves, necrosis, inhibition of protein function or enzyme activity, deficiencies of other essential elements, and disruption of photosynthesis and respiration (Marschner 1995; Yruela 2005).

The strength of the effect of copper toxicity in plants has been linked to the plant growth stage when the high copper concentration is applied (Maksymiec 1997). In brook trout (*Salvelinus fontinalis*), McKim and Benoit (1971) found that high levels of copper ($>17.4 \mu\text{g.L}^{-1}$ (ppb) in the water) resulted in a reduction of both hatchability and the number of viable eggs produced as well as reducing adult brook trout growth and survival. In green crabs (*Carcinus maenas*) and rock crabs (*Cancer irroratus*), Thurberg *et al.* (1973) found that the osmoregulatory function of the crabs was lost with exposure to increasing concentrations of copper.

Copper has also been found to affect the survival and fecundity of many insects. Görür (2006, 2007) found that cabbage aphids (*Brevicoryne brassicae* (Hemiptera: Aphididae)) which were fed copper contaminated cabbages (*Brassica oleracea*) and radishes (*Raphanus sativus*) showed higher fluctuating asymmetry and mortality, and lower fecundity, fitness and reproductive potential, than those fed on non-contaminated control plants. Larvae of the midge, *Chironomus decorus*, experienced lower growth and deformities of their mouthparts when exposed to high levels of copper in their food substrate (Kosalwat and Knight 1987). In tiger mosquitoes, *Aedes albopictus* (Diptera: Culicidae), the effects of exposure to high levels of copper included a reduction in the number of larvae, inhibition of larval growth, increases in larval mortality, and complete prevention of oviposition (Romi *et al.*

2000). Copper was also found to cause reduced feeding, reproduction and survival in *Neochetina eichhorniae* and *Neochetina bruchi* (Newete 2014).

The Palabora Mining Company

Copper is the main product of The Palabora Mining Company, currently the only producer of refined copper in South Africa (PMC 2012). The Palabora Mining Company Limited (PMC), situated in the town of Phalaborwa, Limpopo Province, South Africa, was the country's first open-pit copper mine, with the mining of ore beginning in 1965 (Heinrich 1970). The mine is considered to be world-class and produces 30 000 tonnes of copper ore per day and approximately 60 000 tonnes of refined copper per annum (PMC 2012). The mine also produces a number of by-products, including vermiculite, magnetite, nickel sulphate, phosphates, uranium, zirconium, palladium and small quantities of gold, silver and platinum (PMC 2012).

The Palabora Mining Company shares a border with the Kruger National Park (PMC 2012). This park is one of South Africa's largest national parks and one of Africa's largest conservation areas, as well as being South Africa's largest tourist attraction (Eckhardt *et al.* 2000; PMC 2012). As a result of this shared border, many environmental groups monitor the impact of PMC on its surrounding environment, including government, South African National Parks Board, environmental groups and the local community (PMC 2012). As part of PMC's environmental policy, the company attempts to minimize the negative impact that they have on the environment (PMC 2012).

As with most mining operations, PMC generates waste products. The extraction and refining of copper at PMC involves smelting, with gaseous wastes being released into the environment via the reverberators (smokestacks) after being cleaned (PMC 2012). This reverberator could be a substantial source of pollutants as smelting has been attributed as one of the primary sources of anthropogenic trace metal emissions (van Zyl *et al.* 2014). Solid waste products from PMC's operations are stored in tailings storage facilities (commonly referred to as mine dumps). Tailings facilities have been shown to contain high concentrations of many heavy metals which can be leached by surface water and groundwater and released into the environment (Dudka and Adriano 1997; Concas *et al.* 2006). Plants

growing close to tailings and the organisms that consume these plants would therefore be good indicators of the levels of heavy metals in the system.

Aims and objectives

As mopane trees and mopane caterpillars both occur in abundance in the region of Phalaborwa, where PMC is located, they were selected as bioindicator species for this study. Insects play a vital role in the bioaccumulation and dispersal of pollutants in the environment (Andrahennadi and Pickering 2008), making the mopane caterpillars a good study species for an elemental study such as this. Mopane caterpillars are also easily harvested and have a relatively short generation time, with two generations in a season in this region, further making them a suitable study species. As mopane caterpillars and all other stages of the species life cycle are important sources of food in their natural environment as well as to humans, it is important to understand as much as we can about the life cycle and ecological interactions of this species and to ascertain if toxic concentrations of any of these elements are travelling through the local food web on and around PMC.

The main aim of the study was to measure the concentrations of 25 elements, including copper and several other heavy metals, in both mopane leaves and mopane caterpillars, which provided information on whether or not the trees and caterpillars on and around PMC were taking up potentially toxic levels of these elements. It was expected that concentrations of copper, iron, aluminium and silicon would be higher at the PMC sites than at the control sites as these elements are components of the main products and by-products of PMC. As toxic elements have the potential to harm both the environment and human society, it is important to determine to what extent the environment around the mine is exposed to these substances. For this reason, elemental concentrations were measured at varying distances from the mine's main sources of contamination (the reverberator and the tailings storage facility). It was expected that elemental concentrations would be highest at the reverberator sites, followed by the tailings storage facility sites and control sites respectively. Comparisons of the concentrations of these 25 elements were made between mature mopane leaves, mopane caterpillar bodies and mopane caterpillar gut contents, between sites, between mopane caterpillar bodies and gut contents, and between mature mopane leaves and compensatory growth.

The second aspect of the study examined the carbon and nitrogen isotopic composition of the mopane caterpillars and mopane leaves to ascertain if elemental concentrations have any effect on leaf nutrition and subsequently caterpillar growth. Carbon and nitrogen isotopes were also examined to ascertain if the relative compositions of the isotopes between trophic levels followed the expected trend of a 1‰ increase in $\delta^{13}\text{C}$ and a 2-4 ‰ increase in $\delta^{15}\text{N}$ between trophic levels. A number of carbon and nitrogen isotopic composition comparisons were made, including comparisons of mature mopane leaves to compensatory growth, mopane caterpillar bodies to mature mopane leaves, caterpillar bodies of the five mopane instars to each other, mopane caterpillar bodies to mopane caterpillar gut contents, and lastly comparisons of all these sample types across sites. Correlations between isotopic compositions and elemental concentrations were also made. It was expected that the varying levels of contamination at the study sites would be either positively or negatively correlated with the isotopic composition of the mopane leaves.

The final portion of this study was to examine mopane caterpillars on and around PMC and in Musina (control site), and obtain measurements of headwidths of caterpillars which can be used to assign individual caterpillars to a particular instar. Measurements of mopane caterpillar body mass were also collected and mandible wear scores were allocated to mopane caterpillar mandibles. These two measures were taken to see which if they might be useful tools to further age caterpillars. All three measures were examined to ascertain if they could be used to assess caterpillar growth, and to establish if elemental concentrations may be affecting caterpillar growth. For each instar, an assessment of the relationship between mopane caterpillar headwidth and body mass were made, as well as an assessment of the relationship between mopane caterpillar body mass and mandible wear. It was expected that caterpillars from more contaminated sites would weigh less than those from control sites. It was also expected that the frequency of heavily worn caterpillar mandibles would be higher at contaminated sites than at the control sites.

Study Sites

The field study was largely conducted on and around Palabora Mining Company Limited (PMC) property (23° 57' 21" S 31° 09' 13" E), located just south of the town of Phalaborwa, Limpopo Province, South Africa. The mean monthly minimum temperature in the area during the coldest part of the year (July) is 5.7 °C and while the mean monthly maximum temperature during the hottest part of the year (January) is 38.4 °C (Rutherford *et al.* 2006a). Phalaborwa receives most rainfall between September and May, with an annual mean of 514 mm (Rutherford *et al.* 2006a). The vegetation in this region is Phalaborwa – Timbavati Mopaneveld, with the sandy uplands dominated by *Combretum apiculatum*, *Terminalia sericea* and *Colophospermum mopane* (Rutherford *et al.* 2006a). In the clayey bottomlands, *Colophospermum mopane* becomes more dominant while *Combretum apiculatum* becomes sparse and *Terminalia sericea* is absent (Rutherford *et al.* 2006a).

The two main sources of contamination at PMC are the reverberator (Rev) and the tailings storage facility (TSF) (Figure 1.2). Sampling sites were located at various distances from these sources. Reverberator sites (Rev 1, Rev 2 and Rev 3) were located approximately 0.7 km, 1.7 km and 8.5 km respectively from the reverberator and all lay within the fallout area as determined by the dominant wind direction in the region (typically a south south easterly wind or a southerly wind (WindFinder 2014)) (Figure 1.2). Tailings storage facility sites 1 and 2 (TSF 1 and TSF 2) were located approximately 0.1km and 0.6 km respectively from the closest edge of the tailings storage facility, and 4.22 km and 5.56 km south-east of the reverberator respectively, upwind of the reverberator (Figure 1.2). Two control sites (Con1 and Con 2) were located approximately 5.5 km north and 28 km south-west (24° 5' 3.29" S 30° 53' 48.36" E) of the reverberator respectively (Figures 1.2 and 1.3). Mopane leaf and caterpillar samples from the sites on and around PMC were collected between 26 November and 7 December 2012.

An additional site was located on land portion Runde 592MS, Runde Farm (22° 44' 43.4142" S 29° 48' 0.6984" E), Limpopo Province, South Africa (Figure 1.3), approximately 193 km north west of PMC. The mean monthly minimum temperature in the coldest part of the year (June) in this region

is 0.9 °C while the mean monthly temperature during hottest part of the year (November) is 39.9 °C (Rutherford *et al.* 2006a). The area also receives mean annual rainfall of 334 mm, with most rain received between October and April (Rutherford *et al.* 2006a). The region is part of the Musina Mopane Bushveld vegetation unit (Rutherford *et al.* 2006a). This vegetation unit is dominated in its western section by *Colophospermum mopane* on clayey bottomlands and *Combretum apiculatum* on its hills (Rutherford *et al.* 2006a). The eastern basalt sections of this vegetation unit are dominated by *Colophospermum mopane* and *Terminalia prunioides*, while areas with deep sandy soils are dominated by *Colophospermum mopane*, *Terminalia sericea*, *Grewia flava* and *Combretum apiculatum* (Rutherford *et al.* 2006a). Mopane caterpillar samples from Runde Farm (RF) were collected between 25 December 2012 and 7 January 2013. As no leaf samples were collected from Runde Farm to serve as a reference point for caterpillars collected from here, caterpillars from Runde Farm were omitted from analyses of elemental concentration and isotopes.

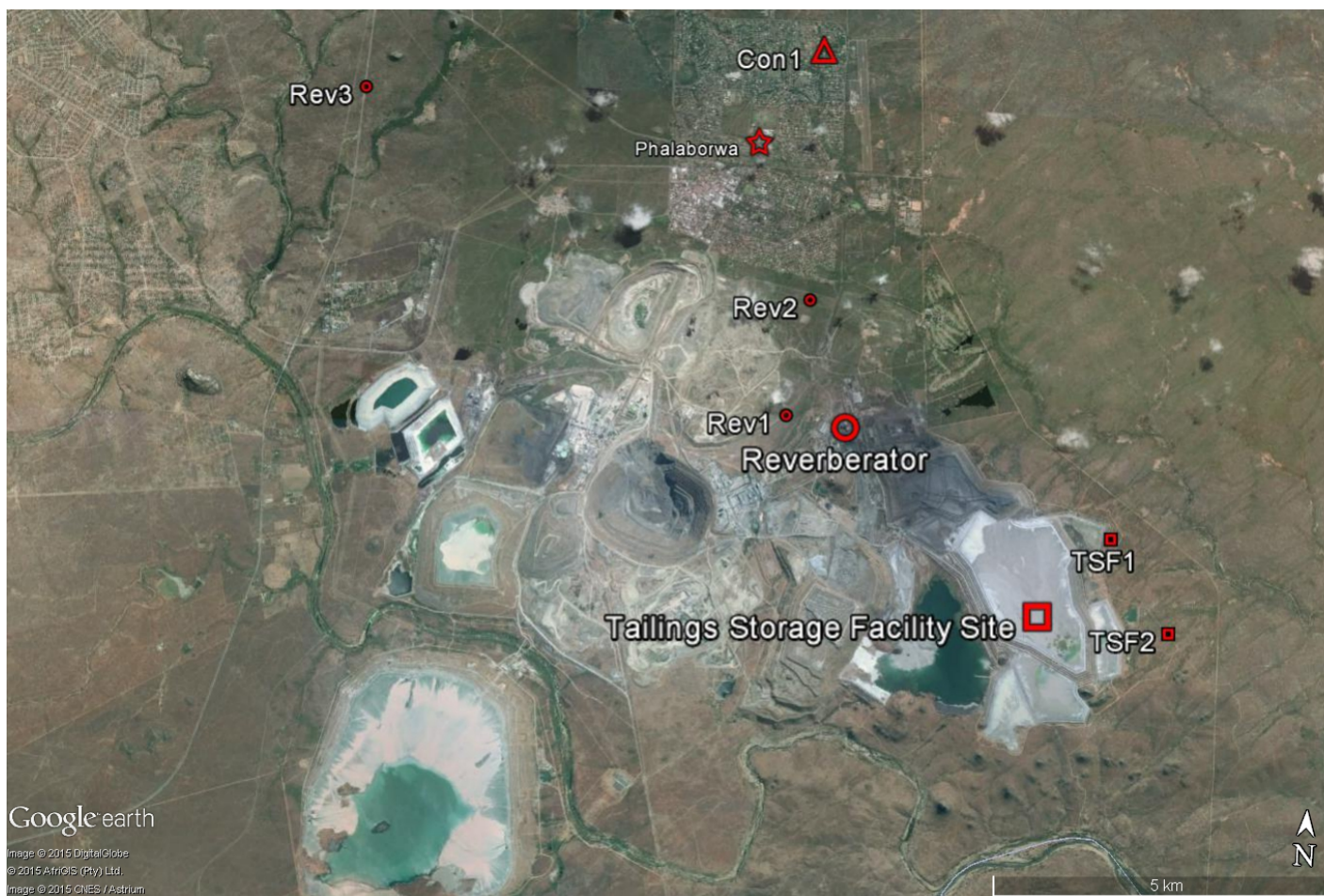


Figure 1.2. Study sites on and around the Palabora Mining Company property. The two sources of contamination are the reverberator (Rev) and the tailings storage facility (TSF) (open circle and open square respectively). There are three Rev sites (solid circles) at increasing distances away from the reverberator, and there are similarly two TSF sites (solid squares) at increasing distances away from the tailings facility. One of the control (Con) sites (open triangle) is located north of the town of Phalaborwa (open star). The prevailing winds in the region are south south easterlies. © 2015 Google Earth; © 2015 CNES/Astrium; © 2015 AfrisGIS (Pty) Ltd.; © 2015 DigitalGlobe.

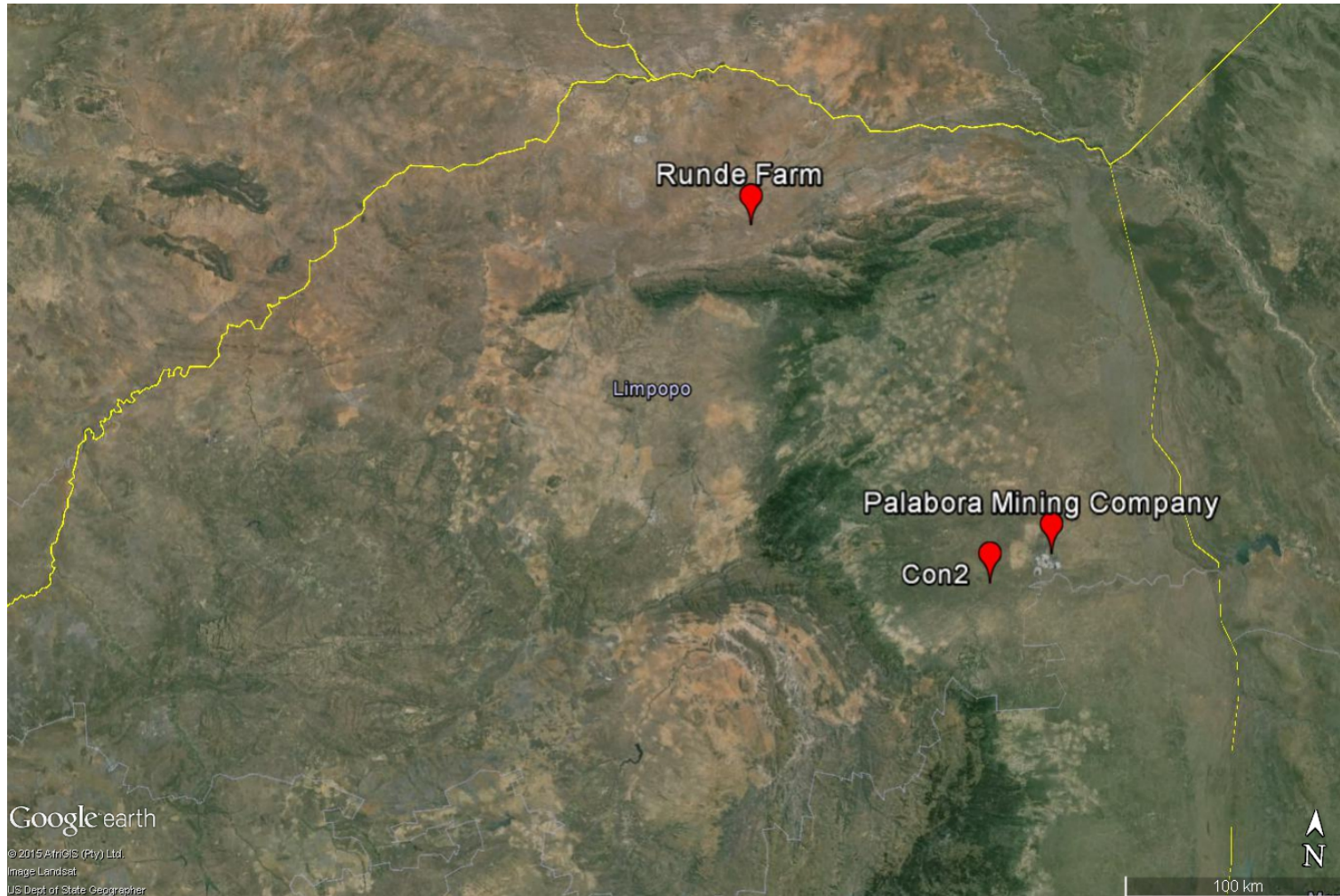


Figure 1.3. Additional study sites were located elsewhere in Limpopo Province. A second Control site (Con 2) is located approximately 28 km south-west of the Palabora Mining Company (PMC) complex, while Runde Farm (RF), an additional control site for mopane caterpillars, is located approximately 193 km north-west of PMC. The yellow line indicates both the northern boundary of Limpopo Province as well as the national border between South Africa (bottom) and its neighbours Botswana, Zimbabwe and Mozambique respectively from left to right. © 2015 Google Earth; © 2015 AfriGIS (Pty) Ltd.; Landsat; US Dept of State Geographer.

Chapter 2

Using mopane caterpillars (*Imbrasia belina*) as an indicator of elemental concentrations in the environment

Abstract

The Palabora Mining Company (PMC) is a copper mine situated in Limpopo Province, South Africa. As with most mining activity, PMC generates both gaseous and solid waste products. Gaseous wastes are released into the atmosphere via a reverberator (Rev) while solid wastes are stored in a tailings storage facility. The study aimed to assess differences in elemental concentrations between mopane leaves (*Colophospermum mopane*) and mopane caterpillars (*Imbrasia belina*), and mopane caterpillar bodies and mopane caterpillar gut contents. This was done to investigate if high concentrations of elements were reaching the environment and humans consuming the caterpillars. The study also aimed to ascertain if these elemental concentrations differed between sites. Mopane leaves and mopane caterpillars were collected from sites around the two sources of contamination as well as from several control sites, and the concentrations of 25 elements were measured in these samples.

Concentrations of copper in the mopane leaves of sites Rev 1 and Rev 2 (42 ppm and 29 ppm), those sites closest to the reverberator, were more than double the copper concentrations found in mopane leaves from the other sites, which ranged between 8 ppm and 14 ppm. Concentrations of elements were not found to be consistently higher or lower in mopane caterpillars than in mopane leaves for all elements measured. Mopane caterpillar gut content elemental concentrations were mostly higher than mopane caterpillar body concentrations of the elements measured. No caterpillars were found at Rev 1 and Rev 2, which could indicate that the dust fallout from the reverberator and the concentrations of elements within the fallout may be too high to allow mopane caterpillars to survive. Of all the sites examined, Rev 1 and Rev 2 had the highest elemental concentrations. Caterpillars from other sites were able to survive despite the elemental concentrations to which they were exposed. The elemental concentrations found in the caterpillars would also pose no health risk to humans who consume them.

Introduction

Environmental impacts of mining

South Africa has a large mining sector with the mining industry directly contributing more than 5% to the country's Gross Domestic Product (GDP) each year (Chamber of Mines of South Africa 2012). Throughout the country, large areas of land have been set aside for mining. Although society has uses for these minerals, there are many environmental costs associated with obtaining them. Most mining activities have adversely affected the environment through land clearing and pollution. Land clearing can have far reaching effects because of the vast amounts of land set aside for the practice of mining. This land clearing results in the loss of habitat for many species and can also cause habitat fragmentation which may result in a number of species going locally extinct (Pimm and Raven 2000; Driscoll 2004). Pollution can also have far reaching effects, as pollutants can be transferred along food webs and in this way can be transported great distances both spatially and temporally (Woodwell 1967; Connolly and Pedersen 1988; Gobas *et al.* 1993).

The form of mine pollution that is often viewed as causing the greatest harm to the environment is Acid Mine Drainage (AMD). The production of AMD occurs when sulphide-bearing rocks react with oxygen and water and form compounds such as sulphuric acid (Johnson and Hallberg 2005; Akcil and Koldas 2006). The acidic water produced provides suitable conditions for heavy metals to dissolve as many metals have increased solubility at low pH (Schindler *et al.* 1980; Bell *et al.* 2001; Sheoran and Sheoran 2006). This heavy metal rich acidic water can then contaminate surface water, groundwater or soil (Akcil and Koldas 2006). These pollutants can then be taken up by plants from the water or the soil.

As plants are the primary producers in terrestrial systems, the majority of the macro- and micro-nutrients needed by organisms at higher trophic levels originate from plants (Boyd *et al.* 2006). A number of metallic elements are categorised as macro- and micro-nutrients, and are vital to the normal functioning of living organisms, filling both nutritional and physiological requirements (Volesky 1990). However the presence of these elements in excess may result in chronic or acute

toxicity or even death (Volesky 1990; Kumar *et al.* 1995; Hall 2002). If these trace elements or other contaminants released into the environment from AMD are bioavailable (referring to the proportion of metals that can be incorporated into the plant and used for metabolic processes (John and Leventhal 1995; Adriano 2001)) to plants, plants have the potential to bioaccumulate large quantities of these substances.

Elemental uptake by plants occurs in a number of ways including, (a) passive transport down a concentration gradient through the plant cell membrane, (b) through the action of plant growth-promoting bacteria and/or (c) through the action of mycorrhizal fungi, which is the predominant route of elemental uptake (Schützendübel and Polle 2002; Glick 2003). If any heavy metals do become bioavailable to plants, organisms at higher trophic levels may then be exposed to even greater amounts of pollutants than the plants through the process of biomagnification, but this does not always occur as pollutants are not always bioaccumulated (Kazimírová and Ortel 2000). If primary consumers feed on plants with high concentrations of heavy metals, secondary consumers that feed on those primary consumers can then accumulate higher concentrations of the pollutants than each primary consumer. When this process is repeated as one moves up the food chain, the result is biomagnification (Laskowski 1991). Some organisms are able to survive high levels of pollutants by excreting or sequestering excess pollutants, while others are able to tolerate high levels of pollutants. If organisms are physiologically unable to excrete, sequester or tolerate the concentrations of harmful substances they have accumulated, this can result in the death of those organisms, or possibly even the death of whole populations or communities within a polluted area.

Heavy metals

One group of pollutants that are often associated with mining activities are a class of elements referred to as heavy metal. The term “heavy metals” does not truly represent the elements making up this group, as most of these elements are not heavy in terms of atomic number, density or atomic weight, and some are not even truly metallic in terms of their properties (Volesky 1990). This categorisation has rather been made because of the potentially toxic properties that all these elements

possess, even in small doses (Boyd *et al.* 2006). Heavy metals tend to have a very large effect on the environment despite them occurring in relatively low proportions in the environment (Volesky 1990). In addition to the negative effects that heavy metals can have on organisms, heavy metals can also remain in the environment for long periods, increasing the likelihood that the heavy metals may cause harm (Kumar *et al.* 1995). Elements considered as heavy metals include arsenic, cadmium, chromium, nickel, lead and copper.

Palabora Mining Company

Copper is the main product of The Palabora Mining Company (PMC), which is situated in the town of Phalaborwa, Limpopo Province, South Africa. Apart from copper, the mine also produces several by-products including vermiculite, magnetite and nickel sulphate (PMC 2012). The extraction of copper and the other by-products of PMC results in the production of gaseous waste products such as sulphur dioxide (SO₂). These gaseous waste products first go through a cleaning process, and are then released into the atmosphere via the reverberators (commonly referred to as smokestacks) (PMC 2012). Small quantities of particulate matter from the products and by-products are also likely to be released into the atmosphere along with the gaseous waste products as a result of the processes used to extract these products from the ore and separate them into different grades. As with most mining operations, PMC also generates solid waste products, many of which are stored in tailings storage facilities (commonly referred to as mine dumps). These tailings are the unwanted materials leftover from mining. Most tailings are reported to leach heavy metals into the environment via AMD, giving many tailings facilities a poor environmental reputation. The reverberator and the copper tailings storage facility are the two main sources of contamination at PMC.

As three of PMC's main products are copper, vermiculite and magnetite, high concentrations of the elements making up these products were expected at the mine sites. Of the elements measured in the current study, those elements that form the products of copper, vermiculite and magnetite, or part thereof, are copper, iron, aluminium and silicon. In order to assess the potential impact of pollutants from PMC on the environment, the plants surrounding the reverberator and the tailings

storage facility, as well as the organisms feeding upon these plants, were considered as indicators of the levels of pollutants in the system.

One of the most dominant trees in the Phalaborwa region where PMC is located is the mopane tree (*Colophospermum mopane* [Kirk ex Benth.]), making this a suitable plant study species of the elemental conditions in the area. In addition to this, the mopane tree is the primary host of the mopane caterpillar (*Imbrasia belina* [Westwood 1849] (Lepidoptera: Saturniidae)). Mopane moths and all stages of their life cycle form part of many trophic links (Gaston *et al.* 1997). Mopane caterpillars are also a staple in the diet of many local people, sometimes even being a preferred meat source, with people in the region harvesting thousands of these caterpillars each year (Ferreira *et al.* 2003; Banjo *et al.* 2006; Akpalu *et al.* 2007; Ghaly 2009; Gahukar 2011). High elemental concentrations in this region originating from the mine can therefore have serious and potentially far reaching consequences not only on the immediate environment but also on locals and people from other regions where mopane caterpillars are exported to. As insects are considered important organisms in the transfer of metals between different trophic levels, anything influencing the metal concentration in the insects could affect the distribution of metals throughout an ecosystem (Lindqvist and Block 1997). Owing to this, and the role that the mopane caterpillars play as a source of food, an assessment of insects in the area surrounding the mine was undertaken. Mopane caterpillars are abundant in the region and have a relatively short life cycle further making them a suitable study species for this region.

Aims and objectives

The aim of this aspect of the study was to measure the concentration of 25 elements in mopane leaves and mopane caterpillars on and around PMC in relation to various sources of contamination in order to assess the mine's impact on the environment. The elements measured were aluminium (Al), arsenic (As), barium (Ba), calcium (Ca), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), mercury (Hg), potassium (K), magnesium (Mg), manganese (Mn), molybdenum (Mo), sodium (Na), nickel (Ni), phosphorus (P), lead (Pb), sulphur (S), selenium (Se),

silicon (Si), strontium (Sr), uranium (U), tungsten (W) and zinc (Zn). The first objective was to measure the concentrations of elements within mopane leaves and mopane caterpillars (bodies and gut contents measured separately) originating from the different sources of contamination, and secondly to compare elemental concentrations between sites. This was done to assess the relative amounts of contamination at each site. Thirdly differences in elemental concentrations between the bodies and gut contents of mopane caterpillars were assessed. This was done to establish if the caterpillars were likely to be storing or eliminating a large proportion of the elements ingested. The fourth objective was to compare the elemental concentrations of mature mopane leaves and mopane leaf compensatory growth (where it could be collected). This was to assess if excess amounts of elements were moved into the new growth perhaps with the goal of elimination from the plant.

Materials and Methods

Experimental design

At each site, excluding Runde Farm, 10 mopane trees were randomly chosen. The GPS coordinates were noted. Five leaf clusters of mature leaves, identified as the tougher, bigger, darker leaves, were selected from each tree. Samples of compensatory growth, identified as the smaller, softer, often reddish leaves, were also collected from trees exhibiting these leaves. From each tree, five mopane caterpillars of each instar were collected where possible and immediately placed in boiling water for a minute. As much of the gut contents were then squeezed from the bodies as possible. Body and gut content samples of each caterpillar were stored separately in plastic bags and all samples were frozen on site. Samples were returned to the laboratory at the University of the Witwatersrand, Johannesburg, where they were then freeze dried. Leaves were not washed before being analysed as mopane caterpillars naturally exposed to these leaves would be exposed to unwashed leaves.

Elemental analyses of mopane leaf and mopane caterpillar samples were done using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Three fifth instar caterpillars were selected for analysis from each of five trees per site for the four sites. Where fifth instar caterpillars were not available, fourth instar caterpillars were instead analysed. Caterpillar body and gut content

samples were analysed separately. A selection of mature leaves and compensatory growth (where available) were selected for analysis from each of the sites where leaves had been collected. No mopane caterpillars or evidence thereof was observed at Rev 1 or Rev 2. Although mopane caterpillars were collected from Control site 2, an insufficient number of fourth instar caterpillars and no fifth instar caterpillars were collected, and as such elemental analysis was not conducted on caterpillars from this site. Elemental data are represented as parts per million (ppm) but can also be read as mg.L^{-1} .

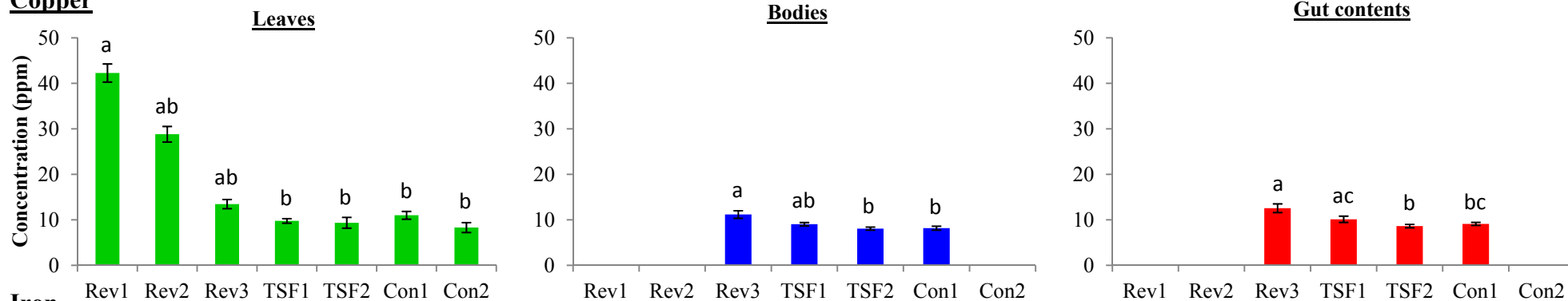
Data analysis

All statistical analyses were completed using R software (R Development Core Team 2013). Kolmogorov-Smirnov tests showed that the elemental data did not follow a normal distribution and non-parametric statistical tests were subsequently used in data analysis. Kruskal-Wallis tests were used to ascertain if there were significant differences between mature leaves, caterpillar bodies and caterpillar gut contents, between sites for each of the elements measured. Kruskal Multiple Comparison post-hoc tests (from the package “pgirmess” (Giraudeau 2011)) were conducted after each Kruskal-Wallis test. Wilcoxon Rank Sum tests were used to establish whether or not there was a difference in concentration of each of the elements between mature leaves and compensatory growth.

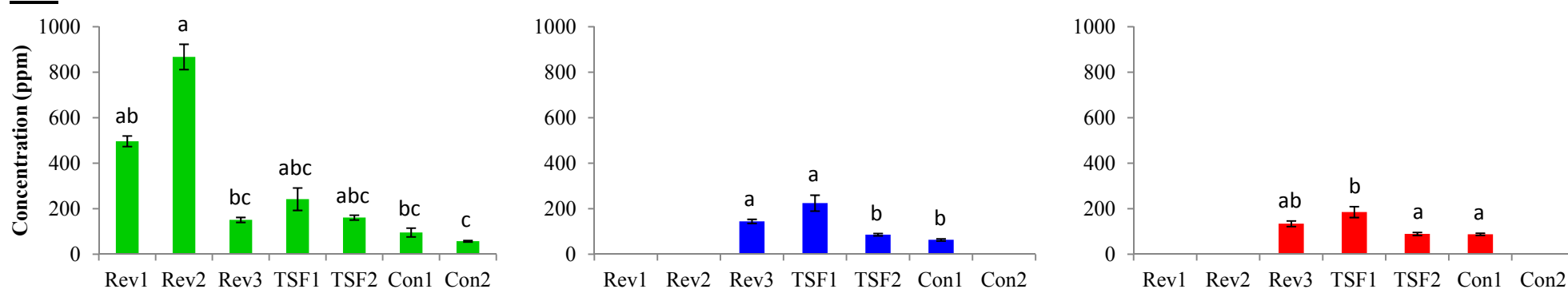
Results

Mean concentrations of Cu in mopane leaves were highest from Rev 1 (43 ppm) and Rev 2 (29ppm) while Cu concentrations of mopane leaves from the remaining sites ranged between 8 ppm and 13 ppm, with leaves from Con 2 having the lowest mean Cu concentration (Kruskal-Wallis $\chi^2_{(6)} = 28.9285$; $n = 34$; $P < 0.0001$) (Figure 2.1). Mean concentrations of Cu in caterpillar bodies were highest from Rev 3 and lowest from TSF 2 but ranged between 8 ppm and 11 ppm across all sites (Kruskal-Wallis $\chi^2_{(3)} = 18.2516$; $n = 60$; $P < 0.0001$) (Figure 2.1). Mean concentrations of Cu in caterpillar gut contents ranged from 9 ppm to 13 ppm, with the highest concentrations from Rev 3 and the lowest concentrations once again from TSF 2 (Kruskal-Wallis $\chi^2_{(3)} = 17.5411$; $n = 60$; $P < 0.001$) (Figure 2.1).

Copper



Iron



Aluminium

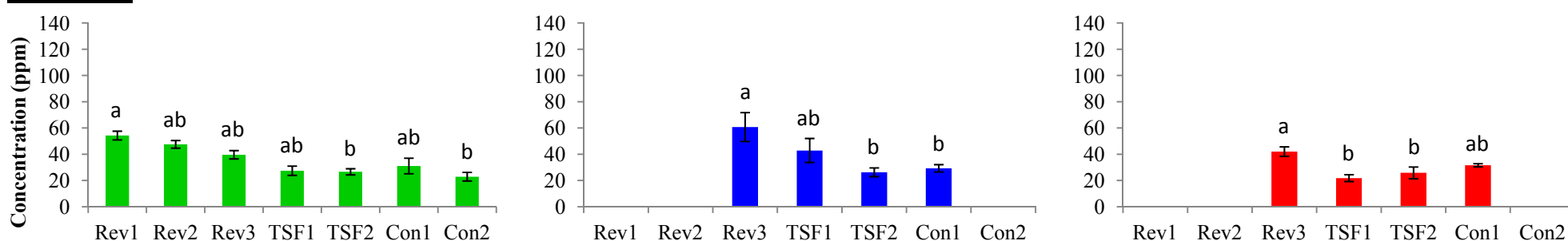


Figure 2.1. Effect of site position on mean concentrations (\pm standard error) of copper, iron and aluminium in mopane leaves (green) (n = 24), mopane caterpillar bodies (blue) (n = 60) and caterpillar gut contents (red) (n = 60) from seven study sites on and around the Palabora Mining Company in Limpopo Province, South Africa. Letters indicate differences between means within sample groups.

Mean concentrations of Fe in mopane leaves from Rev 1 (497 ppm) and Rev 2 (868 ppm) were much higher than from the remaining sites which ranged between 57 ppm and 242 ppm (Kruskal-Wallis $\chi^2_{(6)} = 30.6894$; $n = 34$; $P < 0.0001$) (Figure 2.1). Mean concentrations of Fe in caterpillar bodies were highest from TSF 1 (225 ppm) and Rev 3 (145 ppm) and much lower at TSF 2 (86 ppm) and Con 1 (63 ppm) (Kruskal-Wallis $\chi^2_{(3)} = 40.8155$; $n = 60$; $P < 0.0001$) (Figure 2.1). Mean concentrations of Fe in caterpillar gut contents were highest at TSF 1 (185 ppm) and lowest from TSF 2 (89 ppm) and Con 1 (88 ppm) (Kruskal-Wallis $\chi^2_{(3)} = 17.3327$; $n = 60$; $P < 0.001$) (Figure 2.1).

Mean concentrations of Al in mopane leaves were highest from Rev 1 (54 ppm) and lowest from Con 2 (23 ppm) (Kruskal-Wallis $\chi^2_{(6)} = 23.3982$; $n = 34$; $P < 0.001$) (Figure 2.1). Mean concentrations of Al found in caterpillar bodies ranged between 26 ppm and 61 ppm, but were lowest and highest at TSF 2 and Rev 3 respectively (Kruskal-Wallis $\chi^2_{(3)} = 17.0901$; $n = 60$; $P < 0.001$) (Figure 2.1). The highest mean concentration of Al in caterpillar gut contents was from Rev 3 (42 ppm) while the lowest was from TSF 1 (22 ppm) (Kruskal-Wallis $\chi^2_{(3)} = 20.308$; $n = 60$; $P < 0.001$) (Figure 2.1).

Mean concentrations of Si in mopane leaves from Rev 3 (306 ppm), Rev 1 (257 ppm) and Rev 2 (235 ppm) were much higher than from the remaining sites, which ranged between 104 ppm and 164 ppm, with mopane leaves from Con 1 having the lowest mean Si concentration (Kruskal-Wallis $\chi^2_{(6)} = 20.2398$; $n = 34$; $P < 0.005$) (Figure 2.2). Mean concentrations of Si in caterpillar bodies ranged from 206 ppm to 224 ppm and were lowest and highest at Rev 3 and TSF 1 respectively (Kruskal-Wallis $\chi^2_{(3)} = 0.2101$; $n = 60$; $P > 0.05$) (Figure 2.2). The highest mean concentration of Si in caterpillar gut contents were found from Rev 3 (316 ppm) while the lowest concentrations were found from TSF 2 (219 ppm) (Kruskal-Wallis $\chi^2_{(3)} = 9.929$; $n = 60$; $P < 0.05$) (Figure 2.2).

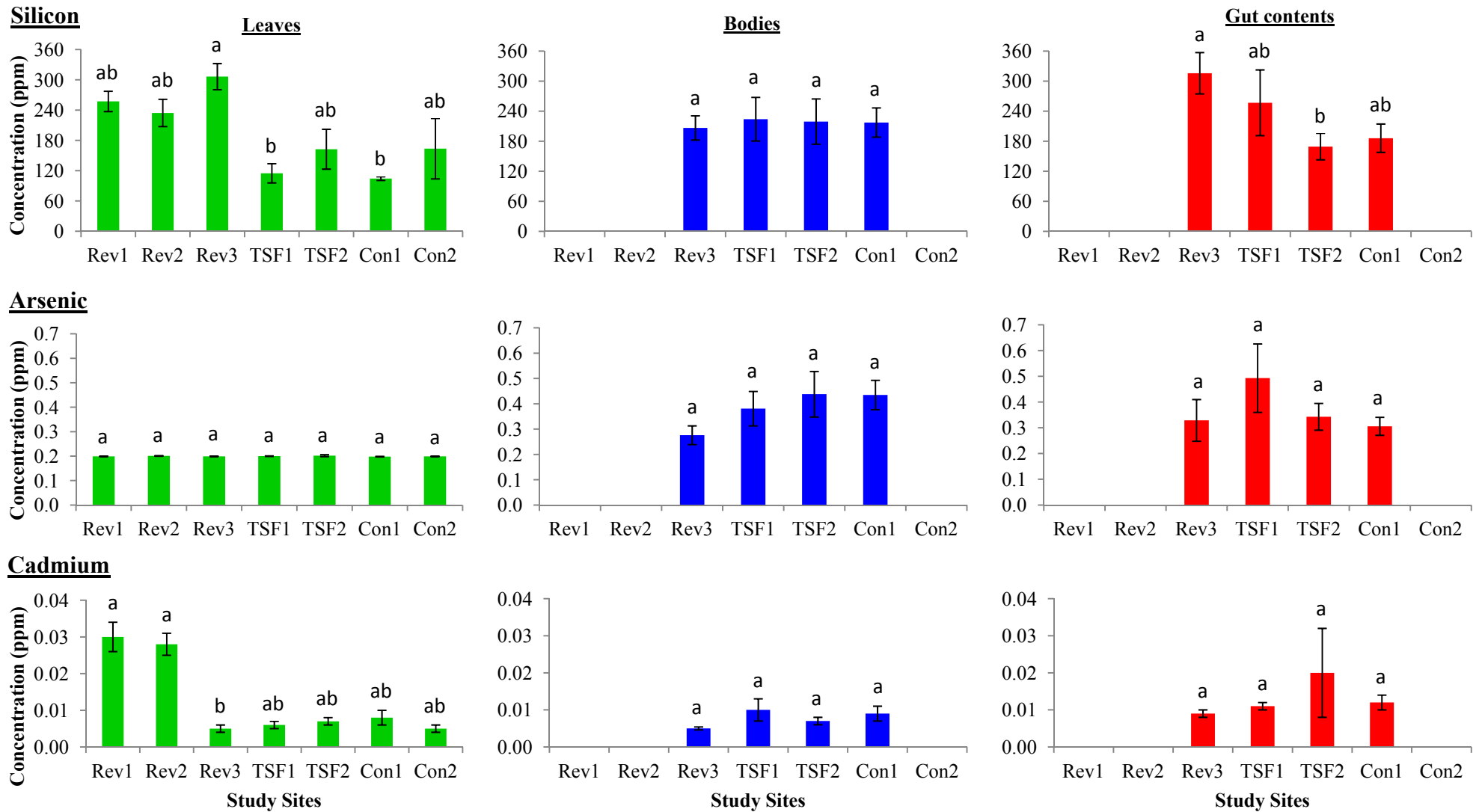


Figure 2.2. Effect of site position on mean concentrations (\pm standard error) of silicon, arsenic and cadmium in mopane leaves (green) (n = 24), mopane caterpillar bodies (blue) (n = 60) and caterpillar gut contents (red) (n = 60) from seven study sites on and around the Palabora Mining Company in Limpopo Province, South Africa. Letters indicate differences between means within sample groups.

Mean concentrations of As in mopane leaves at all sites were approximately 0.2 ppm (Figure 2.2), but the lowest concentrations were from Con 1 while the highest concentrations were from TSF 2 (Figure 2.2). Mean concentrations of As in mopane caterpillar bodies ranged from 0.276 ppm to 0.438 ppm and were lowest and highest respectively from Rev 3 and TSF 2 (Kruskal-Wallis $\chi^2_{(3)} = 4.115$; $n = 60$; $P > 0.05$). Mean concentrations of As in caterpillar gut contents ranged from 0.306 ppm to 0.493 ppm and were lowest and highest from Con 1 and TSF 1 respectively (Kruskal-Wallis $\chi^2_{(3)} = 0.5272$; $n = 60$; $P > 0.05$) (Figure 2.2).

The mean concentrations of Cd found in mopane leaves were highest from Rev 1 (0.03 ppm) and lowest from (0.005 ppm) and Con 2 (0.005 ppm) (Kruskal-Wallis $\chi^2_{(6)} = 25.2718$; $n = 34$; $P < 0.0005$) (Figure 2.2). Mean concentrations of Cd in mopane caterpillar bodies ranged from 0.005 ppm to 0.01 ppm and were lowest and highest from Rev 3 and TSF 1 respectively (Kruskal-Wallis $\chi^2_{(3)} = 5.185$; $n = 60$; $P > 0.05$) (Figure 2.2). Mean concentrations of Cd in mopane caterpillar gut contents ranged from 0.009 ppm to 0.02 ppm with concentrations from Rev 3 being lowest and concentrations from TSF 2 being highest (Kruskal-Wallis $\chi^2_{(3)} = 3.9869$; $n = 60$; $P > 0.05$) (Figure 2.2).

Mean concentration of Cr in mopane leaves from Rev 1 (0.75 ppm) and Rev 2 (0.65 ppm) were much higher than from the remaining sites, which ranged between 0.10 ppm (from Con 2) and 0.25 ppm (from Rev 3) (Kruskal-Wallis $\chi^2_{(6)} = 28.0756$; $n = 34$; $P < 0.0001$) (Figure 2.3). Mean concentrations of Cr in mopane caterpillar bodies ranged from 0.472 ppm to 1.135 ppm and were lowest and highest from Rev 3 and TSF 2 respectively (Kruskal-Wallis $\chi^2_{(3)} = 6.2509$; $n = 60$; $P > 0.05$) (Figure 2.3). Mean concentrations of Cr in mopane caterpillar gut contents were highest from TSF 1 (1.181 ppm) and this was almost double the concentrations found at the remaining sites, which ranged from 0.265 ppm at Con 1 to 0.641 ppm from TSF 2 (Kruskal-Wallis $\chi^2_{(3)} = 6.2509$; $n = 60$; $P > 0.05$) (Figure 2.3).

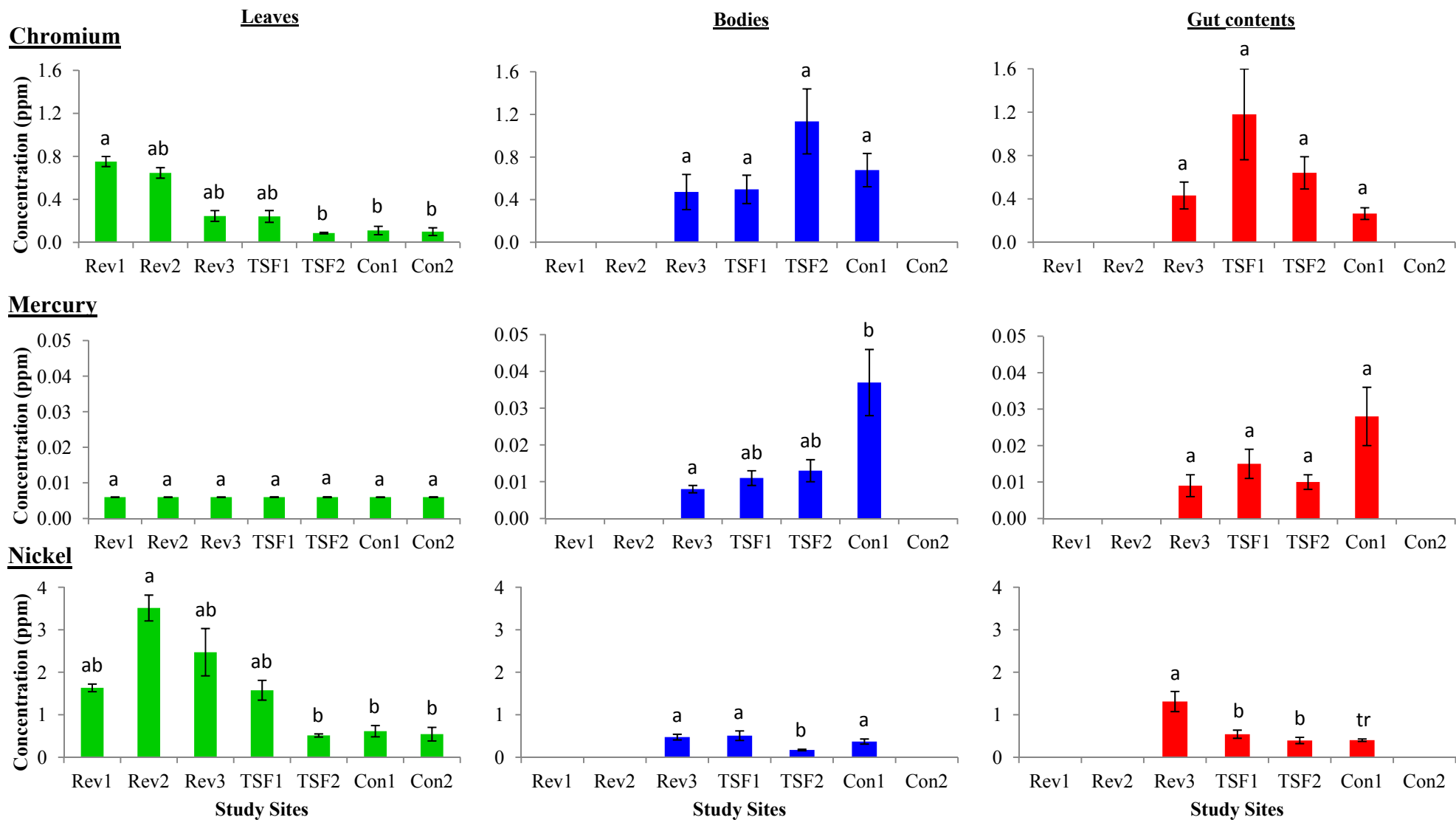


Figure 2.3. Effect of site position on mean concentrations (\pm standard error) of chromium, mercury and nickel in mopane leaves (green) (n = 24), mopane caterpillar bodies (blue) (n = 60) and caterpillar gut contents (red) (n = 60) from seven study sites on and around the Palabora Mining Company in Limpopo Province, South Africa. Letters indicate differences between means within sample groups.

Mature mopane leaves across all sites had mean Hg concentrations of 0.006 ppm (Figure 2.3). Mean concentrations of Hg in caterpillar bodies from Rev 3 (0.01 ppm) were highest while those from Con 1 (0.04 ppm) were lowest (Kruskal-Wallis $\chi^2_{(3)} = 9.2757$; $n = 60$; $P < 0.05$) (Figure 2.3). Similarly the mean Hg concentrations of caterpillar gut contents were lowest from Rev 3 (0.009 ppm) and highest from Con 1 (0.028 ppm) (Kruskal-Wallis $\chi^2_{(3)} = 3.0402$; $n = 60$; $P > 0.05$) (Figure 2.3).

Mean concentrations of Ni in leaves were highest from Rev 2 (4 ppm) and lowest from TSF 2 (0.51 ppm) (Kruskal-Wallis $\chi^2_{(6)} = 25.8529$; $n = 34$; $P < 0.0005$) (Figure 2.3). Mean concentrations of Ni in caterpillar bodies ranged from 0.17 ppm to 0.51 ppm and were lowest and highest at TSF 2 and TSF 1 respectively (Kruskal-Wallis $\chi^2_{(4)} = 20.5899$; $n = 75$; $P < 0.05$) (Figure 2.3). Mean concentration of Ni in caterpillar gut contents were lowest at TSF 2 (0.40 ppm) and highest at Rev 3 (1 ppm) (Kruskal-Wallis $\chi^2_{(4)} = 21.9495$; $n = 75$; $P < 0.0001$) (Figure 2.3).

Mean concentrations of Pb in leaves were highest from Rev 1 (0.32 ppm) and lowest from Con 2 (0.07 ppm) (Kruskal-Wallis $\chi^2_{(6)} = 25.0371$; $n = 34$; $P < 0.0005$) (Figure 2.4). Mean concentrations of lead in caterpillar bodies ranged from 0.08 ppm to 0.15 ppm, with the lowest concentrations from TSF 2 and the highest from TSF 1 (Kruskal-Wallis $\chi^2_{(3)} = 15.7245$; $n = 60$; $P < 0.005$) (Figure 2.4). Mean concentrations of Pb in caterpillar gut contents were highest from TSF 2 (0.09 ppm) and highest from Con 1 (0.15 ppm) (Kruskal-Wallis $\chi^2_{(3)} = 8.458$; $n = 60$; $P < 0.05$) (Figure 2.4).

Mean concentrations of Zn in mopane leaves ranged from 17 ppm to 33 ppm with the lowest concentrations from Rev 1 and the highest from Con 2 (Kruskal-Wallis $\chi^2_{(6)} = 21.8298$; $n = 34$; $P < 0.005$) (Figure 2.4). Mean concentrations of Zn in caterpillar bodies ranged from 134.4 ppm to 160.6 ppm and were lowest and highest at Con 1 and Rev 3 respectively (Kruskal-Wallis $\chi^2_{(3)} = 5.1928$; $n = 60$; $P > 0.05$) (Figure 2.4). Mean concentrations of Zn in caterpillar gut contents were highest from TSF 1 (113 ppm) and lowest at Con 1 (98 ppm) (Kruskal-Wallis $\chi^2_{(3)} = 0.3189$; $n = 60$; $P > 0.05$) (Figure 2.4).

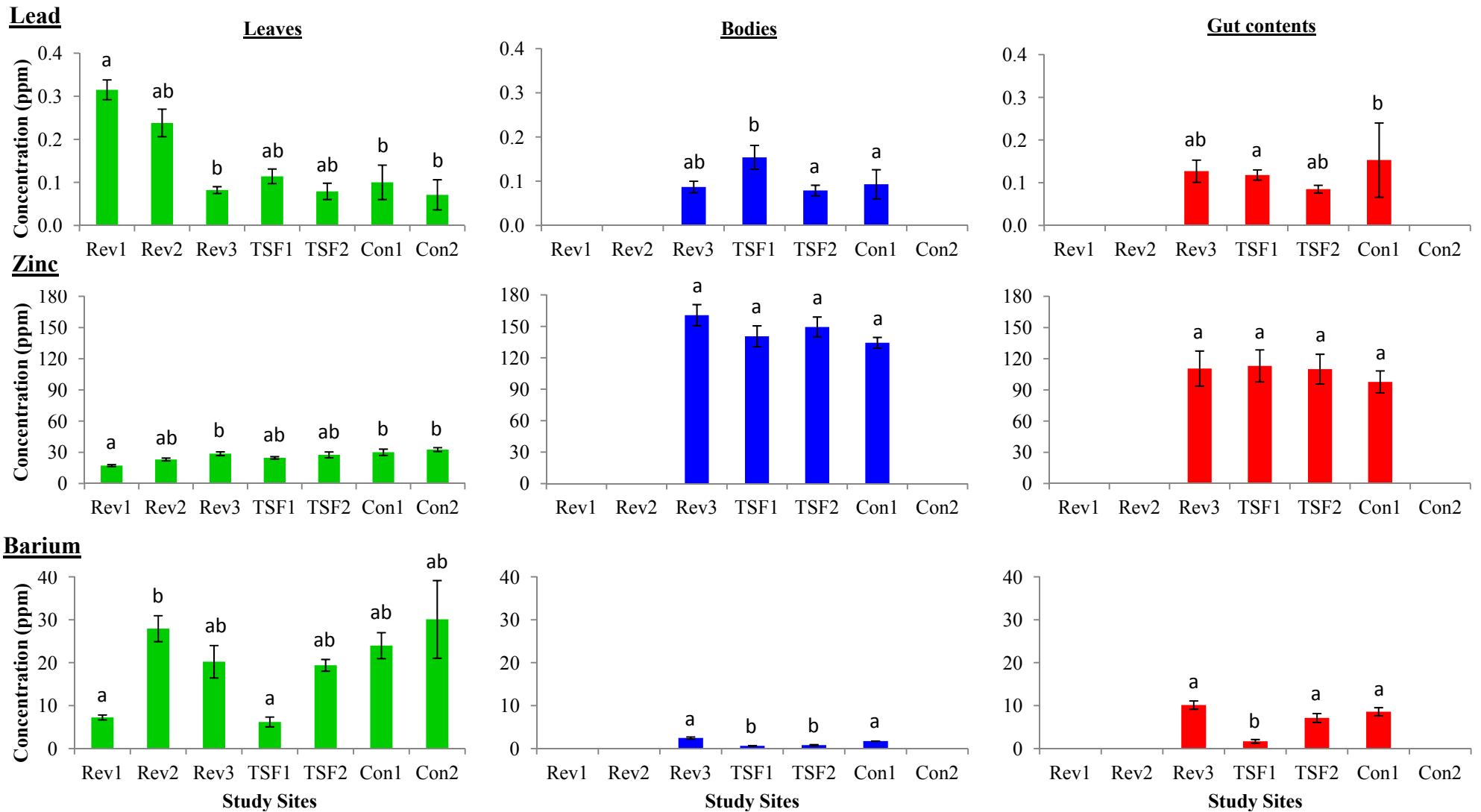


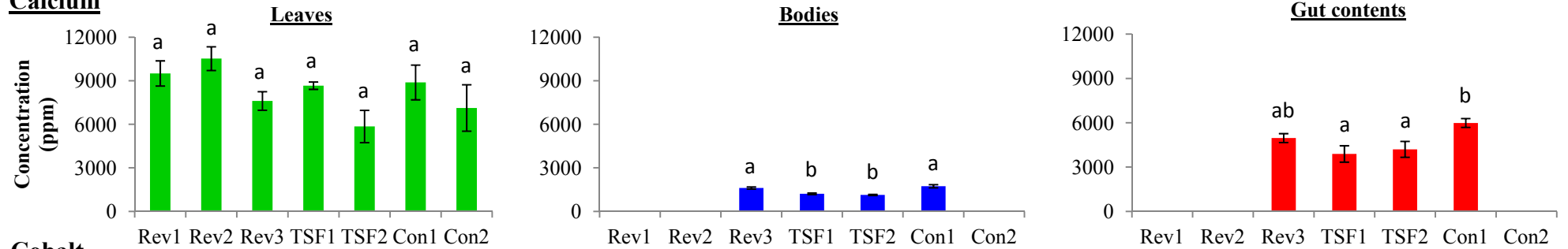
Figure 2.4. Effect of site position on mean concentrations (\pm standard error) of lead, zinc, and barium in mopane leaves (green) ($n = 24$), mopane caterpillar bodies (blue) ($n = 60$) and caterpillar gut contents (red) ($n = 60$) from seven study sites on and around the Palabora Mining Company in Limpopo Province, South Africa. Letters indicate differences between means within sample groups.

Mean concentrations of Ba in mopane leaves from Con 2 (30 ppm) were the highest of all sites, and four times higher than the lowest mean mopane leaf Ba concentration found at Rev 1 (7 ppm) (Kruskal-Wallis $\chi^2_{(6)} = 22.6514$; $n = 34$; $P < 0.001$) (Figure 2.4). The mean concentrations of barium in caterpillar bodies from Rev 3 (2 ppm) were highest while those from Con 1 (2 ppm) were lowest (Kruskal-Wallis $\chi^2_{(3)} = 33.5331$; $n = 60$; $P < 0.0001$) (Figure 2.4). The mean concentrations of Ba in the caterpillar gut contents were much lower from TSF 1 (2 ppm) than at any of the remaining sites (all greater than 7 ppm) with mean caterpillar gut content Ba concentrations from Rev 3 (10 ppm) being highest (Kruskal-Wallis $\chi^2_{(3)} = 32.3561$; $n = 60$; $P < 0.0001$) (Figure 2.4).

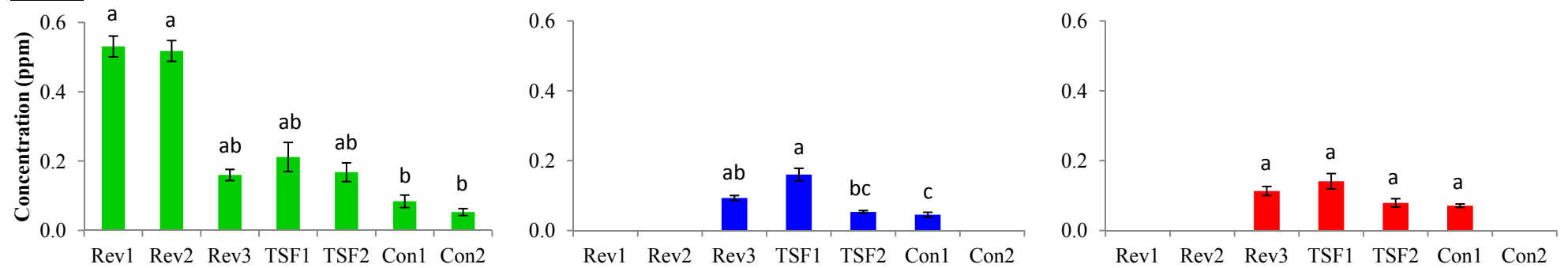
Mean concentrations of Ca in mopane leaves ranged from 5 853 ppm to 10 531 ppm across all sites with the lowest concentrations found at TSF 2 and the highest concentrations found at Rev 2 (Kruskal-Wallis $\chi^2_{(6)} = 12.0017$; $n = 34$; $P > 0.05$) (Figure 2.5). Mean concentrations of Ca in caterpillar bodies from TSF 2 (1133 ppm) were lowest while those from Con 1 (1735 ppm) were highest (Kruskal-Wallis $\chi^2_{(3)} = 29.1193$; $n = 60$; $P < 0.0001$) (Figure 2.5). Mean concentrations of Ca in caterpillar gut contents ranged from 3 890 ppm to 5 986 ppm, with the lowest and highest concentrations from TSF 1 and Con 1 respectively (Kruskal-Wallis $\chi^2_{(3)} = 13.9031$; $n = 60$; $P < 0.005$) (Figure 2.5).

Mean concentrations of Co in mopane leaves were lowest from Con 2 (0.05 ppm) and highest from Rev 1 (0.53 ppm) (Kruskal-Wallis $\chi^2_{(6)} = 28.5238$; $n = 34$; $P < 0.0001$) (Figure 2.5). Mean concentration of Co in caterpillar bodies ranged between 0.04 ppm and 0.16 ppm with the lowest and highest concentrations from Con 1 and TSF 1 respectively (Kruskal-Wallis $\chi^2_{(3)} = 35.3099$; $n = 60$; $P < 0.0001$) (Figure 2.5). Mean concentrations of Co in caterpillar bodies from Con 1 (0.07 ppm) were lowest while those from TSF 1 (0.14 ppm) were highest (Kruskal-Wallis $\chi^2_{(3)} = 8.7789$; $n = 75$; $P < 0.05$) (Figure 2.5).

Calcium



Cobalt



Potassium

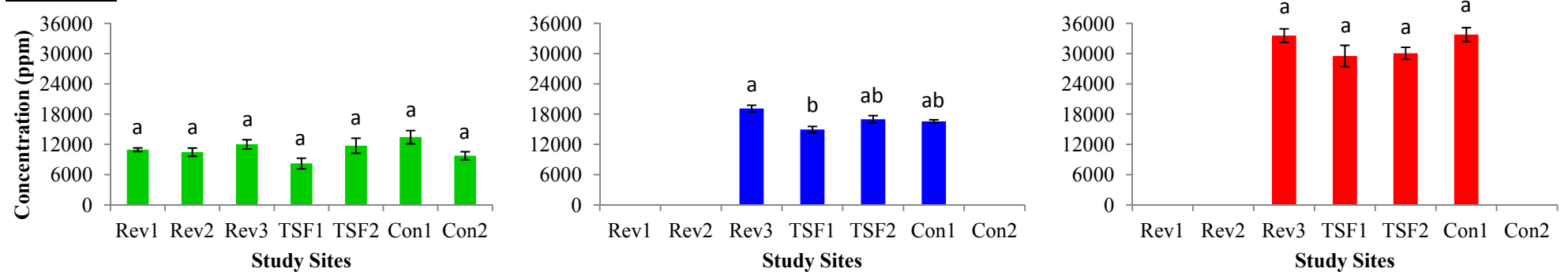


Figure 2.5. Effect of site position on mean concentrations (\pm standard error) of calcium, cobalt and potassium in mopane leaves (green) ($n = 24$), mopane caterpillar bodies (blue) ($n = 60$) and caterpillar gut contents (red) ($n = 60$) from seven study sites on and around the Palabora Mining Company in Limpopo Province, South Africa. Letters indicate differences between means within sample groups.

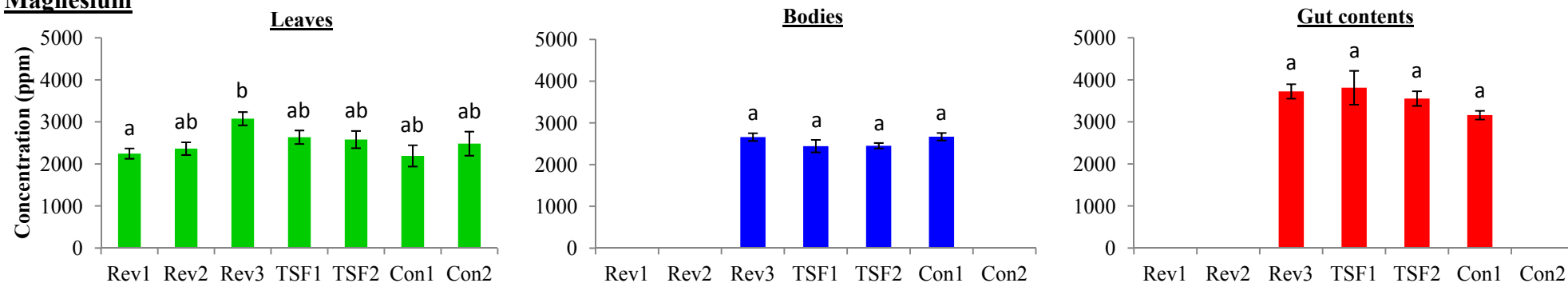
Mean concentrations of K in mopane leaves ranged from 8 224 ppm to 13 447 ppm, with the lowest concentrations found at TSF 1 and the highest concentrations from Con 1 (Kruskal-Wallis $\chi^2_{(6)} = 12.2597$; $n = 34$; $P > 0.05$) (Figure 2.5). Mean concentrations of K in caterpillar bodies from Rev 3 (19 111 ppm) were highest while those from TSF 1 (14 958 ppm) were lowest (Kruskal-Wallis $\chi^2_{(3)} = 18.228$; $n = 60$; $P < 0.0005$) (Figure 2.5). Mean concentrations of K in caterpillar gut contents ranged from 29 533 ppm to 33 783 ppm, with the lowest and highest concentrations from TSF 1 and Con 1 respectively (Kruskal-Wallis $\chi^2_{(3)} = 6.3731$; $n = 60$; $P > 0.05$) (Figure 2.5).

Mean concentration of Mg in mopane leaves from Con 1 (2 193 ppm) were lowest while those from Rev 3 (3 077 ppm) were highest (Kruskal-Wallis $\chi^2_{(6)} = 14.6462$; $n = 34$; $P < 0.05$) (Figure 2.6). Mean concentrations of Mg in caterpillar bodies ranged from 2 444 ppm to 2 669 ppm with the lowest concentrations from TSF 1 and the highest concentrations from Con 1 (Kruskal-Wallis $\chi^2_{(3)} = 4.336$; $n = 60$; $P > 0.05$) (Figure 2.6). Mean concentrations of Mg in caterpillar gut contents ranged from 3 162 ppm to 3 813 ppm with the lowest and highest concentrations from Con 1 and TSF 1 respectively (Kruskal-Wallis $\chi^2_{(3)} = 6.2516$; $n = 75$; $P > 0.05$) (Figure 2.6).

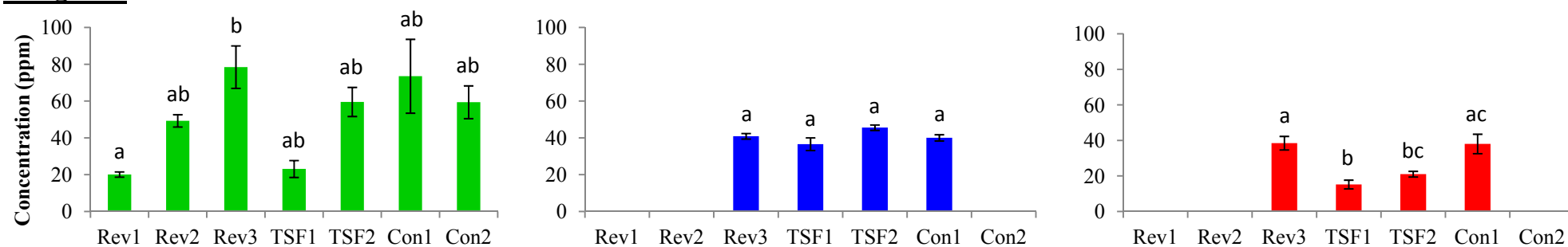
Mean concentrations of Mn in leaves were lowest from Rev 1 (20 ppm) and highest from Rev 3 (78 ppm) (Kruskal-Wallis $\chi^2_{(6)} = 23.1869$; $n = 34$; $P < 0.001$) (Figure 2.6). Mean concentrations of Mn in caterpillar bodies ranged from 37 ppm to 46 ppm with the lowest and highest concentrations from TSF 1 and TSF 2 respectively (Kruskal-Wallis $\chi^2_{(3)} = 5.3987$; $n = 60$; $P > 0.05$). Mean concentrations of Mn in caterpillar gut contents were lowest from TSF 1 (15 ppm) and highest from from Rev 3 (38 ppm) (38 ppm) (Kruskal-Wallis $\chi^2_{(3)} = 30.3443$; $n = 60$; $P < 0.0001$) (Figure 2.6).

Mean concentrations of Mo in mopane leaves were highest from Rev 2 (0.17 ppm) and lowest from Con 1 (0.07 ppm) (Kruskal-Wallis $\chi^2_{(6)} = 13.9338$; $n = 34$; $P < 0.05$) (Figure 2.6). Mean concentrations of Mo in caterpillar bodies ranged from 0.41 ppm to 0.56 ppm, with the lowest and highest concentrations from Con 1 and TSF 1 respectively (Kruskal-Wallis $\chi^2_{(3)} = 6.0728$; $n = 60$; $P > 0.05$) (Figure 2.6). Mean concentrations of Mo in caterpillar gut contents ranged from 0.30 ppm to 0.48 ppm and the lowest and highest concentrations were found at Con 1 and TSF 1 respectively (Kruskal-Wallis $\chi^2_{(3)} = 7.9315$; $n = 60$; $P < 0.05$) (Figure 2.6).

Magnesium



Manganese



Molybdenum

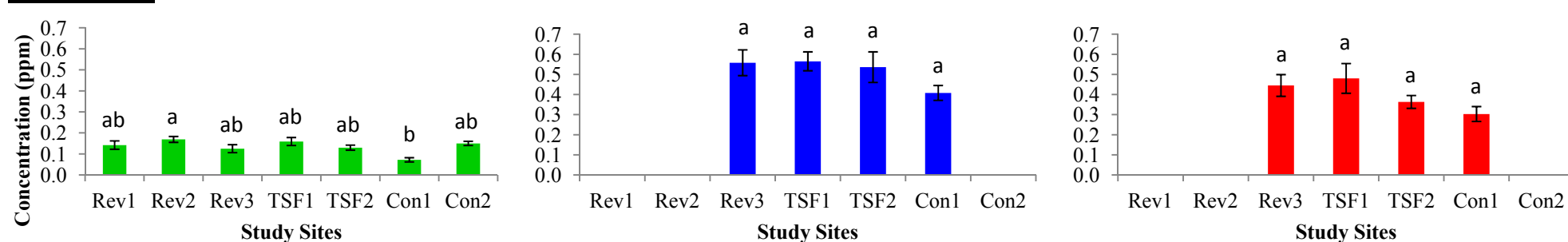


Figure 2.6. Effect of site position on mean concentrations (\pm standard error) of magnesium, manganese and molybdenum in mopane leaves (green) ($n = 24$), mopane caterpillar bodies (blue) ($n = 60$) and caterpillar gut contents (red) ($n = 60$) from seven study sites on and around the Palabora Mining Company in Limpopo Province, South Africa. Letters indicate differences between means within sample groups.

Mean concentrations of Na in mopane leaves ranged between 4 ppm and 6 ppm for most sites, except for Rev 1 (16 ppm) which had a mean Na concentration in the mopane leaves that was more than double that of any other site (Kruskal-Wallis $\chi^2_{(6)} = 11.2663$; $n = 34$; $P > 0.05$) (Figure 2.7). Mean concentrations of Na in caterpillar bodies ranged from 23 ppm to 40 ppm, with the lowest and highest concentrations found at TSF 1 and TSF 2 respectively (Kruskal-Wallis $\chi^2_{(3)} = 7.962$; $n = 60$; $P < 0.05$) (Figure 2.7). Mean concentrations of Na in caterpillar gut contents were lowest from Rev 3 (18 ppm) and highest from TSF 2 (33.8 ppm) (Kruskal-Wallis $\chi^2_{(3)} = 7.5902$; $n = 75$; $P > 0.05$) (Figure 2.7).

Mean concentrations of P in mopane leaves ranged from 1 310 ppm to 2 624 ppm, with the lowest and highest concentrations from Con 2 and Rev 3 respectively (Kruskal-Wallis $\chi^2_{(6)} = 21.6292$; $n = 34$; $P < 0.005$) (Figure 2.7). Mean concentrations of P in caterpillar bodies were lowest from Con 1 (6 635 ppm) and highest from Rev 3 (7 606 ppm) (Kruskal-Wallis $\chi^2_{(3)} = 7.3637$; $n = 60$; $P > 0.05$) (Figure 2.7). Mean concentrations of P in caterpillar gut contents ranged from 6 343 ppm to 7 872 ppm, with the lowest and highest concentrations found at Con 1 and TSF 1 respectively (Kruskal-Wallis $\chi^2_{(3)} = 5.8813$; $n = 60$; $P > 0.05$) (Figure 2.7).

Mean concentrations of S in mopane leaves were lowest from TSF 1 (1 742 ppm) and highest from Rev 2 (2 449 ppm) (Kruskal-Wallis $\chi^2_{(6)} = 19.9255$; $n = 34$; $P < 0.005$) (Figure 2.7). Mean concentrations of S in caterpillar bodies ranged from 5 291 ppm to 5 838 ppm, with the lowest and highest concentrations from TSF 1 and Con 1 respectively (Kruskal-Wallis $\chi^2_{(3)} = 4.2949$; $n = 60$; $P > 0.05$) (Figure 2.7). Mean concentrations of S in caterpillar gut contents were lowest from Con 1 (4 229 ppm) and highest from TSF 1 (5 429 ppm) (Kruskal-Wallis $\chi^2_{(3)} = 2.9991$; $n = 60$; $P > 0.05$) (Figure 2.7).

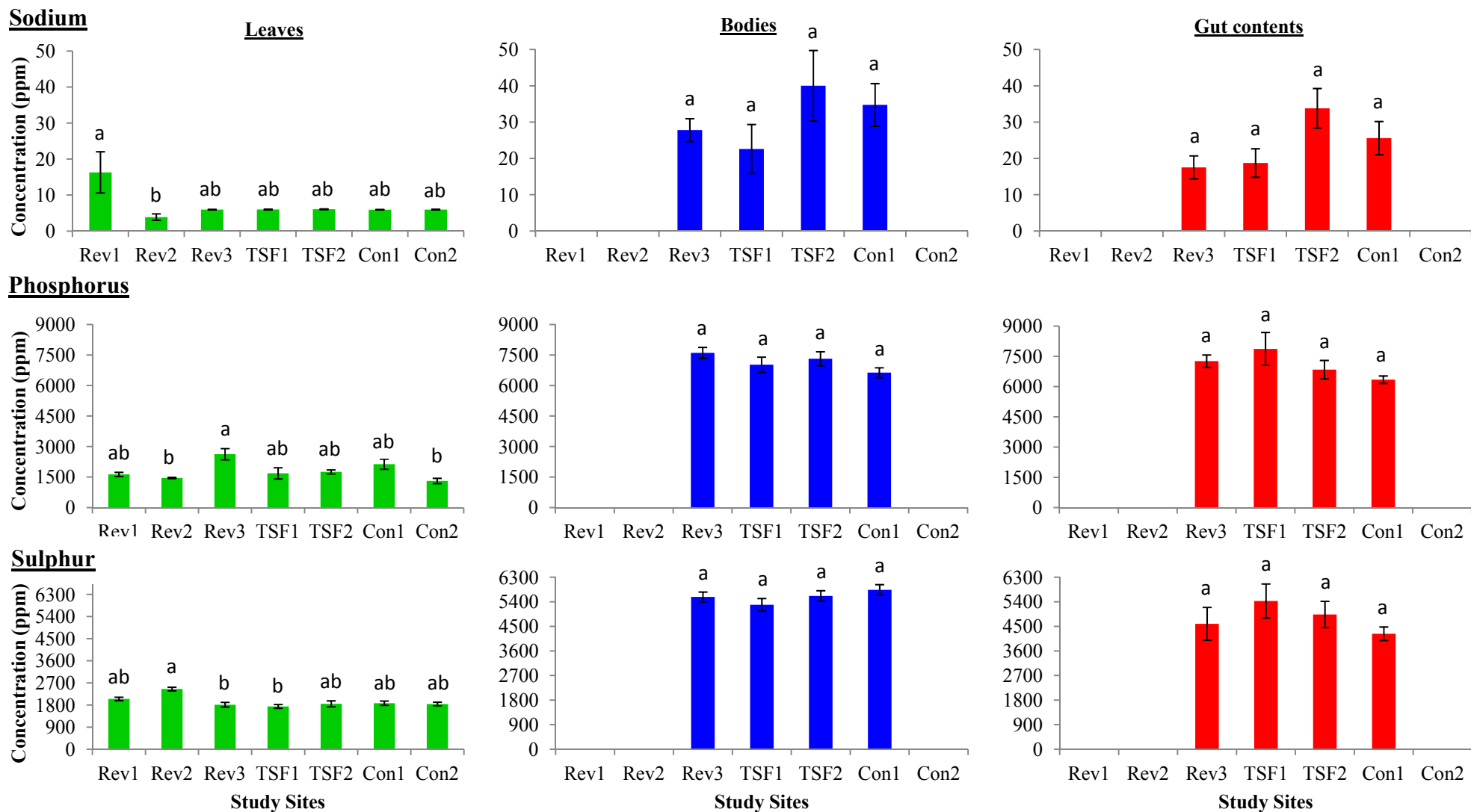


Figure 2.7. Effect of site position on mean concentrations of (\pm standard error) of sodium, phosphorus and sulphur in mopane leaves (green) ($n = 24$), mopane caterpillar bodies (blue) ($n = 60$) and caterpillar gut contents (red) ($n = 60$) from seven study sites on and around the Palabora Mining Company in Limpopo Province, South Africa. Letters indicate differences between means within sample groups.

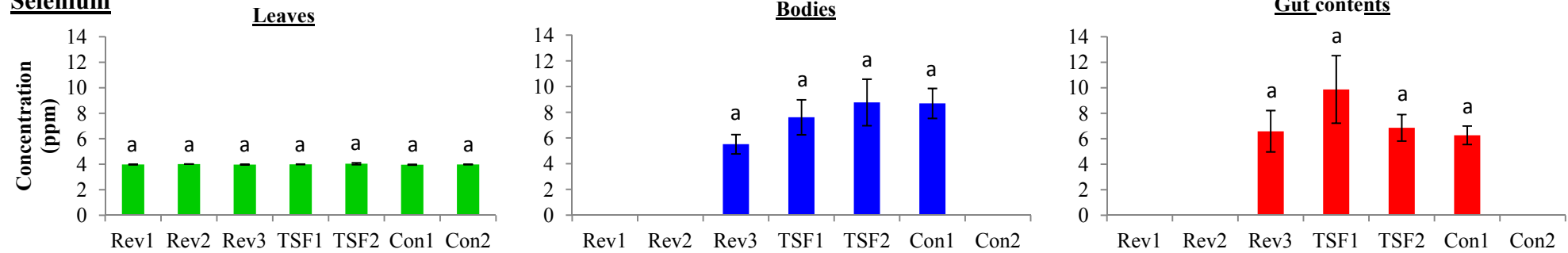
Mean concentrations of Se in mopane leaves ranged from 3.96 ppm to 4.04 ppm, with the lowest and highest concentrations found at Con 1 and TSF 2 respectively (Kruskal-Wallis $\chi^2_{(6)} = 6.3016$ n = 34; P > 0.05) (Figure 2.8). Mean concentrations of Se in caterpillar bodies were lowest at Rev 3 (5.52 ppm) and highest at TSF 2 (8.76 ppm) (Kruskal-Wallis $\chi^2_{(3)} = 4.193$; n = 60; P > 0.05) (Figure 2.8). Mean concentrations of Se in caterpillar gut contents were lowest at Con 1 (6.27 ppm) and highest at TSF 1 (9.87 ppm) (Kruskal-Wallis $\chi^2_{(3)} = 0.4861$; n = 60; P > 0.05) (Figure 2.8).

Mean concentrations of Sr in mopane leaves ranged from 26 ppm to 73 ppm with the lowest and highest concentrations from TSF 1 and Rev 2 respectively (Kruskal-Wallis $\chi^2_{(6)} = 21.1682$; n = 34; P < 0.005) (Figure 2.8). Mean concentrations of Sr in caterpillar bodies were lowest at TSF 1 (3 ppm) and highest at Rev 3 (8 ppm) (Kruskal-Wallis $\chi^2_{(3)} = 33.8319$; n = 60; P < 0.0001) (Figure 2.8). Mean concentrations of Sr in caterpillar gut contents were much lower from TSF 1 (9 ppm) than the other sites, which ranged between 22 ppm and 38 ppm, with Rev 3 having the highest concentration (Kruskal-Wallis $\chi^2_{(3)} = 32.2643$; n = 60; P < 0.0001) (Figure 2.8).

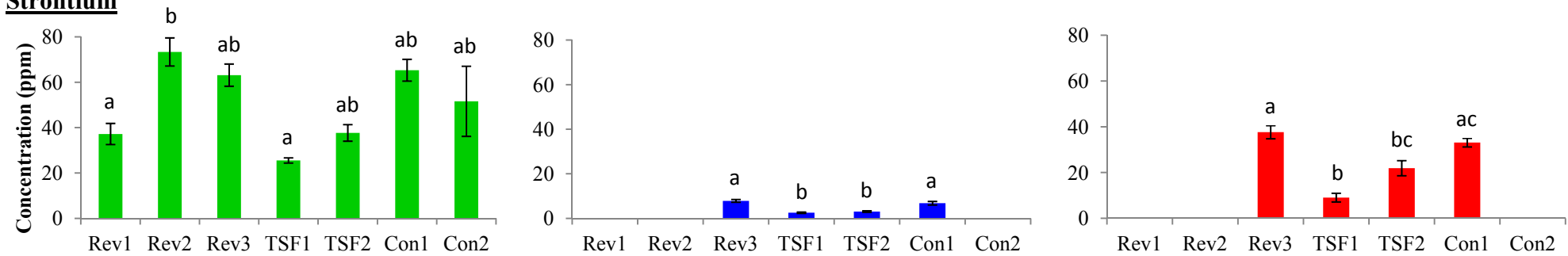
Mean concentrations of U in mopane leaves ranged from 0.003 ppm to 0.086 ppm, with the lowest and highest concentrations found at Con 2 and Rev 2 respectively (Kruskal-Wallis $\chi^2_{(6)} = 27.3095$; n = 34; P < 0.0005) (Figure 2.8). Mean concentrations of U in caterpillar bodies were lowest at Con 1 (0.01 ppm) and highest at TSF 1 (0.03 ppm) (Kruskal-Wallis $\chi^2_{(3)} = 20.2059$; n = 60; P < 0.0001) (Figure 2.8). Mean concentrations of U in caterpillar gut contents were lowest at Con 1 (0.01 ppm) and highest at TSF 1 (0.04 ppm) (Kruskal-Wallis $\chi^2_{(3)} = 16.0724$; n = 75; P < 0.05) (Figure 2.8).

Mean concentrations of W in mopane leaves ranged from 0.001 ppm to 0.003 ppm, with the lowest concentrations found at Con 1 (0.001 ppm) and Con 2 (0.001 ppm), and the highest concentrations found at Rev 2 (0.003 ppm) (Kruskal-Wallis $\chi^2_{(6)} = 5.13$; n = 34; P > 0.05) (Figure 2.9). Mean concentrations of W in caterpillar bodies ranged from 0.003 ppm to 0.012 ppm, with the lowest and highest concentrations from Rev 3 and Con 1 respectively (Kruskal-Wallis $\chi^2_{(3)} = 2.4236$; n = 60; P > 0.05) (Figure 2.9). Mean concentrations of W in caterpillar gut contents were lowest from TSF 2 (0.003 ppm) and were highest at Con 1 (0.009 ppm) (Kruskal-Wallis $\chi^2_{(3)} = 0.8365$; n = 60; P > 0.05) (Figure 2.9).

Selenium



Strontium



Uranium

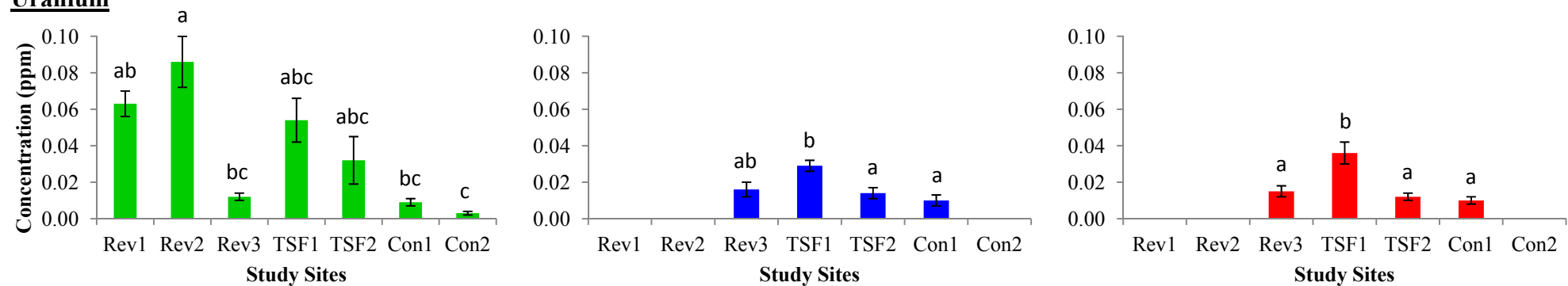


Figure 2.8. Effect of site position on mean concentrations (\pm standard error) of selenium, strontium and uranium in mopane leaves (green) ($n = 24$), mopane caterpillar bodies (blue) ($n = 60$) and caterpillar gut contents (red) ($n = 60$) from seven study sites on and around the Palabora Mining Company in Limpopo Province, South Africa. Letters indicate differences between means within sample groups.

Tungsten

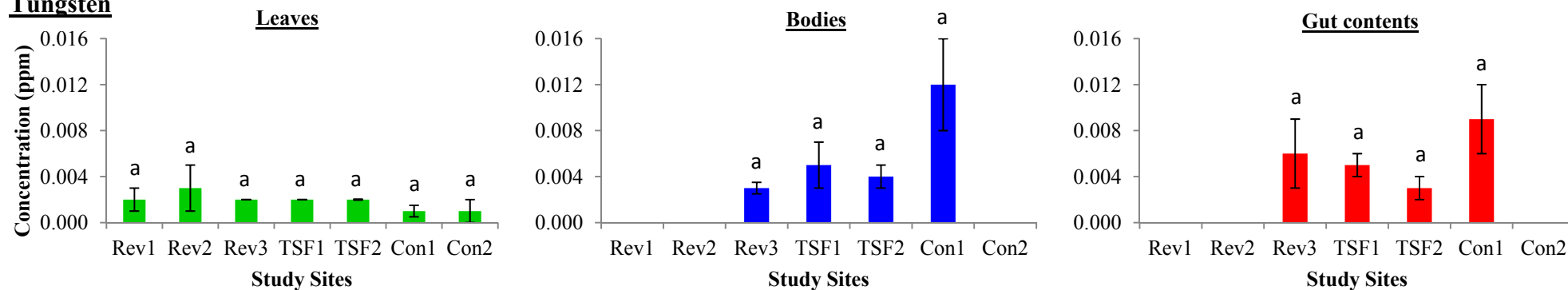


Figure 2.9. Effect of site position on mean concentrations (\pm standard error) of tungsten in mopane leaves (green) ($n = 24$), mopane caterpillar bodies (blue) ($n = 60$) and caterpillar gut contents (red) ($n = 60$) from seven study sites on and around the Palabora Mining Company in Limpopo Province, South Africa. Letters indicate differences between means within sample groups.

Mean concentrations of elements only differed significantly between mopane caterpillar bodies and caterpillar guts for Ca (Wilcoxon Rank Sum Test; $W = 0$; $n = 60$; $P < 0.05$), K (Wilcoxon Rank Sum Test; $W = 0$; $n = 60$; $P < 0.05$), Mg (Wilcoxon Rank Sum Test; $W = 0$; $n = 60$; $P < 0.05$), Sr (Wilcoxon Rank Sum Test; $W = 0$; $n = 60$; $P < 0.05$) and Zn (Wilcoxon Rank Sum Test; $W = 0$; $n = 60$; $P < 0.05$), across all sites (Figures 2.4 – 2.9). Mean concentrations of most elements did not differ significantly between mature mopane leaves and compensatory growth (Table 2.1). Mean Ba concentrations differed significantly between mature mopane leaves (19 ppm) and compensatory growth (3 ppm) across all sites (Wilcoxon Rank Sum Test; $W = 0$; $n = 37$; $P < 0.0005$) (Table 2.1). The mean Ca content of mature mopane leaves (8 649 ppm) differed significantly from those of compensatory growth (1 465 ppm) across all sites (Wilcoxon Rank Sum Test; $W = 0$; $n = 37$; $P < 0.0005$) (Table 2.1). Mean concentrations of K in mature mopane leaves (11 089 ppm) differed significantly from those in compensatory growth (18 460 ppm) across sites (Wilcoxon Rank Sum Test; $W = 101$; $n = 37$; $P < 0.001$) (Table 2.1).

The mean concentrations of Mn in mature mopane leaves (52 ppm) differed significantly from compensatory growth (13 ppm) across all sites (Wilcoxon Rank Sum Test; $W = 1$; $n = 37$; $P < 0.001$) (Table 2.1). Mean concentrations of Na differed significantly between mature mopane leaves (8 ppm) and compensatory growth (50 ppm) across sites (Wilcoxon Rank Sum Test; $W = 100$; $n = 37$; $P < 0.01$) (Table 2.1). The mean P concentrations in mature mopane leaves (1 846 ppm) differed significantly from compensatory growth (2 547 ppm) across sites (Wilcoxon Rank Sum Test; $W = 87$; $n = 37$; $P < 0.05$) (Table 2.1). Mean concentrations of S differed significantly between mature mopane leaves (1 998 ppm) and compensatory growth (2 408 ppm) across sites (Wilcoxon Rank Sum Test; $W = 90$; $n = 37$; $P < 0.05$) (Table 2.1). The mean Sr content of mature mopane leaves (54 ppm) differed significantly from compensatory growth (8 ppm) across sites (Wilcoxon Rank Sum Test; $W = 0$; $n = 37$; $P < 0.0005$) (Table 2.1).

Table 2.1. Mean concentration \pm standard error of 25 elements measured in mature mopane leaves and mopane lead compensatory growth from seven sites on and around Palabora Mining Company, Phalaborwa, South Africa. All concentrations were measured in mg.L^{-1} (ppm).

Element	Mature mopane leaves	Mopane leaf compensatory growth
Al	39.53585 \pm 2.28590	40.56526 \pm 10.59363
As	0.19944 \pm 0.00043	0.19881 \pm 0.00087
Ba	19.14497 \pm 1.91521	2.87783 \pm 0.45198
Ca	8648.75400 \pm 406.33570	1464.53900 \pm 196.13810
Cd	0.01560 \pm 0.00224	0.02367 \pm 0.00760
Co	0.29705 \pm 0.03526	0.44581 \pm 0.11072
Cr	0.38881 \pm 0.04974	0.68360 \pm 0.25717
Cu	21.13287 \pm 2.31675	35.24472 \pm 8.02419
Fe	363.99290 \pm 52.84210	485.22320 \pm 90.17101
Hg	0.00598 \pm 0.00001	0.00596 \pm 0.00003
K	11088.78000 \pm 385.12370	18460.35000 \pm 1001.93600
Mg	2520.29700 \pm 82.67079	1574.31700 \pm 83.84858
Mn	51.63383 \pm 5.06029	13.06862 \pm 1.20402
Mo	0.13698 \pm 0.00802	0.11716 \pm 0.00332
Na	7.67092 \pm 1.36398	50.25621 \pm 13.63378
Ni	1.87287 \pm 0.22577	2.84710 \pm 0.45595
P	1845.98100 \pm 103.25550	2546.78200 \pm 291.10560
Pb	0.16579 \pm 0.01921	0.17570 \pm 0.06445
S	1997.70800 \pm 52.95744	2407.99100 \pm 112.82040
Se	3.98872 \pm 0.00861	3.97626 \pm 0.01741
Si	215.52510 \pm 15.73671	103.88300 \pm 4.54387
Sr	53.57036 \pm 3.56489	7.84669 \pm 0.562700
U	0.04211 \pm 0.00627	0.06581 \pm 0.00852
W	0.00208 \pm 0.00049	0.00192 \pm 0.00007
Zn	25.23908 \pm 1.06604	24.44377 \pm 1.33384

Discussion

Elemental concentrations differed across PMC sites in mopane trees (mature leaves and compensatory leaf growth) as well as in mopane caterpillar body and gut content samples. The concentrations of Al, Ca, Cd, Co, Cr, Cu, Fe, S, Pb and U in mopane leaves were higher at Rev 1 and Rev 2 than at the other sites. In mopane leaves, concentrations of Na were highest at Rev 1, concentrations of Ba, Ni and W were highest at Rev 2, and concentrations of Mg, Mn, P and Si were highest at Rev 3, and were some of the lightest of the elements sampled in terms of atomic mass. Of the 25 elements measured, leaves from Rev 1 and/or Rev 2 had the highest concentrations of 14 of

these elements. When all three reverberator sites are considered, they have the highest mopane leaf concentration of 18 of the 25 elements measured, suggesting that the reverberator sites are much more polluted than the other sites. For the most part the lighter elements (in terms of atomic mass), travelled further and were higher at sites that were further away, while the highest concentrations of the heavier elements generally occurred closest to the reverberator. This does show that concentrations of elements are usually highest closest to the reverberator, and decrease progressively further from the reverberator, as was found by Cartwright *et al.* (1977), Kuo *et al.* (1983), and McMartin *et al.* (1999). There does not appear to be a general trend of concentrations of elements increasing or decreasing with increased distance from the tailings storage facility or with increased distance from PMC. The reverberator sites are subjected to dust fallout from the reverberator and dust particles paired with the high concentrations of elements found at this site could be the reason for no mopane caterpillars being found at Rev 1 and Rev 2.

Alexander *et al.* (1944), Kalmus (1944), Wigglesworth (1944), Korunic (1998) and Glenn (1999) all suggest that some dusts can damage the cuticle of insects through abrasion and adsorption, thereby making the cuticle more susceptible to water loss which increases insects' likelihood of drying out and subsequently dying. Insects with bodies that are hairy or rough can collect more dust particles per unit area and can consequently undergo greater damage to the cuticle (Korunic 1998). As mopane caterpillars have hair and spines on their bodies as well as a rough body surface, dust could easily collect on the caterpillars which could make the mopane caterpillars particularly susceptible to desiccation. This may be why the mopane caterpillars have not established at Rev 1 and Rev 2 despite the abundance of suitable food at these sites.

The reason for no eggs being laid at Rev 1 and Rev 2 may be as a result of the dust film that accumulates on the leaves at these two high dust fallout sites. Glenn *et al.* (1999) has suggested that a particle film barrier could make host plants unrecognizable either tactually or visually. Knight *et al.* (2000) and Unruh *et al.* (2000) found that female obliquebanded leafrollers, *Choristoneura rosaceana*, and codling moths, *Cydia pomonella*, respectively, oviposited less on host plants treated with a particle film than on those host plants with no particle film. Dusts containing silica or

aluminium have been found to be effective insecticides in the past (Alexander *et al.* 1944; David and Gardiner 1950; Ebeling and Wagner 1959). As aluminium and silicon concentrations were high in leaves from Rev 1 and Rev 2, the high concentrations of these elements could be an additional reason for mopane moths being deterred from these sites and not laying eggs here. Elemental concentrations found at the tailings storage facility and control sites however were similar to each other for most elements. This would suggest that the flora and fauna in the areas surrounding the tailings storage facility are able to tolerate the concentrations of elements present at these sites.

Caterpillar body and gut content elemental concentrations were much higher than the corresponding mopane leaf values for a number of elements (As, Cr, Hg, K, Mo, Na, P, S, Se, W, and Zn) most of which were heavy metals or macronutrients. This follows Lindqvist (1992) who found that concentrations of metals were usually lowest in leaves, moderate in the larvae of phytophagous insects, and highest in the adults of these insects. This could indicate that the caterpillars are bioaccumulating these elements. In order to assess if the caterpillars are bioaccumulating certain elements, the concentrations of elements could be measured in each caterpillar several times through an instar, as done by Judy *et al.* (2012) examining bioaccumulation in the tobacco hornworm (*Manduca sexta*). If the concentrations of these elements increase at each successive time interval, this would indicate that the caterpillars are bioaccumulating those elements. Even if the caterpillars do appear to bioaccumulate within an instar, they may then eliminate excess elements when moulting occurs, so shed material should be collected and measured as well to ascertain if they are bioaccumulating elements or eliminating them when they moult.

The concentrations of elements are similar in the caterpillar bodies and gut contents for many of the elements. In the cases where gut content concentrations of an element were higher than the body concentrations, there was probably an excess of that element, that instead of being absorbed into the body, remained in the gut to be eliminated as frass. This process was observed by Aoki and Suzuki (1984) who found that the fleshfly (*Sarcophaga peregrina*) excreted excess amounts of cadmium after eclosion from the pupae. Raubenheimer and Simpson (2004) also found generalist locusts (*Schistocerca gregaria*) were able to eliminate excess nutrients by excreting them. Similarly

Lee *et al.* (2002) found that a generalist caterpillar (*Spodoptera littoralis*) was able to regulate the nutrients in its body after ingesting these nutrients from their diet. Lindqvist and Block (1995) found that mealworms (*Tenebrio molitor*) were able to eliminate excess cadmium but eliminated more cadmium during metamorphosis than at any other point in the life cycle. The reason for this is that during the process of metamorphosis, many organs are broken down and new ones built from embryonic cells (Wigglesworth 1974), whereas with moulting, parts of the old cuticle are reabsorbed by the larvae (Chapman 1998). Concentrations of elements in frass from this instar, and in pupae would have to be measured and compared to each other and to final mopane instars measured, to ascertain if mopane caterpillars may be getting rid of any excess elements via frass or after eclosion.

Hrabar *et al.* (2009) showed that compensatory growth in mopane trees defoliated by mopane caterpillars resulted in a much smaller leaf and shoot size, which was also seen in the current study (personal observation). Despite these leaves being smaller, the concentrations of Al, Ba, Ca, Cd, Co, Cr, K, Mn, Na, P, Pb, S, Sr and U were higher in the compensatory growth than in mature leaves, but not significantly higher for all these elements except Ba, Ca and K. The higher concentrations of copper in compensatory growth may be because photosynthetic rates are high in newer leaves, and Cu plays a vital role in photosynthesis (Dučić and Polle 2005; Yruela 2005). Alternatively, this could have been the result of Cu chelation, whereby excess Cu from plants is buffered by phytochelatins, metallothioneins, amino acids or organic acid, and transported by chaperones to metal-acquiring proteins, vacuoles, or other organelles (Fernandes and Henriques 1991; Cobbett 2000; Polle and Schützendübel 2004; Dučić and Polle 2005).

As with Cu, the mopane compensatory growth also had higher concentrations of Ca, Fe, K, Ni, P, and S than the mature mopane leaves, which is likely also because of the importance of these elements to plant growth (Marschner 1995). Smith (1962) states that the mineral compositions of tissues are dynamic, with some elements being present in high concentrations in young leaves and these concentrations decreasing as the leaves age, while other elements are at first present at low concentrations and these concentrations increase with leaf age. The concentrations of Al, Cd, Co, Cr, Na, Pb and U could be elements naturally present in high concentrations in young mopane leaves.

Mopane leaves of various ages would need to be sampled from additional sites to examine if this is the case.

Marschner (1995) suggests that Cu becomes toxic to plants when concentrations reach 20-30 ppm, which indicates that the mopane leaves from Rev 1 (42.3 ppm) and Rev 2 (28.8 ppm) contained toxic concentrations of Cu. The trees however looked healthy, which suggests that the plants must either be able to tolerate these levels of Cu, or excess Cu may be eliminated at a later stage, possibly through Cu chelation. Assuming a single caterpillar to weigh 5g (dry weight), one caterpillar from PMC would contain less than 0.6 mg of Cu. If 10 caterpillars were eaten each day that would indicate that 6 mg Cu would be consumed by a person each day, and acute copper toxicity in humans at concentrations lower than $10 \text{ mg} \cdot \text{day}^{-1}$ has been suggested to occur only in sub-populations of the general population that are more susceptible to copper toxicity and in children (EFSA 2006). If consumed by a person of average weight (60 kg), that would equate to $0.01 \text{ ppm} \cdot \text{day}^{-1}$, which is in line with the $0.01 \text{ ppm} \cdot \text{day}^{-1}$ indicated by the ATSDR's (2014) as being the minimal risk level for acute-duration copper toxicity when ingested orally. This would suggest that the ingestion of the caterpillars from PMC and its surrounds would result in minimal risk in terms of Cu content.

Markert (1992) indicate that the average range of Fe in plants is 5 to 200 (ppm), which suggests that there is a possibility that the mopane leaves from Rev 1 (496.9 ppm) and Rev 2 (867.8 ppm) of the current study contain harmful levels of Fe for the plants. As the trees appeared to be healthy however, it would suggest that the trees are either able to tolerate these concentrations of Fe or are able to eliminate any excess Fe. In humans, Wood and Ronnenberg (2006) indicate that Fe toxicity can occur with the ingestion of substances containing Fe concentrations ranging from 20 to 60 ppm. If calculated similarly to Cu, this would suggest that the levels of Fe found in the mopane caterpillar bodies would be equivalent to 0.19 ppm in a person of average weight, and caterpillars from on and around PMC therefore contain less Fe than the recommended daily allowance.

Aluminium toxicity in plants has been noted at concentrations ranging from 0.1 to 30 ppm (Markert 1992), suggesting that the Al concentrations of mopane leaves at Rev 1 (54.2 ppm), Rev 2

(47.5 ppm), Rev 3 (39.6 ppm) and Con 1 (31 ppm) may possibly be toxic to the plants. Again however the plants looked healthy, which indicates that they are able to tolerate these Al concentrations or eliminate any excess Al. In humans, 1 ppm.day⁻¹ has been suggested as the minimal risk level for oral exposure of an intermediate duration (ATSDR 2011, 2014). For an individual weighing 60 kg, this suggests that the mopane caterpillars from PMC and its surrounds would contain the equivalent of 0.02 ppm.day⁻¹ of Al, which would not exceed the recommended daily allowance of Al for humans.

Other elements found in the mopane leaf and mopane caterpillar samples that are of importance, and need consideration in this study are As, Cd, Cr, Hg, Ni, Pb and Zn, as these elements, along with Cu, are all heavy metals (Sheoran and Sheoran 2006). The concentrations of all these elements within the mopane leaves fall within or below the normal levels for plants (Markert 1992), suggesting that none of these heavy metals pose any risk to the mopane trees on and around PMC. Campbell (1926) showed that 0.00001 ppm of As was fatal to silkworms. Assuming each caterpillar weighs 5 g, the most As contained in the mopane caterpillars was equivalent to approximately 0.002 ppm. This suggests that if mopane caterpillars are as susceptible to As as the silkworms, they contain harmful concentrations of As. The caterpillars are however able to survive despite these levels of As, so these levels of As are probably not harmful to them. Ilijin *et al.* (2010) indicate that concentrations of Cd exceeding 100 µg reduced fitness components in the gypsy moth (*Lymantria dispar*). The mopane caterpillars contained a maximum of 0.05 µg of Cd, assuming a caterpillar to weigh 5 g (dry weight). If mopane caterpillars are similarly susceptible to Cd, this would suggest that the mopane caterpillars did not contain harmful levels of Cd.

Concentrations of U found in the mopane leaves (0.003 ppm to 0.086 ppm) fall above the normal plant range (0.005 ppm to 0.06 ppm) only for those leaves sampled from Rev 1 (0.063 ppm) and Rev 2 (0.086 ppm), while other leaves sampled fall within the normal range. This could suggest that mopane leaves from Rev 1 and Rev 2 contained concentrations of U that may cause harm to these plants. These plants were however able to survive and appeared to be healthy, suggesting that they are able to tolerate these concentrations of U or they are able to eliminate excess concentrations.

Despite the levels of elemental concentrations at the sites where mopane caterpillars were found, the caterpillars are able to persist as the mopane moths and caterpillars continue to be observed each season. The concentrations of the 25 elements measured in the mopane caterpillars therefore appear not to pose any immediate detrimental effect to the caterpillars, if any at all. At most of the sites sampled, the elemental concentrations found have thus far not shown any adverse effects on mopane trees and mopane caterpillars. The concentrations of elements in mopane caterpillars also do not exceed the recommended daily allowances suggested for humans for the elements measured.

Rev 1 and Rev 2 however, despite having safe concentrations of many heavy metals and most other elements, did have the highest concentration of more than 50 % of the elements analysed, which indicates that these two sites are more contaminated than the other sites. These two sites also appear to be unable to support the mopane caterpillar's life cycle. In order to test this, caterpillars could be introduced to this site to see if they can survive at these two sites. If they can survive, and if the species is able to persist for several generations, then the elemental concentrations at these sites could be ruled out as the reason why the caterpillars were not found at these sites during data collection.

As no caterpillars were found at Rev 1 and Rev 2, the threat of people consuming caterpillars containing potentially harmful concentrations of elements is unlikely. As the concentrations of elements are higher at the reverberator sites when compared to other sites in many cases, harvesting of plants or animals, and access by animals to this region should be limited. This may prevent possible harmful effects of these elements travelling further than these two reverberator sites.

Additional methods of gaseous waste disposal at the reverberator sites could perhaps be made in an attempt to reduce the amount of dust released into the environment. One such addition could be the introduction of secondary hoods to trap fugitive gas emissions and particulate matter, or the addition of further hoods if secondary hoods are already present. This could help to reduce the quantities of gases and particulate matter that are released into the environment. If this is done, mopane caterpillars may have increased chances of surviving at these sites. To better assess the effects elemental concentrations may have on mopane caterpillars however, assessments of mopane

caterpillar growth was required. This was assessed by examining the food consumed by the caterpillars and by examining morphological features of the caterpillars themselves, and is addressed in Chapters 3 and 4.

Chapter 3

Assessing carbon and nitrogen isotopes in mopane caterpillars (*Imbrasia belina*) and mopane leaves (*Colophospermum mopane*) on and around a copper mine

Abstract

The movement of elements through the environment has been studied for many years, but a method for tracking these movements that is still in its infancy is that of stable isotope analysis. Two elements that ecologists typically focus on with stable isotope analyses are carbon and nitrogen, as they can provide us with information on the diet and trophic structure of an organism's food web. The purpose of this study was to measure the carbon and nitrogen isotopic content of mopane leaves

(*Colophospermum mopane*) and mopane caterpillars (*Imbrasia belina*) that were collected on and around the Palabora Mining Company, Limpopo Province, South Africa. This could provide information on whether or not elemental concentrations at these sites may be affecting the carbon and nitrogen content of leaves and subsequently caterpillar growth which is used as a proxy for food quality. If the isotopic compositions of leaves differed greatly between sites, elemental concentrations may have been affecting the carbon and nitrogen isotopic composition of the leaves, which could in turn affect how much carbon and nitrogen the caterpillars ingest, and eventually affect the growth of the caterpillars. Comparisons of carbon and nitrogen isotopic compositions were made between mopane leaves and mopane caterpillars, between mopane caterpillars of different ages, and between mopane caterpillar bodies and caterpillar gut contents. Comparisons of carbon and nitrogen isotopic signatures were also made between sites for all sample types. Although the $\delta^{15}\text{N}$ content of the mopane caterpillars was higher than that of the mopane leaves as would be expected, the $\delta^{15}\text{N}$ content of the caterpillars, particularly the oldest caterpillars (fifth instar), was not 2-4 ‰ higher than that of the mopane leaves as would be expected with an increase in trophic level, but less than 2 ‰ higher than the $\delta^{15}\text{N}$ of the leaves. Older mopane caterpillars may thus be able to assimilate nitrogen better than younger mopane caterpillars. These data also demonstrated that elemental concentrations do not appear to be affecting the carbon and nitrogen content of leaves, as there was little variation in

isotopes between sites, so there is unlikely to be an effect of food quality on caterpillar growth as the leaves from all sites had similar carbon and nitrogen compositions.

Introduction

Introduction to stable isotopes

Ecosystems are complex and involve many interactions between organisms and their environment, including both biotic and abiotic factors. The working of the biosphere, and in particular the movement of elements throughout the biosphere, has received much attention. A useful way to monitor the movements of elements through the environment is with the use of stable isotopes (Fry 2006).

Isotopes refer to multiple forms of an element, where each form has a different number of neutrons in the nucleus (Rubenstein and Hobson 2004; Fry 2006). Stable isotopes refer to those isotopes that are not radioactive and do not decay, and it is these isotopes that many scientists use to understand biogeochemical processes (Peterson and Fry 1987). One of the main ways that ecologists use isotopes to monitor the movements of elements through an ecosystem is through the use of isotope tracers (Fry 2006). Isotopes of an element occur naturally at different abundances in various regions of the biosphere (Dawson *et al.* 2002; Rubenstein and Hobson 2004; Fry 2006). The process of isotopic tracing involves adding a known amount of certain isotopes to a natural system, and periodically measuring the abundance of the various isotopes of that element throughout the system to which the tracer was added.

The way in which the abundance of the isotopes is measured is through the use of a mass spectrometer (Peterson and Fry 1987; Rubenstein and Hobson 2004). The results of the mass spectrometry are typically reported as a delta (δ) value, which represents a measurement of the difference between the sample and the standard against which each sample is measured (Bender 1971; Handley and Raven 1992; Fogel and Cifuentes 1993; Roth and Hobson 2000; Evans 2001; Cerling *et al.* 2006). As a result of the differences between the samples and the standards typically being very small, the calculation of the δ value involves a multiplication by 1 000 (Evans 2001; Dawson *et al.*

2002; Fry 2006). The δ value thus signifies a “parts per thousand” measure and this is represented as “‰”, “per mil” or “permil” (Cerling *et al.* 2003; Rubenstein and Hobson 2004; Cerling *et al.* 2006).

The calculation of the δ value incorporates the relative proportions of the heavy and light isotopes of the element being considered in both the sample and the standard (Handley and Raven 1992; Fogel and Cifuentes 1993; Cerling *et al.* 2003). The higher or the more positive the δ value, the greater the amount of the heavy isotope present in the sample, while the lower or more negative the δ value, the lower the amount of the heavy isotope present in the sample (Fogel and Cifuentes 1993; Dawson *et al.* 2002; Fry 2006). A standard would have a δ value of 0 ‰ as the δ value is calculated by comparing a sample to the standard, so in this case comparing the standard to itself, so the difference is zero (Handley and Raven 1992; Fry 2006).

Uses of stable isotopes in ecology

The elements most commonly used in stable isotope research, which are also the main elements involved in most of the elemental cycles that ecologists are most interested in, are hydrogen, oxygen, sulphur, carbon and nitrogen. Two of these elements which are of particular interest to scientists studying food webs are carbon and nitrogen. Carbon isotope analyses can assist scientists in identifying what sources of food contribute to the diet of organisms (Cerling *et al.* 2003; Cerling *et al.* 2006; Fry 2006). Carbon isotopes are particularly useful in identifying what plants contribute to an organism’s diet because C₃, C₄, and CAM plants typically have different carbon isotope compositions (Cerling *et al.* 2003; Cerling *et al.* 2006; Fry 2006). In addition to this, carbon isotope analyses, along with oxygen isotope analysis, can also further our understanding of the movement of carbon (specifically carbon dioxide) throughout the biosphere which is very important in light of our ever-changing climate (Ciais *et al.* 1995; Rayner *et al.* 1999; Fry 2006).

Nitrogen isotope analyses can provide us with some insight into the cycling of nitrogen in the environment (Fry 2006). As nitrogen often limits plant productivity in terrestrial systems, nitrogen isotope analyses can be helpful in identifying how the nitrogen isotopes travel through plants including how nitrogen is gained and lost by plants (as well as the isotopic signatures of these gains

and losses) (Evans 2001; Fry 2006). Nitrogen isotope analyses are also very useful as they can be used to indicate where organisms lie within the trophic web, with 2-4 ‰ $\delta^{15}\text{N}$ enrichment expected at each successive trophic level (Minagawa and Wada 1984; Peterson *et al.* 1985; Robinson 2001; Dawson *et al.* 2002; Post 2002; McCutchan *et al.* 2003; Fry 2006).

An organism considered an important part of the trophic web in the region where it occurs is the mopane caterpillar, the larval stage of the Anomalous Emperor Moth (*Imbrasia belina* [Westwood 1849] (Lepidoptera: Saturniidae)) (Banjo *et al.* 2006; Hrabar 2006; Akpalu *et al.* 2007). This species has been shown to have formed at least 70 trophic links, with various stages of the species' life cycle being consumed by birds, mammals and other invertebrates (Styles 1995; Styles and Skinner 1996; Gaston *et al.* 1997; Hrabar 2006). Mopane caterpillars are also an important source of food and income to many people who live in regions where the caterpillars are present (Frears *et al.* 1997; Barany *et al.* 2001; Akpalu *et al.* 2007; Ghaly 2009). Not only are the caterpillars a cheap and rich source of protein for those people living in and around areas where the caterpillars occur, but the caterpillars are also considered a delicacy in many places, so the caterpillars are often sold or bartered (Dreyer 1968; Dithlogo 1996; Frears *et al.* 1997; Stack *et al.* 2003; Banjo *et al.* 2006; Akpalu *et al.* 2007; Dube and Dube 2010; Gondo *et al.* 2010).

These mopane caterpillars subsist on a number of plants but their primary food source is the mopane tree (*Colophospermum mopane*) (Pinhey 1972; Dithlogo 1996; Gaston *et al.* 1997; Illgner and Nel 2000; Mpuchane *et al.* 2000; Picker *et al.* 2004). Mopane caterpillars have been known to completely defoliate mopane trees during periods of large caterpillar populations (Frears *et al.* 1997; Gaston *et al.* 1997; Frears *et al.* 1999; Picker *et al.* 2004; Hrabar 2006). Following such defoliation, mopane trees sometimes produce compensatory growth, which has been found to be nutritious and contain a lower tannin and total phenol content than older mopane leaves (Styles and Skinner 1997; Hrabar 2006).

One region of South Africa where both mopane caterpillars and mopane trees co-exist is in abundance is in Limpopo Province, South Africa (Picker *et al.* 2004; Rutherford *et al.* 2006a). This

region is also well known for its many mining operations, and includes one of the world's largest mineral repositories, the Phalaborwa Complex, mined by the Palabora Mining Company (PMC) and Foskor (Council for Geoscience 2011; PMC 2012). The Palabora Mining Company shares a border with Kruger National Park, and people are often allowed to harvest mopane caterpillars from both these areas during the caterpillars' growth period.

Aims and objectives

As mopane caterpillars are valued by people in this region as well as being a pivotal part of the food web in the Phalaborwa region, it is important to investigate how the elemental concentrations from PMC might be affecting growth of the caterpillars. One factor that could provide this information would be the carbon and nitrogen content of the caterpillars' food which can be used as a proxy for the quality of the food. This information may then be able to provide some insight into the caterpillars' growth. The purpose of this aspect of study was to measure the carbon and nitrogen isotopes of both mopane caterpillars and mopane trees (specifically the leaves) in the region. This was done to gain some insight into the trophic interaction between the mopane caterpillars and mopane leaves at an isotopic level. It also provided a means of establishing if elemental concentrations may be affecting leaf nutrition and subsequently caterpillar growth. The first objective was to assess if information regarding the species' relative trophic levels could be discerned using nitrogen and carbon isotopes, in order to establish if the usual isotopic pattern holds for mopane caterpillars. Secondly we wanted to ascertain if there were any differences in carbon and nitrogen isotopes between mature mopane leaves and the compensatory growth to ascertain if there was any difference in the food quality of the two types of leaves. These sample types were all compared between the various study sites on and around PMC and Limpopo Province to establish if relative elemental concentrations at each of the study sites may have affected the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ compositions of the mopane leaves.

Materials and Methods

Experimental design

Where present, mopane caterpillars and mature mopane leaves were collected from 10 mopane trees at each site. Five caterpillar bodies (MB) and five clusters of mature mopane leaves (LM) were collected from each tree. Where possible, five individuals of each of the five mopane instars were collected from each tree, but most trees yielded only one or two instars of mopane caterpillars. Mopane leaf and mopane caterpillar body samples from each of two to four trees per site were selected to be analysed for carbon and nitrogen isotopes. At one of the sites, Rev 3, where all mopane caterpillar instars were collected, five bodies of each instar were also measured for carbon and nitrogen isotopes. In addition to this, a further two fifth instar caterpillars' bodies (MB-5*) and gut contents (MG-5*), from the same tree at Rev 3, were separately analysed for both nitrogen and carbon isotopes.

As the compensatory growth (LC) on mopane trees is quite small in area, only enough material for a single sample was available for each of the three trees from which compensatory growth was collected. These compensatory growth samples were also analysed for carbon and nitrogen isotopes. The samples selected were all freeze dried and then ground using a ceramic pestle and mortar. The ground samples were then sent to IsoEnvironmental cc in the Department of Botany at Rhodes University, Eastern Cape Province, South Africa, where the carbon and nitrogen isotopes of the samples were analysed using a mass spectrometer.

Data analysis

Descriptive statistics were performed using Microsoft Excel (2007). Means and standard errors of the carbon and nitrogen compositions were calculated for mature mopane leaves (LM), mopane leaf compensatory growth (LC), mopane caterpillar bodies (MB) (for each instar) and mopane caterpillar gut contents (MG) per tree selected. Spearman Rank Correlation tests were used to measure the relationship between the concentrations of each of the 25 elements measured in Chapter 2, and $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ independently to ascertain if any of these elements may be affecting the carbon or nitrogen

content of the leaves. Further statistical analyses were not conducted on the carbon and nitrogen isotope data owing to the very small sample sizes available here.

Results

Mature mopane leaves from Rev 1 had higher $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ ($\delta^{13}\text{C}$: -25.21 ± 0.28 ; $\delta^{15}\text{N}$: 1.01 ± 0.18) values than the compensatory growth ($\delta^{13}\text{C}$: -26.46 ± 0.13 ; $\delta^{15}\text{N}$: 0.57 ± 0.32) from Rev 1 (Figures 3.1 and 3.2). This indicates that the mature mopane leaves contained more of the heavy isotopes of carbon and nitrogen respectively than the compensatory growth. Similarly the mature mopane leaves from Rev 2 also had higher $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ ($\delta^{13}\text{C}$: -25.81 ± 0.10 ; $\delta^{15}\text{N}$: 0.49 ± 0.26) values than the compensatory growth ($\delta^{13}\text{C}$: -26.00 ; $\delta^{15}\text{N}$: 0.15) from Rev 2 (Figures 3.1 and 3.2).

All the mopane caterpillar bodies and gut contents from Rev 3 had higher $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values than the mature mopane leaves (Figure 3.2). For instars 1 to 3 of Rev 3, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were higher and lower respectively between successive instars (Figure 3.2). The $\delta^{13}\text{C}$ values were lower in instar 4 caterpillar bodies ($\delta^{13}\text{C}$: -23.86 ± 0.04) than in instar 3 caterpillar bodies ($\delta^{13}\text{C}$: -23.53 ± 0.07), and lower still in instar 5 caterpillar bodies ($\delta^{13}\text{C}$: -24.09 ± 0.11) when compared to instar 4 caterpillar bodies ($\delta^{13}\text{C}$: -23.86 ± 0.04) (Figure 3.2). Values of $\delta^{15}\text{N}$ in caterpillar bodies however were higher in instar 4 caterpillar bodies ($\delta^{15}\text{N}$: 2.66 ± 0.17) than in instar 3 caterpillar bodies ($\delta^{15}\text{N}$: 2.62 ± 0.03), but were again lower in instar 5 caterpillar bodies ($\delta^{15}\text{N}$: 2.51 ± 0.15) when compared to instar 4 caterpillar bodies ($\delta^{15}\text{N}$: 2.66 ± 0.17) (Figure 3.2).

Two additional fifth instar mopane caterpillars had both their bodies and gut contents analysed separately (indicated by MB-5* and MG-5* respectively; Figure 3.2). For these two caterpillars, the $\delta^{13}\text{C}$ values of the caterpillar gut contents ($\delta^{13}\text{C}$: -24.83 ± 0.04) were lower than those of the caterpillar bodies ($\delta^{13}\text{C}$: -24.06 ± 0.05), indicating that the caterpillar gut content contained less of the heavy isotope of carbon than the caterpillar bodies. Conversely however, the $\delta^{15}\text{N}$ values of the caterpillar gut contents ($\delta^{15}\text{N}$: 3.30 ± 0.01) were higher than those of the caterpillar bodies ($\delta^{15}\text{N}$: 2.59 ± 0.01), indicating that the caterpillar gut contents contained more of the heavy isotope of nitrogen than the corresponding caterpillar bodies (Figure 3.2).

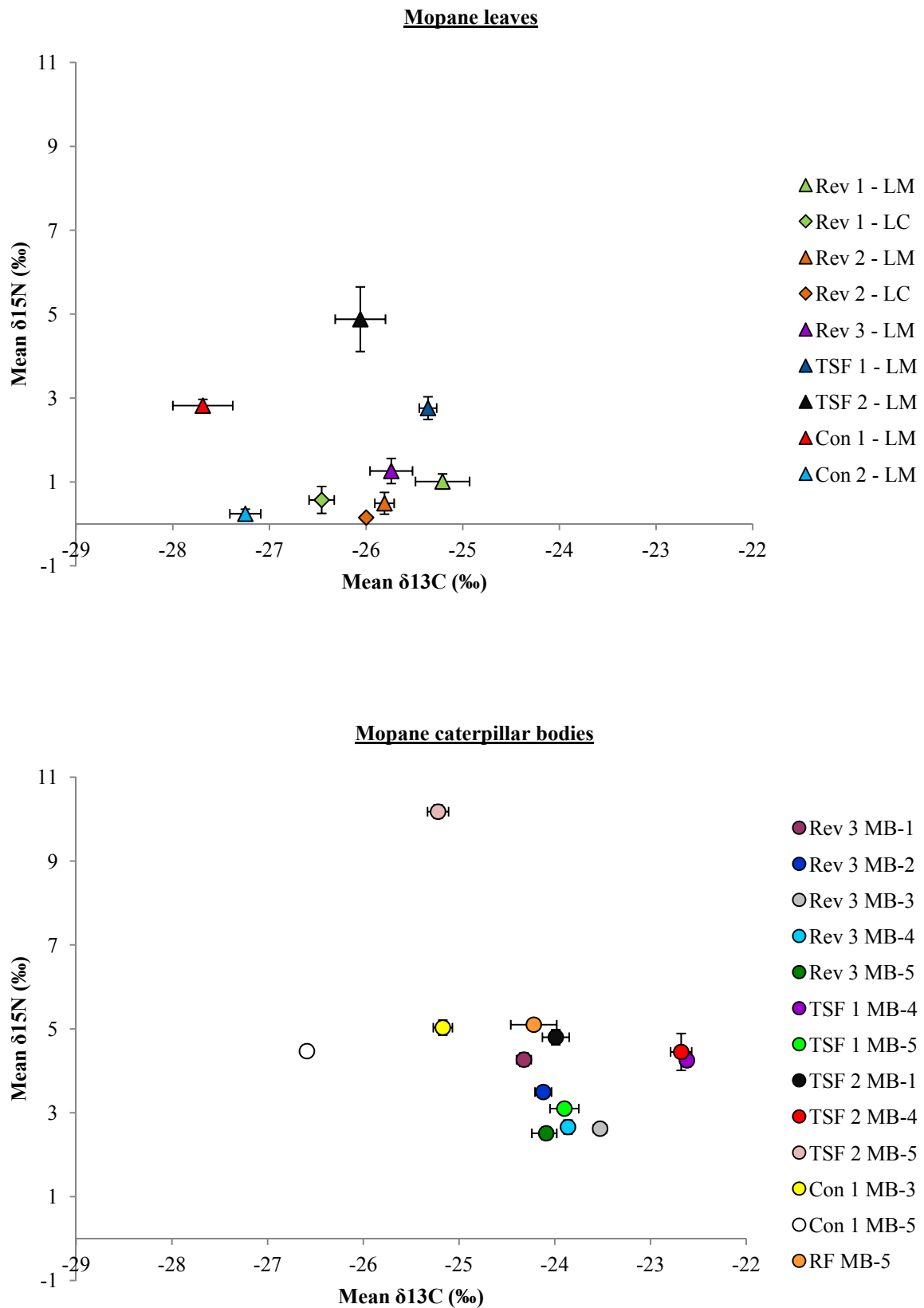
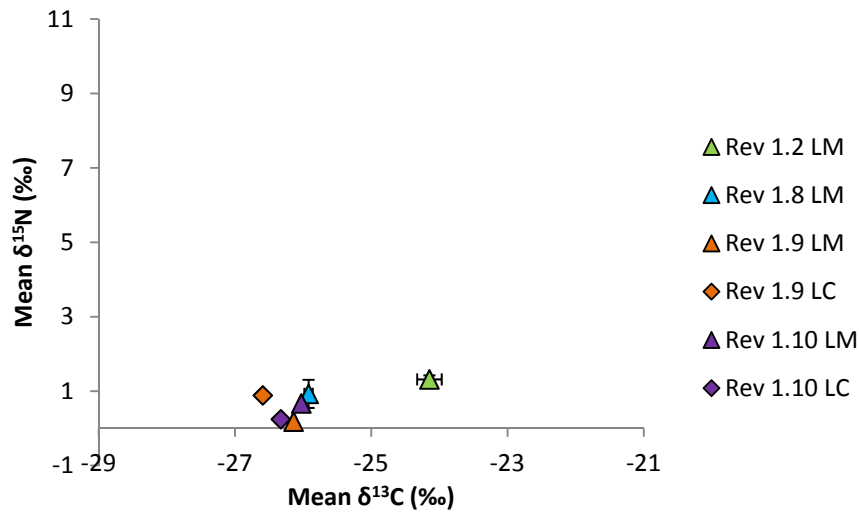
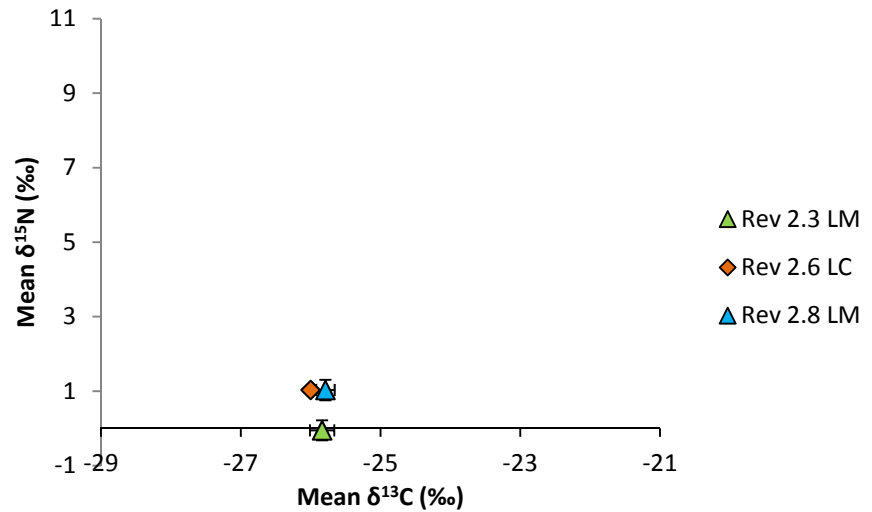


Figure 3.1. The effect of site position on mean (\pm standard error) stable isotope measures of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of mature mopane leaves (LM) (triangles), mopane leaf compensatory growth (LC) (diamonds) and mopane caterpillar bodies (MB) (circles) from eight sites in Limpopo Province, South Africa. The number following “MB” indicates the caterpillar instar.

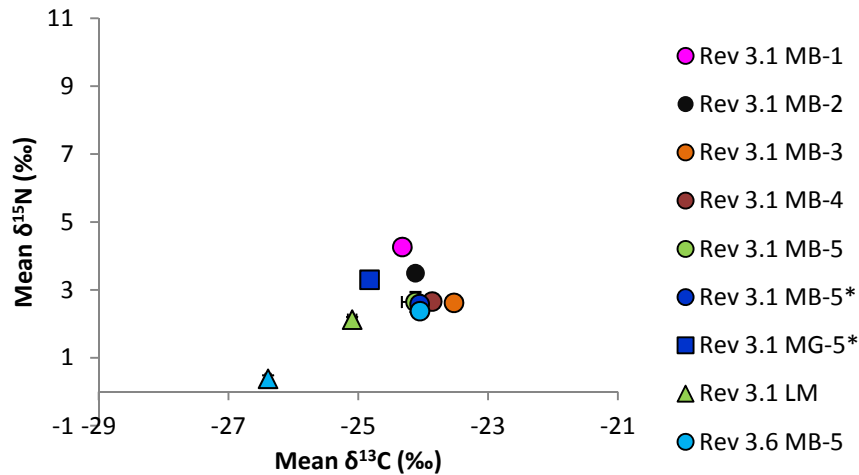
Reverberator site 1



Reverberator site 2



Reverberator site 3



Tailings storage facility site 1

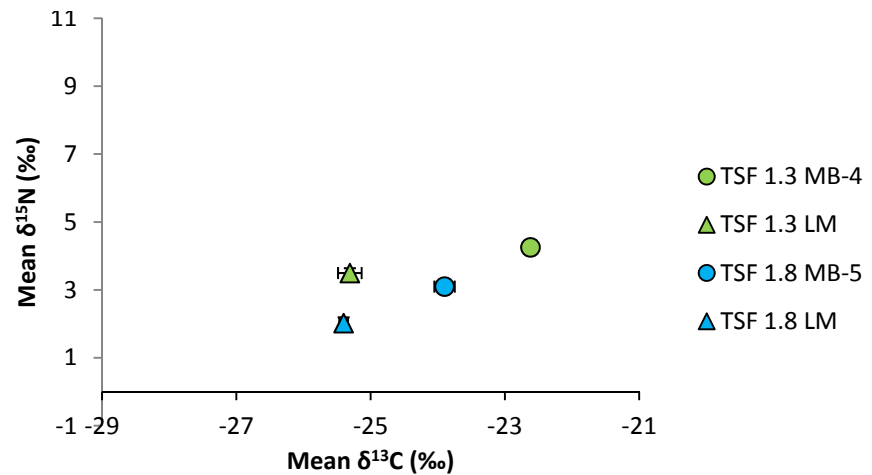


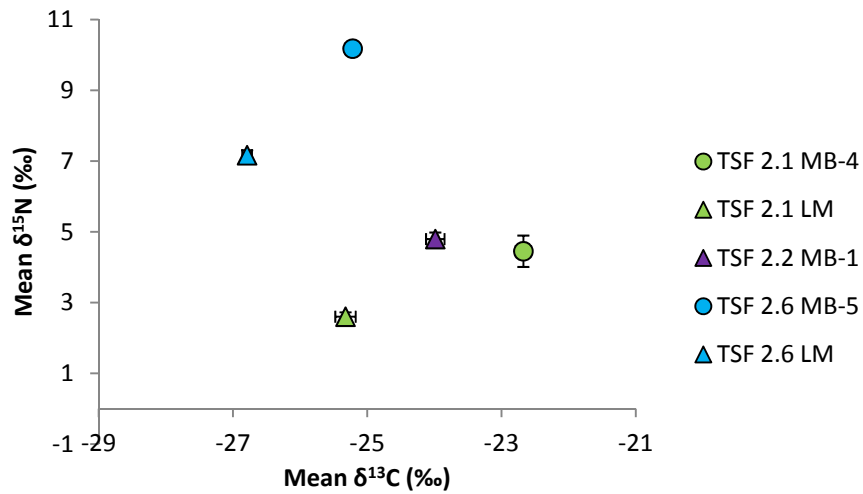
Figure 3.2. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ (mean \pm standard error) of mature mopane leaves (LM) (triangles), mopane leaf compensatory growth (LC) (diamonds), mopane caterpillar bodies (MB) (circles) and mopane caterpillar guts (MG) (squares) from four sites in Limpopo Province, South Africa. Numbers following site in the legend indicate tree number, while the number following “MB” indicates the caterpillar instar. The “*” indicates caterpillar bodies and guts from the same individuals.

At TSF 1, both the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the caterpillar bodies were higher than the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the mature mopane leaves from this site (Figure 3.2). The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of the first ($\delta^{13}\text{C}$: -23.99 ± 0.14 ; $\delta^{15}\text{N}$: 4.80 ± 0.18) and fourth ($\delta^{13}\text{C}$: -22.68 ± 0.11 ; $\delta^{15}\text{N}$: 4.45 ± 0.44) instars mopane caterpillar bodies from TSF 2 were higher than one of the trees sampled ($\delta^{13}\text{C}$: -25.33 ± 0.15 ; $\delta^{15}\text{N}$: 2.60 ± 0.12) but lower than that of the second tree sampled ($\delta^{13}\text{C}$: -26.79 ± 0.07 ; $\delta^{15}\text{N}$: 7.17 ± 0.14), which had much higher $\delta^{15}\text{N}$ values than the other mopane leaves from this site (Figure 3.3). The $\delta^{15}\text{N}$ content of this second tree, tree 6, was much higher than that of the other mopane leaves from this site. The mopane caterpillars sampled from this tree did however have higher $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values than the leaves of the tree from which they were collected (Figure 3.3). This shows that despite changes in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in tree 6 of TSF 2, the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ content of the mopane caterpillars closely resembles that of the mopane leaves. The $\delta^{15}\text{N}$ composition of both the mature mopane leaves and the mopane caterpillar bodies from tree 6 of TSF 2 were approximately double that of any other mopane leaves and mopane caterpillar bodies respectively from all sites sampled (Figure 3.1).

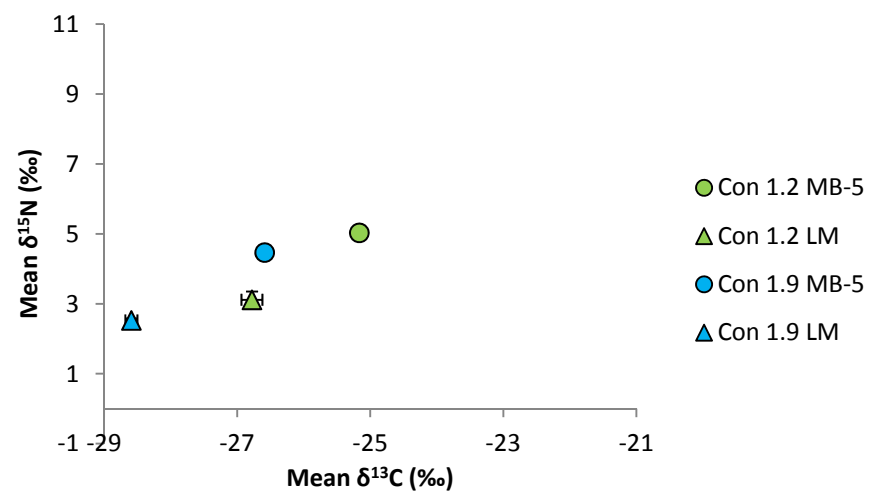
At Con 1, the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the mopane caterpillar bodies were higher than those of the mature mopane leaves (Figure 3.2). Con 1 also had mopane leaves with the lowest $\delta^{13}\text{C}$ value of all the sites ($\delta^{13}\text{C}$: -27.69 ± 0.31) (Figure 3.1). The mature mopane leaves from Control site 2 had the second lowest $\delta^{13}\text{C}$ values ($\delta^{13}\text{C}$: -27.25 ± 0.16) and the lowest $\delta^{15}\text{N}$ values ($\delta^{15}\text{N}$: 0.24 ± 0.11) of all the sites (Figure 3.1). The mopane caterpillar bodies from Runde Farm had the highest $\delta^{15}\text{N}$ values ($\delta^{15}\text{N}$: 5.10 ± 0.11) apart from those mopane caterpillars from tree 6 of TSF 2 which had much higher $\delta^{15}\text{N}$ compositions.

The mopane leaves across all the sites, ranged on average from -28 ‰ to -25 ‰ for $\delta^{13}\text{C}$ and 0 ‰ to 3 ‰ for $\delta^{15}\text{N}$ (Figure 3.1) with the exception of the outlying tree from TSF 2. The mopane caterpillar bodies from all sites however ranged on average from -27 ‰ to -22 ‰ for $\delta^{13}\text{C}$ and 2 ‰ to 6 ‰ for $\delta^{15}\text{N}$, again with the exception of the fifth instar caterpillars from the outlying tree of TSF 2 (Figure 3.3). The $\delta^{13}\text{C}$ values of the mature mopane leaves were similar across most of the sites, but were lowest at the two control sites (Figure 3.1). The $\delta^{15}\text{N}$ values of the mature mopane leaves were

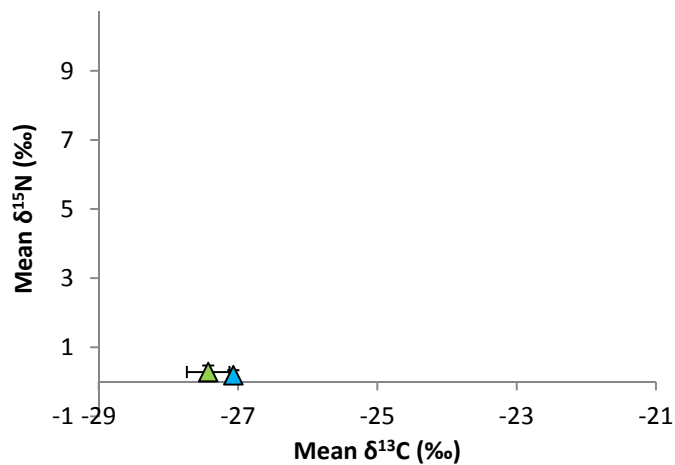
Tailings storage facility site 2



Control site 1



Control site 2



Runde Farm

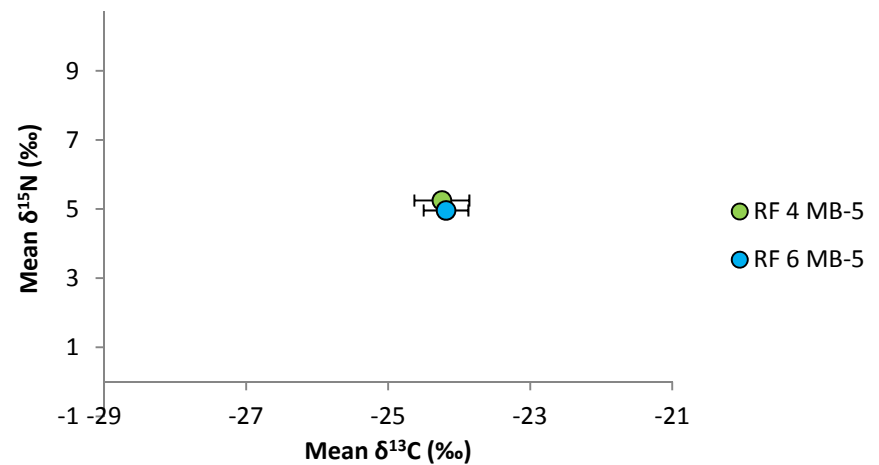


Figure 3.3. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ (mean \pm standard error) of mature mopane leaves (LM) (triangles), mopane caterpillar bodies (MB) (circles) and mopane caterpillar guts (MG) (squares) from four sites in Limpopo Province, South Africa. Numbers following site in the legend indicate tree number, while the number following “MB” indicates the caterpillar instar.

lowest at Con 2, and higher $\delta^{15}\text{N}$ at the reverberator sites, TSF 1, Con 1 and TSF 2 respectively (Figure 3.1). Similar to the mature mopane leaves, the mopane caterpillar bodies from Con 1 had the lowest $\delta^{13}\text{C}$ values, along with the caterpillars from tree 6 of TSF 2, while those from the other sites were quite similar to each other (Figure 3.1). The $\delta^{15}\text{N}$ values however of the mopane caterpillar bodies were highest at RF, followed closely by Con 1 (Figure 3.1). Although the carbon and nitrogen isotopes of the caterpillars were closely linked to the mopane leaves (Figures 3.2 and 3.3), the relative carbon and nitrogen compositions of each site did not follow the same trend for mopane leaves as was found for mopane caterpillars across the sites.

Of all the elements sampled in the mopane leaves in Chapter 2, only two elements were found to be correlated with the $\delta^{13}\text{C}$ signatures of the leaves from those sites, while no elements in the mopane leaves were correlated with the $\delta^{15}\text{N}$ signatures of the mopane leaves. The $\delta^{13}\text{C}$ compositions of the mopane leaves were found to be positively correlated with Co ($r = 0.79$; $n = 8$; $P = 0.048$) and negatively correlated with Zn ($r = -0.79$; $n = 8$; $P = 0.048$).

Discussion

Overall the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ compositions of mopane caterpillar bodies were higher than their diet, mature mopane leaves, as was expected. The $\delta^{15}\text{N}$ composition of the caterpillars relative to the mopane leaf values were however less than the 2 ‰ to 4 ‰ increase in $\delta^{15}\text{N}$ expected of a consumer's $\delta^{15}\text{N}$ relative to its diet (Peterson *et al.* 1985; Robinson 2001; Dawson *et al.* 2002; Post 2002; McCutchan *et al.* 2003; Fry 2006). Mopane caterpillars are however primary consumers, and McCutchan *et al.* (2003) found that smaller differences in $\delta^{15}\text{N}$ between consumer and diet will be found in primary consumers versus organisms consuming high protein diets. Fry (2006) says that small differences in $\delta^{15}\text{N}$ between consumers and their diet could indicate that those organisms are able to assimilate nitrogen more efficiently than other organisms. Behar *et al.* (2005) found that the fruit fly (*Ceratitis capitata*) contained enterobacteria which mediated nitrogen fixation, and suggest that nitrogen fixation may also occur in other insects. If this is the case with mopane caterpillars, the

older mopane caterpillars may contain greater amounts of bacteria as a result of the larger size of the caterpillar, allowing older caterpillars to assimilate more $\delta^{15}\text{N}$ than younger caterpillars.

The caterpillar bodies had different isotopic compositions to the caterpillars gut contents. The gut contents of the caterpillars showing higher $\delta^{15}\text{N}$ values than the mature mopane leaves suggest that the nitrogen from the leaves is processed in the gut of the caterpillars, or the gut contents would have displayed a similar isotopic composition to the leaves themselves. As seen in plants and other animals, the constituent organs of organisms usually have different isotopic signatures, which is due to elements being metabolised and incorporated at different rates in different tissues (Tieszen *et al.* 1983; Roth and Hobson 2000; Evans 2001, Dawson *et al.* 2002; Gratton and Forbes 2006). This could be the reason for the difference in $\delta^{15}\text{N}$ between the leaves and the caterpillars being smaller than expected, as whole caterpillars were not analysed.

Both the mature mopane leaves and compensatory growth had $\delta^{13}\text{C}$ values within the typical range of $\delta^{13}\text{C}$ for C_3 plants (-30 ‰ to -20 ‰) (Bender 1971; Fogel and Cifuentes 1993; Fry 2006). The $\delta^{13}\text{C}$ values of the caterpillars did not show a consistent pattern relative to the mopane leaves on the trees from which they were collected, as was found by Hobson *et al.* (1999) when examining monarch butterflies (*Danaus plexippus*) in relation to their diet. The $\delta^{15}\text{N}$ values calculated for both the mature mopane leaves and compensatory growth ranged mostly between -1 ‰ and 4 ‰, well within the usual range for plant $\delta^{15}\text{N}$ (-10 ‰ to 10 ‰) (Evans 2001). Outliers in terms of mopane leaf $\delta^{15}\text{N}$ were however calculated for the mature mopane leaves from tree 6 of TSF 2. These leaves had $\delta^{15}\text{N}$ values of around 7.5 ‰, which were higher than the rest of the mopane leaves, and were closer to the upper end of the usual $\delta^{15}\text{N}$ range.

Craine *et al.* (2009) found foliar $\delta^{15}\text{N}$ to increase with increasing availability of nitrogen. The high $\delta^{15}\text{N}$ levels in tree 6 of TSF 2 could have been caused by that tree being exposed to more nitrogen than the trees in the surrounding area. This tree was also in a separate cluster of mopane trees that were a short distance away from trees 1 and 2, and different factors at work in each of these two areas, such as differing geology and fauna, may have resulted in the difference in nitrogen content

between tree 6 and the other trees from TSF 2. Of all the sites examined, the tailings storage facility sites had some of the highest $\delta^{15}\text{N}$ levels. A possible reason for this could be that nitrogen from the explosives used in the mining operations at PMC may be reaching the tailings storage facility, and this high $\delta^{15}\text{N}$ content may then be filtering out to the plants and soil surrounding the tailings storage facility.

Compensatory leaf growth of the mopane trees had $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ patterns on the lower end of the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ ranges seen for all the mopane leaves analysed. This indicates that there were less of the heavy isotopes of both carbon and nitrogen in these samples. This suggests that lighter isotopes of both carbon and nitrogen are deposited in compensatory leaf growth. Additional carbon and nitrogen analyses of compensatory growth should be conducted using a bigger sample size. The isotopic composition of compensatory growth should also be compared to that of mature mopane leaves from the same tree.

Based on this study, the expected difference in nitrogen isotopes between trophic levels, in this case between the mopane leaves and mopane caterpillars, was not found. This may indicate that different patterns of nitrogen isotope increases may be found in invertebrate herbivores than were found in other groups, as shown by Zanden and Rasmussen (2001) and McCutchan *et al.* (2003). It was thought that the varying levels of contaminants at each site could be affecting the isotopic compositions of the mopane leaves and subsequently the mopane caterpillars in this region. Of the 25 elements analysed however, only Co and Zn concentrations of mopane leaves were found to be correlated with the $\delta^{13}\text{C}$ composition of the leaves. This suggests that elemental concentrations are not greatly affecting plant nutrition, and are thus unlikely to be affecting caterpillar growth. The next step in investigating the potential effects of the elemental concentrations on the caterpillars was to assess the physical growth of the caterpillars by looking at morphological features of the caterpillars.

Chapter 4

Headwidth and mandible wear as a tool to age mopane caterpillars

(Imbrasia belina)

Abstract

As caterpillars grow, their bodies expand and their mandibles need to grow proportionally in order to maintain a certain rate of food intake. As a caterpillar's head capsule is rigid and cannot expand as its body does, the only way for a caterpillar to increase the size of its head capsule, and thus of the mandibles, is by moulting. This increase in head capsule can thus be used as an indication of the age of an individual caterpillar of a particular species. As caterpillars grow in size and wear down their mandibles when they feed at each instar, body mass and mandible wear could be used as tools to further age the caterpillars within an instar. The purpose of this study was to measure the headwidth, body mass and mandible wear of mopane caterpillars (*Imbrasia belina*) collected from Limpopo Province, South Africa. This information would provide identifying morphological characteristics of each instar which could be used to assess caterpillar growth, and compare this growth between sites. Headwidths would be able to indicate the instar of an individual while the body mass and mandible wear were assessed to see which of these could provide information regarding the age of a caterpillar within an instar. Headwidths and body mass of caterpillars were also compared between different sources and intensities of contamination. This was done to ascertain if elemental contamination from a mine may be affecting caterpillar growth. Five clusters of headwidth measurements were found for the mopane caterpillars, and these correspond to the five instars of mopane caterpillars. Headwidth was found to be fairly constant between sites while body mass was very variable, which suggests that elemental concentrations had little or no effect on the growth and body size of caterpillars. Mandible wear was correlated with body mass, suggesting that within an instar, heavier individuals are more likely to be older. Mandible wear however was concluded to be the more reliable means of aging caterpillars within an instar.

Introduction

Mopane Caterpillars

Mopane caterpillars, commonly referred to as mopane worms, are the larval stage of the Anomalous Emperor moth or Mopane moth (*Imbrasia belina* [Westwood 1849] (Lepidoptera: Saturniidae)) (Ditlhogo 1996). The species is widespread throughout central and Southern Africa and occurs in abundance in Namibia, Zimbabwe, Botswana, Zambia, Angola and South Africa (Picker *et al.* 2004; Banjo *et al.* 2006; Akpalu *et al.* 2007; Gondo *et al.* 2010). Although the name mopane worm is derived from the caterpillars' tendency to defoliate their primary host, the mopane tree (*Colophospermum mopane*) (Gaston *et al.* 1997; Mpuchane *et al.* 2000), mopane caterpillars also feed on other plant species such as *Carissa macrocarpa*, *Diospyros* spp., *Ficus* spp., *Searsia* spp., *Sclerocarya* spp., *Commiphora glandulosa*, *Acacia tortilis*, *Cassia abbreviata*, *Terminalia* spp. and *Trema orientalis* (Pinhey 1972; Ditlhogo 1996; Illgner and Nel 2000; Picker *et al.* 2004).

Adult mopane moths live for just a few days with the sole purpose of reproducing (Hrabar 2006; Gondo *et al.* 2010). After mating has occurred, female moths lay single clusters of eggs on leaves or around twigs, with a cluster containing 30 to 335 eggs (Hrabar 2006; Akpalu *et al.* 2007; Gondo *et al.* 2010). Approximately 10 days after the eggs have been laid, small brown/black larvae hatch from the eggs and begin their 4-6 week long larval stage, during which time the larvae pass through five larval growth stages, known as instars (Ditlhogo 1996; Klok and Chown 1999). The first two larval stages are usually light brown in colour and the last three stages are black with aposematically coloured scales which are yellow, white and red in colour (although the colouration of the caterpillars does not seem to deter predators) (Gaston *et al.* 1997). During this larval period, mopane caterpillars undergo a rapid increase in body size, with fifth instar caterpillars usually weighing 12-13g and measuring approximately 80mm in length (Frears *et al.* 1999; Hrabar 2006). This immense increase in body size makes it necessary for the caterpillars to moult between instars as chitin is rigid and cannot stretch to accommodate an increase in body size (Merzendorfer and Zimoch 2003; Gondo *et al.* 2010). It has however been suggested that discrete growth is restricted to

sclerotized structures, and that cuticle expansion occurs continuously during the intermoult period, not just before and after moulting (Williams 1980; Nijhout 1998).

At the end of the fifth instar the larvae descend from the host tree and burrow into the ground where they then pupate (Ditlhogo 1996). As mopane moths are bivoltine throughout most of their distribution, there are usually two generations each year, but in some regions the species is univoltine (Hrabar 2006). For bivoltine populations, pupae will remain underground for either six to seven weeks (first generation) or experience a period of diapause where they remain underground for six to seven months (second generation) depending on which generation they are from (Gondo *et al.* 2010). The first emergence of mopane moths during their breeding season occurs between October and December while the second emergence of moths usually occurs between February and April the following year (Frears *et al.* 1997; Gaston *et al.* 1997; Hrabar 2006).

Tools for mopane caterpillar age determination

Headwidths of successive lepidopteran larval stages have been found to follow a regular geometric progression which is said to be roughly constant for all species (Dyar 1890; Hansen *et al.* 1981). Many previous studies on caterpillars have used headwidth measurements as a tool to identify individual larvae to an instar (Hansen *et al.* 1981; Daly 1985; Freitas 1993; Floater 1996; Singtripop *et al.* 1999; Stavridis *et al.* 2003). The head capsule is used as it is rigid and remains constant in size over the course of a single instar (Dyar 1890; Nijhout 1975; Daly 1985). In order to go a step further and identify the age of caterpillars within an instar, an aspect of a caterpillar's morphology that changes within an instar would need to be identified.

The body mass of caterpillars increases within an instar as the caterpillars are continuously eating and growing. Body mass could therefore be one method of ascertaining the age of a caterpillar within an instar. Larvae also moult between instars, primarily to increase the size of the mouthparts as this is what limits food intake in larvae (Nijhout 1981; Hutchinson *et al.* 1997; Etilé and Despland 2008). This is partially because the size of the mouthparts becomes limiting for food intake and reduces the rate of body growth, but also because the mandibles themselves are worn down by the

action of the larvae eating, and can eventually no longer cut, tear or grind food as efficiently (Dockter 1993; de Boer 1995). This is most likely caused by the abrasive action of substances such as silica contained in plant material that act as a physical plant defence and have been shown to cause increased mandible wear (Massey and Hartley 2009; Reynolds *et al.* 2009). As this wearing down of the mandible would occur gradually between moulting events, the level of mandible wear could provide a means for larval aging within an instar.

Aims and objectives

The purpose of this aspect of the study was to examine the morphological characteristics of mopane caterpillars that could be used to age an individual caterpillar and provide some information on the growth of that individual. The first objective was to measure the headwidth and body mass of mopane caterpillars which would provide a means of identifying a caterpillars' instar and a possible means of indentifying a caterpillar's age within an instar. The second objective was to ascertain if there was a difference in these measurements at different sites. If there were differences in these measures between sites, this may indicate that chemicals present in the area, originating from the mine's activities, could be affecting the growth of the caterpillars. The next objective was to assess mandible wear of the caterpillars. As there is existing evidence of mandibular wear in other insect larve, this objective served to establish if this process also occurs in mopane caterpillars. If so, mandible wear can be used as a tool to classify individual mopane caterpillars into the early or late stages within an instar. These three morphological features (headwidth, body mass and mandible wear) of the caterpillars could then be used as a means of comparing caterpillar growth between sites. Such growth comparisons could subsequently give some indication of whether or not chemical contamination from the mine's activities could be affecting caterpillar growth. Being able to more accurately assess the age of an individual caterpillar may also be used to ascertain if caterpillars are bioaccumulating elements or eliminating elements when moulting, as chemicals concentrations could be measured at different ages within an instar.

Materials and Methods

Experimental design

Ten trees were chosen at each site and the GPS coordinates of each tree was noted. From each tree, 5-10 caterpillars of each instar were collected where possible. All caterpillars were frozen in a portable freezer and returned to the laboratory at the University of the Witwatersrand, Johannesburg, where measurements were taken for each individual. Headwidth and body mass (wet weight) measures were taken. Headwidth was measured in millimetres using dial callipers accurate to one decimal place, while body mass, measured in grams, was measured using a Sartorius 2007Mp balance accurate to four decimals.

In order to assess mandible wear, caterpillar heads were first detached from the bodies and the mandibles then dissected out. The mandibles were then examined under a dissecting microscope ((Novel NSZ-606) up to x1000 magnification). An index was created based on the wear to the grinding surface of each mandible, and this index was then used to assign a mandible wear score to each mandible, with possible scores ranging from 1 to 3. A mandible wear score of 1: indicated mandibles with high ridges; 2: mandibles moderately worn with mandible ridges having been worn down slightly but that were still raised and obvious; 3: mandibles had been completely worn down where ridges could no longer be seen, or where only the outline of where ridges had been could be seen.

Data analysis

All statistical analyses were completed using R software (R Development Core Team 2011). Shapiro-Wilk tests showed that the data did not follow a normal distribution and thus non-parametric statistical tests were used. Kmeans cluster analysis (from the package “fBasics” (Wuertz 2010)) was used to sort clusters of headwidths and allocate these to instars. The number of clusters chosen for this analysis was decided upon by considering both the clustering of the data itself, as well as the number of instars indicated by the literature. The average growth ratio of the caterpillars (the average increase in headwidth between successive instars) was calculated by dividing the average headwidth of one

instar (calculated by averaging the headwidths of all the individuals of a particular instar) by that of the previous instar, and then averaging these numbers.

Means and standard errors (from the package “plotrix” (Lemon 2006)) were calculated for both headwidth and body mass per instar and per site. For each instar, Kruskal-Wallis tests were used to ascertain if there were statistical differences in body mass and in headwidth between instars as well as between sites. Kruskal Multiple Comparison post-hoc tests (from the package “pgirmess” (Giraudoux 2011)) were carried out after each Kruskal-Wallis test to show where any differences lay. Spearman Rank Correlation tests were then used to measure the relationship between body mass and headwidth per site as well as for the overall data set from all six sites. Confidence intervals (from the “psychometric” package (Fletcher 2010)) for the above mentioned correlation tests were also calculated. Spearman Rank Correlation tests were also performed to show the relationship between mandible wear and body mass, and this was done for both left and right mandibles.

Results

A total of 733 caterpillars were sampled across the sampling sites but no caterpillars were found at Rev 1 and Rev 2. As there was only one site at which mopane caterpillars of a wide size range (multiple instars) were found, the data from all sites were considered together to distinguish headwidths per instar, but statistical analyses were still conducted between sites to identify possible site differences. When the log of body mass was plotted against headwidth, five clusters of headwidths were evident (Figure 4.1), so five clusters were chosen to conduct the kmeans analysis. The clusters allocated by the kmeans analysis were then labelled as instars 1 to 5 and are referred to as such hereafter.

Headwidths across all sites ranged from 0.5 mm to 8.3 mm (Table 4.1), while body mass across all sites ranged from 0.0004 g to 14.57 g (Table 4.1). An average headwidth growth ratio of 1.65 between successive instars was calculated. Although there was overlap between all the instars in terms of body mass (Table 4.1), there was a significant difference in body mass between each instar across all sites (Kruskal-Wallis $\chi^2_{(4)} = 619.8472$; $n = 733$; $P < 0.0001$).

Table 4.1. Measures of mopane caterpillar (*Imbrasia belina*) body mass (g) and headwidth (mm) from six study sites in Limpopo Province, South Africa. The allocation to instars was based on kmeans clustering where five clusters had been specified.

Instar	Average headwidth (mm)	Headwidth range (mm)	Headwidth growth ratio	Average body mass (g)	Body mass range (g)
1	1.0	0.5 - 1.4	-	0.0073	0.0004 - 0.1940
2	1.8	1.5 - 2.3	1.80	0.0483	0.0072 - 0.2420
3	3.2	2.7 - 3.7	1.78	0.2880	0.0400 - 0.7800
4	5.0	4.2 - 6.0	1.56	1.7100	0.0700 - 8.9600
5	7.2	6.2 - 8.3	1.44	5.2030	0.1500 - 14.5700

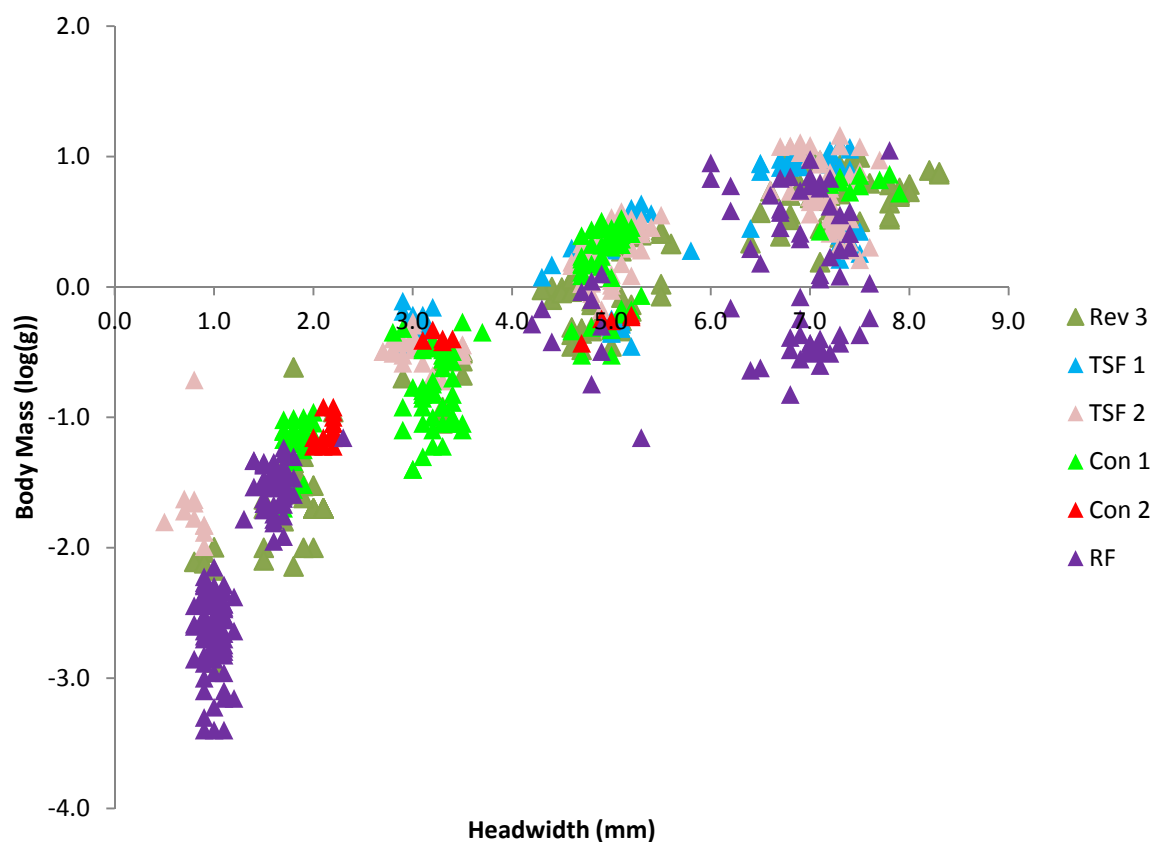


Figure 4.1. The relationship between body mass (g) and headwidth (mm) for *Imbrasia belina* caterpillars collected from six study sites in South Africa.

Instar 1 caterpillars were collected only at Rev 3, TSF 2, and RF (Figure 4.2). The mean headwidth of instar 1 caterpillars from TSF 2 (0.8 mm) were smallest while those from RF (1 mm) were widest (Kruskal-Wallis $\chi^2_{(2)} = 23.2606$; $n = 107$; $P < 0.0001$) (Figure 4.2). Conversely the mean body mass of instar 1 caterpillars was highest from TSF 2 (0.0334 g) and lowest from RF (0.0042 g) (Kruskal-Wallis $\chi^2_{(2)} = 42.2881$; $n = 107$; $P < 0.0001$) (Figure 4.2).

Instar 2 caterpillars were collected at all sites except the two tailings storage facility sites (Figure 4.2). Mean headwidths of instar 2 caterpillars from RF (1.6 mm) were the smallest, while those from Con 2 (2.2 mm) were the widest (Kruskal-Wallis $\chi^2_{(3)} = 89.7296$; $n = 138$; $P < 0.0001$) (Figure 4.2). Mean body mass of instar 2 caterpillars from RF (0.0329 g) were the smallest while those from Con 2 (0.084 g) were the largest (Kruskal-Wallis $\chi^2_{(3)} = 74.5336$; $n = 138$; $P < 0.0001$) (Figure 4.2).

Instar 3 caterpillars were found at all sites except RF (Figure 4.2). Mean headwidths of instar 3 caterpillars were smallest at TSF 1 (3 mm) and widest at Rev 3 (3.3 mm) (Kruskal-Wallis $\chi^2_{(4)} = 20.9187$; $n = 102$; $P < 0.0005$) (Figure 4.2). The mean body masses of instar 3 caterpillars from Con 1 (0.1937 g) were the lightest, while those from TSF 1 (0.5677 g) were heaviest (Kruskal-Wallis $\chi^2_{(4)} = 53.8322$; $n = 102$; $P < 0.0001$) (Figure 4.2).

Instar 4 caterpillars were collected at all six sites (Figure 4.2). Mean headwidths of instar 4 caterpillars from Rev 3 (4.9 mm) were the smallest while those from TSF 1 (5.1 mm) were the widest (Kruskal-Wallis $\chi^2_{(5)} = 18.8052$; $n = 194$; $P < 0.005$) (Figure 4.2). Mean body masses of instar 4 caterpillars were smallest from Con 2 (0.538 g) and largest at TSF 2 (2.0733 g) (Kruskal-Wallis $\chi^2_{(5)} = 46.1836$; $n = 194$; $P < 0.0001$) (Figure 4.2).

Instar 5 caterpillars were collected at all sites except Con 2 (Figure 4.2). Mean headwidths of instar 5 caterpillars were smallest at RF (7 mm) and widest at Con 1 (7.5 mm) (Kruskal-Wallis $\chi^2_{(4)} = 24.5414$; $n = 192$; $P < 0.0001$) (Figure 4.2). Mean body mass of instar 5 caterpillars from RF (2.4567 g) were smallest while those from TSF 1 (7.3769 g) were the largest (Kruskal-Wallis $\chi^2_{(4)} = 53.5142$; $n = 192$; $P < 0.0001$) (Figure 4.2).

Of all the sampling sites, Rev 3 had the strongest positive correlation ($r = 0.93$; $CI = 0.90 - 0.94$; $n = 158$; $P < 0.0001$) between body mass and headwidth. The poorest correlation between body mass and headwidth was seen with individuals from TFS 1, but this was still a strong positive correlation ($r = 0.71$; $CI = 0.57 - 0.81$; $n = 68$; $P < 0.0001$). A very strong positive correlation was also seen between body mass and headwidth for individuals from TSF 2 ($r = 0.87$; $CI = 0.82 - 0.90$; $n = 137$; $P < 0.0001$), Con 1 ($r = 0.88$; $CI = 0.84 - 0.92$; $n = 140$; $P < 0.0001$) and Con 2 ($r = 0.87$; $CI = 0.72 - 0.94$; $n = 25$; $P < 0.0001$), and RF ($r = 0.88$; $CI = 0.84 - 0.90$; $n = 205$; $P < 0.0001$).

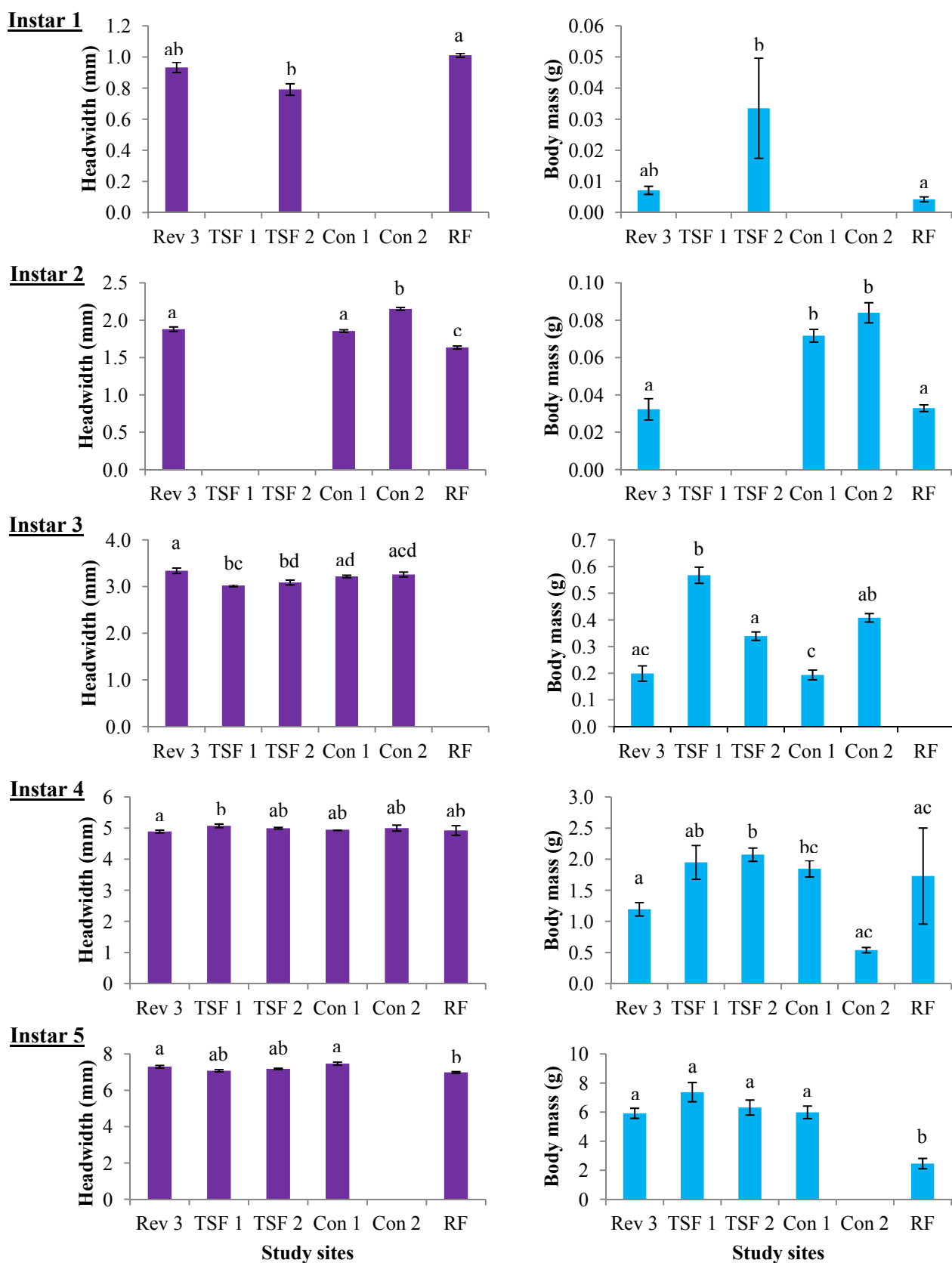


Figure 4.2. Effect of site position on mean (\pm standard error) headwidth (mm) and mean (\pm standard error) body mass (g) of all mopane caterpillar (*Imbrasia belina*) instars collected from six sites in Limpopo Province, South Africa. Shared letters above the bars in each graph indicate sites that were not significantly different.

Of the mandibles examined, 80.7 % were assigned a mandible wear score of 3, 13 % assigned a mandible wear score of 2, and 6.3 % assigned a mandible wear score of 1 (Figure 4.3). At least 65 % of samples at each of the sites were assigned mandible wear scores of 3 (Figure 4.3). TSF 2 and RF however had the highest percentages of caterpillars with a mandible wear score of 3, while Rev 3 had the lowest percentage of caterpillars with a mandible wear score of 3 (Figure 4.3). The highest percentage of caterpillars with mandibles wear scores of 1 were found at TSF 1 (Figure 4.3).

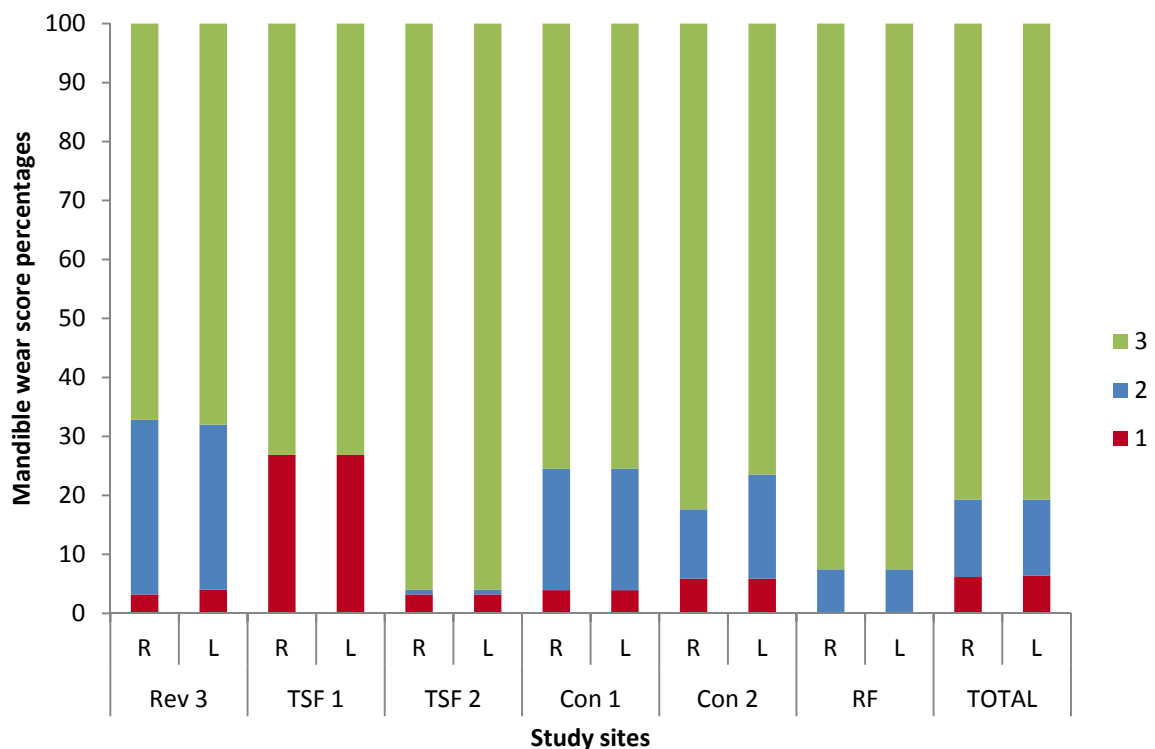


Figure 4.3. Mandible wear of mopane caterpillar (*Imbrasia belina*) mandibles of instars 2 to 4 (both right (R) and left (L)), from six study sites in Limpopo Province South Africa. A mandible wear score of 1 indicates minimal wear; 2 indicates moderate wear; and 3 indicates high wear.

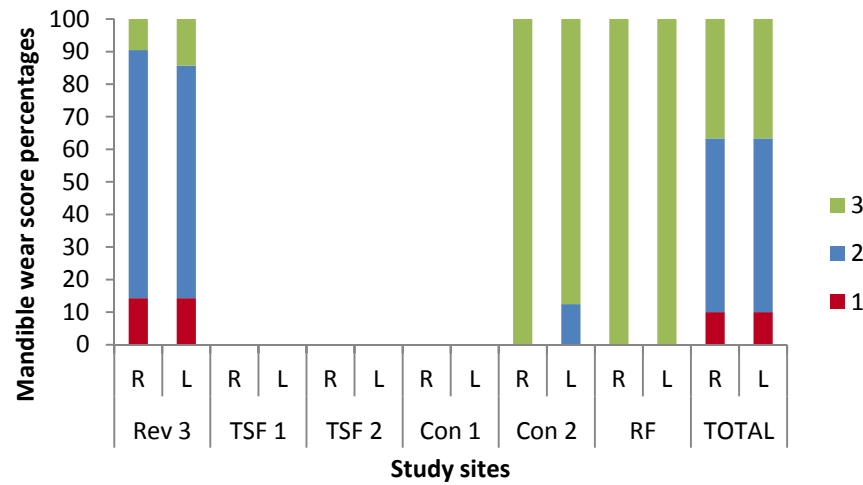
For instar 2 caterpillars, the majority of mandibles sampled (~70 %) from Rev 3 were assigned mandible wear scores of 2, while almost 100 % of caterpillars from Con 2 and RF were assigned mandible wear scores of 3 (Figure 4.4). All instar 3 caterpillars sampled from TSF 1, TSF 2 and Con 2 were assigned mandible wear scores of 3 (Figure 4.4). Approximately 30 % of instar 3 caterpillars from Rev 3 and Con 1 were assigned mandible wear scores of 2, while the majority of the remaining instar 3 caterpillar mandibles from these sites were assigned mandible wear scores of 3 (Figure 4.4).

At all sites except Con 2, at least 50 % of instar 4 caterpillars were assigned mandible wear scores of 3 (Figure 4.4). Approximately 50 % and 35 % of instar 4 caterpillars sampled from Con 2 and Rev 3 respectively were assigned mandible wear scores of 2, while approximately 45 % of instar 4 caterpillars from TSF 1 were assigned mandible wear scores of 1 (Figure 4.4). No less than 75 % of instar 5 caterpillars were assigned a mandible wear score of 3 at each site, but approximately 25 % of instar 5 caterpillars sampled from TSF 1 were assigned a mandible wear score of 1 (Figure 4.4).

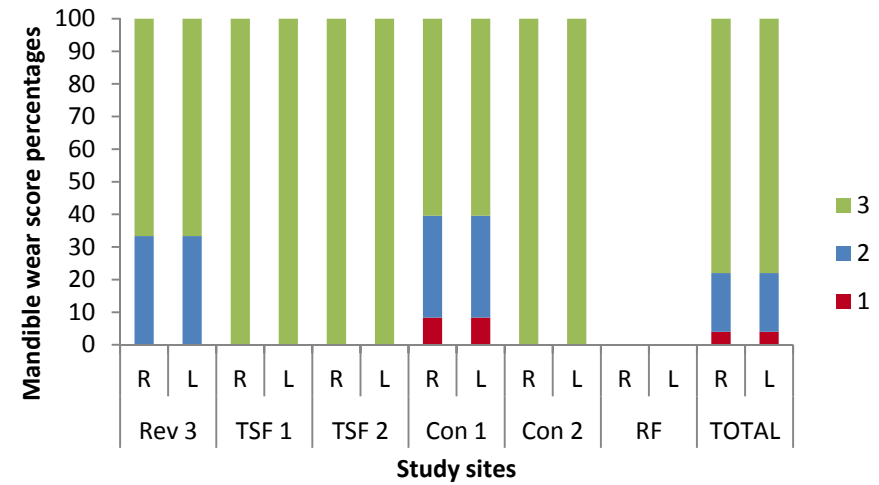
Mandible wear was positively correlated with body mass across all six sites where caterpillars were sampled for both the left mandibles ($r = 0.42$; $CI = 0.34 - 0.49$; $n = 503$; $P < 0.0001$) and the right mandibles ($r = 0.42$; $CI = 0.34 - 0.49$; $n = 503$; $P < 0.0001$). At Rev 3, mandible wear was positively correlated with body mass for both the left mandibles ($r = 0.69$; $CI = 0.59 - 0.78$; $n = 125$; $P < 0.0001$) and the right mandibles ($r = 0.72$; $CI = 0.62 - 0.79$; $n = 125$; $P < 0.0001$). At TSF 1, there was also a positive correlation between mandible wear and body mass for both the left mandibles ($r = 0.44$; $CI = 0.23 - 0.62$; $n = 67$; $P < 0.005$) and the right mandibles ($r = 0.44$; $CI = 0.23 - 0.62$; $n = 67$; $P < 0.005$).

At Con 1, there was a positive correlation between mandible wear and body mass for both the left mandibles ($r = 0.61$; $CI = 0.47 - 0.72$; $n = 102$; $P < 0.0001$) and the right mandibles ($r = 0.61$; $CI = 0.47 - 0.72$; $n = 102$; $P < 0.0001$) for the 102 caterpillars sampled at this site. At Con 2 however, there was a negative correlation between mandible wear and body mass for both the left mandibles ($r = -0.55$; $CI = -0.82 - -0.10$; $n = 17$; $P < 0.05$) and right mandibles ($r = -0.63$; $CI = -0.85 - -0.22$; $n = 17$; $P < 0.05$), but there was a low sample size at this site. Lastly at RF, a poor positive correlation between mandible wear and body mass was found for both the left mandibles ($r = 0.27$; $CI = 0.03 - 0.48$; $n = 67$; $P < 0.05$) and right mandibles ($r = 0.27$; $CI = 0.03 - 0.48$; $n = 67$; $P < 0.05$).

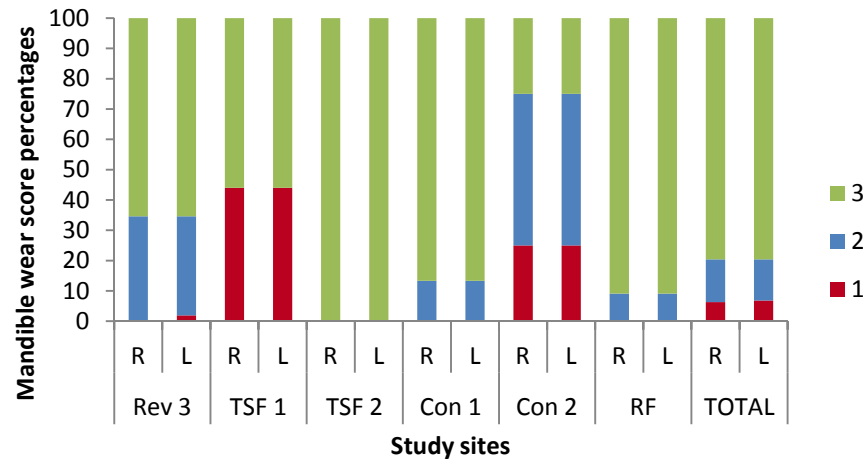
Instar 2



Instar 3



Instar 4



Instar 5

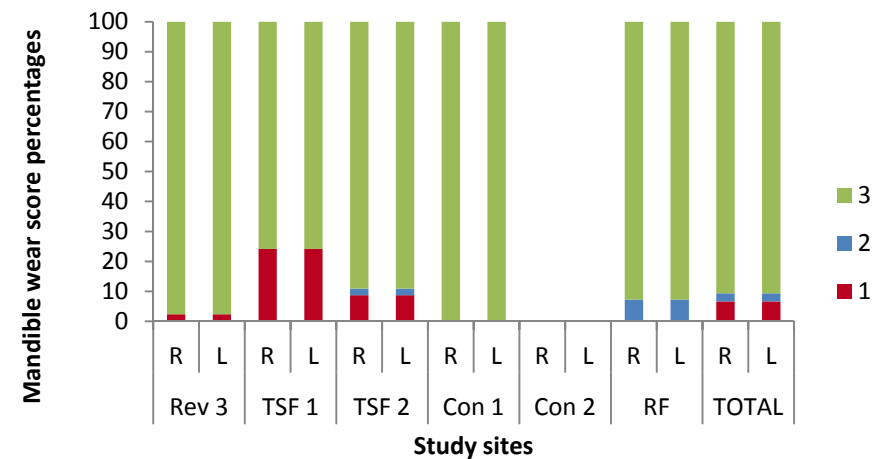


Figure 4.4. Mandible wear of mopane caterpillar (*Imbrasia belina*) mandibles (both right (R) and left (L)), of instars 2 to 4, from six study sites in Limpopo Province South Africa. A mandible wear score of 1 indicates minimal wear; 2 indicates moderate wear; and 3 indicates high wear.

Discussion

There was little variation in headwidth for each instar across the sites, with the headwidths being grouped into five distinct size classes. There was however some variation in the ranges of headwidth measures at each of the different sites per instar but these ranges were similar at each of the sites. Although using headwidths as a tool in larval aging of caterpillars is not novel (Hansen *et al.* 1981; Freitas 1993; Stavridis *et al.* 2003), the five headwidth size classes found in this study provide further evidence that there are indeed five larval instars of mopane caterpillars (Ditlhogo 1996; Klok and Chown 1999; Gondo *et al.* 2010).

Mopane caterpillars in this study were also found to have an average growth ratio of 1.65 with growth ratios between successive instars ranging from 1.44 to 1.8. This disagrees with those studies that refer to Dyar's Rule (1890) and state that the head capsule of caterpillars increases by a constant ratio in the region of 1.4 between successive instars (Wigglesworth 1942; Hansen *et al.* 1981; Irigaray *et al.* 2006). This study is instead in agreement with those studies which prove there to be a variable growth ratio between successive instars within a species (Gaines and Campbell 1935; Beck 1950; Calvo and Molina 2008). Studies by Ripley (1923), Gaines and Campbell (1935), Byrne (1988), Jobin *et al.* (1992) and Calvo and Molina (2008) have actually found that the growth ratio tends to decrease overall between successive instars as larvae grew older, which corresponds with what was found here with the mopane caterpillars.

In addition to providing an average growth ratio for mopane caterpillars, this study also provides ranges and averages for headwidths of each of the five mopane instars, which may be a useful tool in identifying the age of mopane caterpillars in future studies. As headwidth was fairly consistent between sites for each instar, this measure seems to provide a fairly simple and accurate method of aging caterpillar larvae to instars. As the headwidths are quite consistent between sites, this also indicates that elemental concentrations at the sites do not seem to be affecting caterpillar growth.

As the caterpillars progressed through the five instars, there was an exponential increase in body mass. This was expected as caterpillars increase substantially in size over the duration of their

larval growth (Gondo *et al.* 2010). The masses of caterpillars at the two tailings storage facility sites were often the heaviest within each instar but caterpillars from the two control sites were also heavier than the instars from Rev 3 and RF for some instars. Caterpillars from RF however were usually the smallest per instar. There was large variation in body mass, in instar 4 caterpillars from RF, shown by the large standard error. A possible reason for this great variation could have been the difference in diet between the caterpillars from RF and those from the other sites, as most of the caterpillars collected from RF were not collected on mopane trees, but on other tree species. This suggestion is corroborated by Gaines and Campbell (1935) who found that many factors, such as the caterpillars' diet, can result in variability of caterpillar growth. Although caterpillar body mass was seen to be positively correlated with caterpillar headwidth across all sites, the high variability of body mass within and between instars makes this a fallible measure to age caterpillars within an instar on its own.

Mandible wear was used to age individuals within an instar. It was initially expected that mopane caterpillars would have scissor-like mandibles such as those found in *Eldana saccharina* (Smith *et al.* 2007; Kvedaras *et al.* 2009). Instead it was found that mopane caterpillars have molar-like, ridged, grinding mandibles. Although Bernays and Janzen (1988) found this in other Saturniid caterpillars, they classified Saturniid caterpillars as having snipping versus chewing mandibles because there was no further processing of food once bitten off. Observations in the current study however indicate that mopane caterpillars may mechanically process leaf particles after biting off pieces of leaves.

Unlike the Saturniids examined by Bernays and Janzen (1988) that bit off leaf particles that were strongly correlated with the size of the mandibles of the caterpillars, the leaf material found between the mouthparts of the mopane caterpillars was often smaller than the size of the caterpillars' mandibles. Saturniid caterpillars are known to feed on mature, tough leaves (Bernays 1998), so if the mopane caterpillars do mechanically process leaves further before they are digested, it may be in reaction to the mopane leaves being particularly hard. Bernays and Janzen (1988) did find that there was variability in the types of mandibles within a family, so unlike the majority of Saturniidae that

that Bernays and Janzen (1988) found to have snipping mandibles, mopane caterpillars may have evolved differently to other members of the family to better process the tough leaves on which they feed. This would however have to be further examined.

Mopane caterpillars from Rev 3 were expected to have the highest percentage of individuals with high mandible wear as it was thought that the dust fallout here from the reverberator would adversely affect their mandibles by making them more brittle or causing them to wear faster. Caterpillars from this site however had the lowest percentage of high mandible wear compared to the other sites. If the pollutants were positively affecting mandible wear by strengthening the hardness of mandibles, the highest percentage of individuals with low mandible wear would have been expected to be found at Rev 3, but this was also not the case. This suggests that the level of pollutants that the mopane caterpillars were exposed to did not affect the wear of the caterpillars' mandibles.

The positive correlation between mandible wear scores and caterpillar body mass at most sites indicates that the size of an individual caterpillar of a particular instar could act as a proxy for mandible wear and thus age of an individual within an instar. Individuals that had recently moulted (indicated by the soft, lighter heads and often ecdysed skin still attached to the body in places) were seen to have mandibles that were highly ridged albeit still not fully hardened. As it was known that these individuals had very recently moulted, it was certain that those individuals were at the early stages of that particular instar and the ridged mandibles indicated a new set of mandibles. These newly moulted caterpillars, although accounting for some of the individuals with the lowest mass within an instar, were not the least heavy individuals within each instar. Although caterpillar body mass was correlated with mandible wear, the high variability of body mass and the differences in body mass of caterpillars found between sites makes body mass an unreliable measure of within instar age. Mandible wear on the other hand is a more consistent measure to age caterpillars within an instar as it cannot vary as much as body mass can.

In individuals preparing to moult (indicated by head capsules being easily removed from the caterpillar and being much thinner and more brittle than other individuals) a similar observation was

made, where a worn down, smooth pair of mandibles was found on the head capsule itself, but a second pair of larger mandibles were found beneath the head capsule and these underlying pairs of mandibles were highly ridged. This demonstrates that an individual in the later stages of an instar has mandibles which are smooth and visibly worn in comparison to the highly ridged mandibles of caterpillars early in an instar which corresponds with studies by Dockter (1993), de Boer (1995) and Kvedaras *et al.* (2009). This would indicate that mandible wear, along with headwidth, can be very useful tools in ascertaining how far along within an instar an individual mopane caterpillar may be.

Chapter 5

Conclusion

Elemental concentrations at The Palabora Mining Company (PMC) were found to be highest close to the reverberator for almost half the elements measured, with elemental concentrations decreasing with increased distance from the smelter for many of these elements. This is in line with evidence presented by Cartwright *et al.* (1977), Kuo *et al.* (1983), McMartin *et al.* (1999) and Belskaya and Vorobeichik (2013) regarding other smelters. As the two sites closest to the reverberator were less than 2 km away from the reverberator itself, it was expected that these two sites would also have low numbers of mopane caterpillars present as a result of the high levels of dust in this region from the reverberator, as particulate pollutants have previously been linked to the decline of insect numbers (Alstad and Edmunds 1982; Korunic 1998; Glenn 1999).

No caterpillars were found at these two reverberator sites, which could be as a result of the dust (particulate pollutants) present at these sites, as previous studies have found that this dust can result in desiccation and sometimes even death in insects (Alexander *et al.* 1944; Kalmus 1944; Wigglesworth 1944). Further tests would however need to be conducted to ascertain if dust did play a role in the absence of mopane caterpillars at Rev 1 and Rev 2. The fact that mopane leaves from the two reverberator sites closest to the reverberator had the highest mean concentrations of 14 of the 25 elements measured, and that no caterpillars were found at these two sites, but were found at all the other sites sampled, strongly suggests that the levels of pollution at these two sites, in terms of the elemental concentrations or amount or type of dust, may be too high to sustain the mopane caterpillars. Mopane caterpillars were however found at other sites on and around PMC and were able to survive at these sites, which suggests that the levels of pollution at these sites are not high enough to have caused in any adverse effects on the caterpillars which may have resulted populations of the species to decline.

The concentrations of the elements measured in the mopane caterpillars were found to be within acceptable levels for human consumption (EFSA 2006; Wood and Ronnenberg 2006; ATSDR

2011, 2014), indicating that the potential pollutants at these sites where the caterpillars were found, would not pose a risk to humans consuming caterpillars from these areas. As for the caterpillars themselves, if elemental concentrations were affecting the caterpillars' growth, headwidth would be expected to vary between sites in relation to the elemental concentrations at each site. This was seen in studies by Savopoulou-Soultani and Tzanakakis (1990) and Calvo and Molina (2008) who found that diet affected the headwidths of *Lobesia botrana* and *Streblote panda* caterpillars respectively. There was however very little variation in headwidth between sites, which suggests that the elemental concentrations in those regions were not adversely affecting the caterpillars' growth.

In addition to this, the quality of food available to the caterpillars (using $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ as a proxy for food quality) did not appear to differ greatly between sites, also suggesting that growth should have been similar between sites, as was found. Isotopic compositions measured were also not significantly correlated with elemental concentrations, except for two elements that were correlated with $\delta^{13}\text{C}$. These results seem to suggest that the elemental concentrations of the mopane leaves at the sites on and around PMC had no noticeable effect on the quality of the caterpillars' food, or in turn the growth of the caterpillars' themselves. If the different elemental concentrations at the different sites had some effect on the mopane leaves, we would again have expected differences in the development of the caterpillars as found by Savopoulou-Soultani and Tzanakakis (1990) and Calvo and Molina (2008).

Mandible wear was another physical feature of the mopane caterpillars expected to be affected if elemental concentrations or dust (particulate pollutants) had any negative impact on the caterpillars. It was expected that mopane caterpillars from the Rev 3 area would have the highest percentage of highly worn mandibles as the reverberator sites are exposed to most of the dust fallout, and particles such as silica have been found to cause mandible wear (Massey and Hartley 2009; Reynolds *et al.* 2009). The highest mean concentration of silicon in the mopane leaves was also found at Rev 3, further suggesting that mandible wear may have been high at this site. The majority of mandibles sampled across all PMC sites however were highly worn, suggesting that dust and elemental concentrations were not specifically affecting mandible wear, which could have

subsequently affected caterpillar growth. If mandibles had been more worn down at some sites, this would have reduced the efficiency of an individual caterpillar to ingest food, and would thus likely result in caterpillars of smaller mass at those sites (Dockter 1993; de Boer 1995).

With regards to caterpillar body mass, some of the heaviest caterpillars collected for this study were from sites on and around PMC. As caterpillars collected from the RF control site often weighed less than caterpillars of the corresponding instar collected on and around PMC, the elemental concentrations on and around PMC may even be promoting growth in mopane caterpillars. Zvereva *et al.* (1995) and Scheirs *et al.* (2006) suggest that moderate levels of pollution may benefit phytophagous insects. The large weights measured in the mopane caterpillars at the two TSF sites could thus be as a result of exposure to moderate levels of elemental concentrations. This large caterpillar size would translate into larger female moths, and larger female body size in insects has been linked to greater fecundity (Honěk 1993; McIntyre and Hutchings 2003). This suggests that the mopane moths at the two TSF sites, which were moderately polluted in comparison to the reverberator sites, may express increased fecundity than at sites with low or high levels of elemental concentrations.

This study was able to provide data on the physical characteristics of individual mopane caterpillar instars, which will be a very useful tool for further studies examining mopane caterpillar morphology. Although large quantities of data were gathered to measure both the elemental concentrations and physical caterpillar characteristics, further strengthening the validity of the findings related to those data, multiple additional replicates of these data as well as more data relating to isotopes and compensatory growth should be gathered in order to supply more conclusive results on these topics. As demonstrated by Luyssaert *et al.* (2003), this will decrease the variance in the data, as well as allowing for a more precise assessment of what the minimum effect of some ecological significance may be. In addition to this, a number of further studies could be conducted to make more in depth assessments of the elemental concentrations around the PMC region, as well as in other areas, using the tools provided in the current study, such as the tools for aging caterpillar instars.

Being able to distinguish between early- and late-stage instars provides a useful tool with which to assess whether or not mopane caterpillars bioaccumulate elements or if they eliminate excess amounts of elements. If elemental concentrations of caterpillars increase from early within an instar to later in the same instar, as well as from one instar to the next that would suggest that the caterpillars may be bioaccumulating these elements. If however the elemental concentrations of caterpillars in the early stages of an instar are lower than they were in the late stage of the previous instar, that would suggest that the caterpillars are eliminating excess elements, as was shown by Raubenheimer and Simpson (2004) and Lee *et al.* (2002) who found that a generalist locust (*Schistocerca gregaria*) and a generalist caterpillar (*Spodoptera littoralis*) respectively were able to eliminate excess nutrients. These data would be of great value, as they could provide more information on what elements may be bioaccumulated by the mopane caterpillars. As there could subsequently be biomagnification of these elements along trophic levels (Woodwell 1967; Connolly and Pedersen 1988; Laskowski 1991; Gobas *et al.* 1993), other species that form part of the same food web as the mopane caterpillars should also be examined to measure the elemental concentrations in their tissues.

As Rev 1 and Rev 2 are located less than 2 km from the reverberator, the high dust fallout must be considered as a possible reason for the species not being able to establish at these two sites, as previous studies have shown it to affect insect survival (Alexander *et al.* 1944; Kalmus 1944; Wigglesworth 1944; Korunic 1998; Glenn 1999). To investigate this, mopane caterpillars could be reared in a laboratory and fed washed and unwashed mopane leaves collected from these two reverberator sites. If the caterpillars are able to survive in both the washed and unwashed leaf treatments, and the species is able to persist, then the dust fallout and not the elemental concentrations themselves could be the reason that the caterpillars do not survive at these sites. If only caterpillars from the washed leaf treatment are able to survive and persist, the presence of dust in general, not specifically airborne dust could be affecting the species survival. If no caterpillars from either of these treatments survived, it could then be that it is simply the elemental concentrations of their food source that is affecting their survival. This type of experiment would be similar to studies conducted by Yoshida *et al.* (1995), Tillman *et al.* (2002), and Lahtinen *et al.* (2004), who tested for various

substances on the surface of leaves, and if these had any effect on aspects such as insect preference, growth and survival.

If this appears to be the case, each element could then be tested separately to establish which of the elements present at these two sites may be causing the species not to survive here. This would provide information on the elements that need to be targeted for future revisions to gaseous waste release from the reverberator. The caterpillars could be fed concentrations that range between the lower of the two concentrations found in the mopane leaves from Rev 1 and Rev 2, and the highest of the concentrations found in the mopane leaves at all other sites. This would then provide threshold values, for each of the 25 elements measured, at which mopane caterpillars can or cannot survive. If none of these elements are found to be limiting to the caterpillars, other elements and other leaf attributes, such as the amount of dust present on leaves, could then be examined.

Overall it is clear that Rev 1 and Rev 2 are the two sites with highest elemental concentrations, and were also the two sites where no mopane caterpillars were collected. Further research as suggested above should be undertaken as they could provide further insight into the possible reasons for why mopane caterpillars were not found at these two sites. If corrective actions to reduce elemental concentrations from PMC's activities were to be considered, these should likely be focused at the sites where mopane caterpillars have been found in order to minimise the caterpillars' exposure to contaminants at these sites in the future.

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