MANIPULATION OF DUNG DURING FEEDING AND NESTING BY DUNG BEETLES (COLEOPTERA: SCARABAEIDAE).

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MSc Dissertation

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Abstract

Dung beetle feeding is still not a widely understood behaviour. Feeding on the waste of other animals creates a challenge of acquiring the necessary nutrients for growth, development and for survival. Because of their soft mouthparts, it has always been thought that adult dung beetles cannot chew the hard parts of dung but only select smaller particles to feed on. Dung beetle larvae on the other hand can chew harder components, but where they get the nutrients required for development has not yet been discovered. The aim of this study was to discover which dung particles adult dung beetles select when feeding, and when creating a nest for their offspring, and also how much they can alter the dung nutrient content in the process. Foregut and hindgut contents were dissected out of the dung beetle *Scarabaeus goryi*, and their contents compared with untreated dung from the cow and the excreta of adult beetles. Brood balls and maternal gifts of *Euoniticellus intermedius* were also dissected. Nitrogen and carbon content, the carbon to nitrogen ratio, and results of the carbon and nitrogen stable isotopes were compared between all samples. When feeding dung beetles selected particles which were bigger than 800 µm in diameter and had three times more nitrogen than raw dung, and used particles over 2000 µm in size in the maternal gift packed for their offspring. Nitrogen content in the selected dung was increased from 1.40 % to 5.14% by adult dung beetle feeding and up to 2.62% in dung selected for the larvae to feed on. The carbon to nitrogen ratio also decreased when feeding adults and in the brood balls made for the larvae to feed on. Both carbon and nitrogen were absorbed from ingested dung during digestion. The heavier nitrogen isotope was selected for when feeding. The heavier nitrogen isotopes and the lighter carbon isotope were selected for during absorption. Dung beetles increase the nitrogen content of the dung on which they feed by careful selection of particles of a certain size and nitrogen content, which is probably the limiting nutrient when feeding and nesting. The increase in the nitrogen content is not only from the change in particle size but also in selection of particles with higher nitrogen content.

Declaration

I declare that this MSc dissertation is my own, unaided work. It is being submitted for the fulfilment of the Degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted for any degree or examination before at any other university.

Signature:

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Chapter 1: General Introduction

1.1 Introduction

1.1.1 Dung beetle life history

Coprophagy, feeding on excreta is a fundamental feature of most dung beetles (Coleoptera: Scarabaeinae) (Halffter and Matthews 1966). It defines their distribution, behaviour, morphology and development. The majority of dung beetles feed on the dung of herbivorous and omnivorous mammals (Simmons and Ridsdill-Smith 2011).

The term dung beetle refers to members of the Scarabaeidae which use dung as the food source for both the adult and the larvae (Halffter and Edmonds 1982). Dung beetles belong to the subfamilies; Scarabaeinae and Aphodiinae (Holter and Scholtz 2007). The Scarabaeinae includes about 5700 species in 227 genera (Davis *et al*. 2008). Dung beetles are found all over the world except in Antarctica, and their species richness and ecological importance increases with decreasing latitude (Hanski and Cambefort 1991).

Scarabaeine dung beetles are divided into 3 ecological groups: telecoprids, paracoprids and endocoprids. Telecoprids, also called rollers, form a dung ball which they roll away from the dung pat, bury and then use for either feeding or breeding. Paracoprids, commonly called tunnellers, make tunnels under the dung which they use to transport the dung from the dung pat to a nest which is usually at the end of a tunnel. Endocoprids feed and make a nest inside the dung pat for their larvae. For this study the focus will be on telecoprids and paracoprids because they create nests for their larvae.

1.1.2 Ecosystem Services

Through relocation of dung, feeding and breeding, dung beetles play a very important role in nature. They transfer excrement underground from the soil surface which prevents the loss of nitrogen through ammonia volatilization and enhances soil fertility by increasing the amount of nitrogen in the soil (Nichols and Gómez 2013). Soils exposed to dung beetle show an activity increase in soil nutrients like N, P, K, Ca and Mg (Bertone 2004; Yamada *et al*. 2007; Nichols and Gómez 2013).

Dung beetles also increase water permeability and the soil water holding capacity through their tunnels and underground chambers (Brown *et al*. 2010). They create tunnels which can go down to about 1.3 meters. These tunnels allow the water to permeate further into the soil and allow aeration in upper soil layers (about 10 cm) (Edwards and Aschenborn 1987; Nichols and Gómez 2013). It has been found that plants grown in soil which has been exposed to dung beetle activity have increased biomass, plant height, nitrogen content and protein levels (Bang *et al.* 2005; Lastro 2006). Some studies showed that dung beetles outperformed chemical fertilizers; plants exposed to dung beetle activity showed an increase in height, leaf production and yield (Fincher *et al*. 1981; Miranda *et al*. 2000).

Dung beetles not only improve soil conditions, but are also of direct economic importance. Through their feeding and relocation of dung, dung beetles control the population of dung-breeding and dung-dispersed pests and parasites like flies, nematodes and protozoa which reduce livestock productivity and hide quality (Haufe 1987; Nichols and Gómez 2013). Dung beetles increase fly mortality by disturbing and damaging eggs and larvae of flies during dung relocation and feeding (Bishop *et al.* 2005). In addition, the removal of dung when making brood balls generates increased resource competition among fly larvae (Ridsdill-Smith and Hayles 1987; 1990). For example, dung beetles were introduced into Australia to control fly populations which were causing significant economic loss as a result of the increased cattle farms and fly impacts on the cattle (Ridsdill-Smith and Hayles 1987; 1990).

1.1.3 Importance of dung as food source

Little research has been done on dung beetle feeding (Holter 2016). Little is known about dung beetle feeding on dung, the most fundamental aspect of dung exploitation by both the adult dung beetle and the larvae (Holter 2016). The dung itself is a metabolic end product, as it is made up of partially digested, undigested and undecomposed food particles like plant cell walls (Holter 1974; Endrödy-Younga 1982). The dung of different species of animals differs due to the differences in digestive physiology of the animals and the food that those animals eat (Sitters *et al*. 2013). The main differences are nutrient content (nitrogen, carbon and phosphorus) and moisture (Sitters *et al.* 2013). Dung beetles not only use dung for feeding, they also use it to provide for their offspring. Paracoprid and telecoprid dung beetles remove some dung from the dung pat and use it to make shelters for their eggs and also to provide food for their offspring.

Although carbohydrates are the major source of energy for most insects, many insects do not have specific carbohydrate compound requirements for their diet (Nation 2008). They can utilise any carbohydrate compound when feeding. Because plants are mainly carbohydrates, nitrogen often becomes the limiting nutrient (Mattson 1980). Nitrogen in plant tissue ranges between 0.03 to 7.0%, and in dung it ranges from 1.14 to 3.49% (Chown and Nicholson 2004; Holter and Scholtz 2007). Insects need nitrogen, which they get from amino acids and proteins, for growth and reproductive maturation (Nation 2008). Despite this, the nutritional requirements for dung beetles are still not known (Holter 2016).

1.1.4 Food provisioning by parent insects

Dung beetles show sub-social behaviour by packing food into brood balls which is critical when the food is limited and ephemeral (Halffter *et al*. 1996; Hunt and House 2011). Some species of dung beetles go further, giving parental care to their offspring (Hunt and House 2011). Within the species that give this care, it has been proven that parental care increases the longevity of the offspring

(Halffter *et al*. 1996; Sowig 1996). Halffter and Matthews (1966) found that in *Copris fricator* Fabricius offspring survival decreased from 93% to 59% and in *Copris diversus* Waterhouse, Tyndale-Biscoe (1984) found that survival decreased from 76% to 36% when the parents were excluded from the nest. For *Kheper nigroaeneus* Boheman, Edwards (1988) found that there were no survivors if the mothers were excluded.

A study by Moczek (1998) suggested that adult dung beetles are able to measure the resource quality of the dung when they provision for their offspring. He found that in the laboratory when *Onthophagus taurus* Schreber was reared on horse dung which was of higher quality the beetles used less (both in wet and dry weight) dung in making brood balls compared to when they were reared using cow dung which was of relatively lower quality. With high quality dung the larvae will meet the nutritional needs more quickly compared to when feeding on dung of lower quality hence less is used during the broodball construction. The amount and quality of the dung can influence offspring size which is directly related to the male reproductive success, and survival and fecundity in females (Hunt and Simmons 1997; 2000; 2001; Hunt *et al*. 2002).

In *Euoniticellus intermedius* there is no parental care, after laying the egg the maternal parent seals the brood ball and leaves it underground. The only way of ensuring the proper development of the larval beetle is by making sure that the offspring will have enough food during development. It has been previously shown that dung beetles lower the C: N ratios and increase the nitrogen content of the dung in brood ball (G Lindsay-Smith unpublished data). The C: N ratio was decreased from 33:1 in bulk dung of cattle to 17:1 in brood balls. It is not exactly known how dung beetles increase the nitrogen content in the brood balls, Holter and Scholtz (2007) suggest that adult dung beetles use selection of smaller particles to increase the nutrient content of the dung on which they feed.

The increase in the nitrogen content of brood balls could also be caused by the addition of the “maternal gift” (Fig. 1) to the brood ball. The maternal gift is a smooth paste of finer dung particles laid there by the maternal dung beetle, which is used to anchor the egg inside the egg chamber

(Byrne *et al*. 2013). Davis (1977) described the maternal gift as the 'fine-grained matrix' which supports the egg, and is 'easily utilisable by a small larva' which is "made squeezing out moisture from the dung inside the egg chamber". A recent study by Byrne *et al*. (2013) showed that the maternal gift is not essential for larval development nor is it used to transfer microbes which could help with digestion of cellulose in the gut, from the maternal dung beetle to the larva, although more recent molecular work might require a reassessment of the role of microbes in dung beetle larval development (Shukla *et al*. in press).

1.1.5 Larval feeding

Unlike adult dung beetles, dung beetle larvae have hard mouth parts which are used for chewing dung particles (Holter *et al*. 2009). Dung beetle larvae feed on the dung in the brood ball which the adult beetle (usually the female) has selected from the dung pile, apparently using both finer and coarse particles. It appears that dung beetle larvae are able to break down dung fibres which they feed on, probably excrete and then re-eat it again (Byrne *et al*. 2013). During the brood ball creation, some species of bacteria are vertically transferred from the maternal dung beetle to the larvae through the brood ball (Estes *et al*. 2013). These bacteria are claimed to be responsible for breaking down the cellulose from the fibres (Estes *et al*. 2013), and thought to be essential for the development of the larvae, but Byrne *et al*. (2013) found that they were not required for dung beetle development. This point of contention is still not settled. It is still unknown which component of dung; mammalian digestive cells, saprophytic fungi, bacteria, partially digested plant material or maternal secretions are responsible for larval growth and development (Byrne *et al*. 2013).

1.1.6 Dung Beetle gut structure

Dung beetles have a gut structure of typical insects. The gut is divided into three regions: foregut, midgut and hindgut. The foregut is made up of the pharynx, oesophagus, crop, gastric caeca and proventriculus, and is mainly used for secreting salivary enzymes (Nation 2008). The midgut is made up of the gastric caeca, in most insects this is the area of secretion of digestive enzymes, digestion and absorption (Nation 2008). The hindgut is made up of malphigian tubules, ileum, and rectum, the main function is the absorption of water, salts and other molecules (Gullan and Cranston 2010).

Figure 1.1. The egg on top of the maternal gift, the shiny smooth paste in the opened brood ball of *Euoniticellus intermedius* Reiche.

Adult dung beetles have a gut that is specialised for eating fresh, wet dung of herbivores and omnivores (Holter and Scholtz 2013). The midgut is long and coiled and the hindgut is shorter, and the width is approximately similar between the midgut and hindgut (Holter and Scholtz 2013). The dung beetle gut is lined by a peritrophic membrane which prevents damage by food particles that are passing through the gut, reducing exposure of the gut to large toxin molecules and assisting concentration of food and digestive enzymes (Engel and Moran 2013).

1.2 Aims and Objectives

The purpose of this study was to improve the understanding of feeding and nesting by dung beetles which live on wet dung. The objectives are: (1) to test if dung beetles select particles by size for feeding or nesting, (2) to test if dung beetles increase the nutrient content of their food by particle selection, (3) to determine which particles dung beetles feed on or use to make nests (brood ball).

1.3 Study Species

1.3.1 Morphology

Two study species were used*,* *Euoniticellus intermedius* Reiche (Fig. 1.2) and *Scarabaeus goryi* Calstelnau (Fig. 1.3)(Coleoptera: Scarabaeidea). *Euoniticellus intermedius* is a small 10 mm long) paracoprid species that is found in areas with different ecological conditions and can adapt to a wide range of soil and climatic conditions (Endrödy-Younga 1982). As a result it is widely distributed throughout the Afrotropical region and is found from the Sahel to Southern Africa. This is one of the dung beetle species which was exported from Africa to Australia and from Australia to the USA to control flies which breed in the dung (Bertone 2004). *Scarabaeus goryi* is found in Southern Africa and some parts of Central and West Africa. Little is known about *S. goryi*.

Figure 1.2. Adult *Euoniticellus intermedius* Reiche dung beetles with female on the left and male on the right. Adapted from Shukla *et al*. In press

1.3.2 Nesting behavior

Euoniticellus intermedius is a diurnal paracoprid dung beetle. The female burrows under the dung and digs tunnels for nesting. Females become sexually mature in about two weeks and start digging tunnels for nests (Pomfret and Knell 2006). Females push or pull the dung underground to the end of each tunnel and where they form a brood ball (Pomfret and Knell 2006).

One egg is laid in each brood ball, which hatches in about two days (Pomfret and Knell 2006). It takes about four to six weeks to develop from egg to adult during warmer seasons (Pomfret and Knell 2006). The life span is about one to two months (Pomfret and Knell 2006). *Scarabaeus goryi* is (30 mm) nocturnal telecoprid dung beetles. Both males and females pack dung into a ball and roll it away from the dung pat for feeding or for breeding. The ball is buried a few centimetres underground if used for feeding (Edwards and Aschenborn 1989). Little is known about the nest building behaviour of this species, but if it behaves like its close relatives, the brood ball is more deeply buried where it is reworked by the female (Edwards and Aschenborn 1989). She then lays an egg into the ball and guards it until the new adult emerges, which can take several weeks or months (Edwards and Aschenborn 1989). In both species the male deserts the female after she has laid her eggs.

Figure 1.3. An adult male *Scarabaeus goryi* Calstelnau dung beetle in dung.

1.4 Dung beetle Collection

Euoniticellus intermedius individuals were collected in Thengwe Village, Limpopo province (22° 42' 56'' S, 30° 33' 24'' E) on the 2nd and 3rd of January 2014. The vegetation of the area is Lowveld Sour Bushveld (Mucina and Rutherford 2006). This is a summer rainfall area with very hot summers and the winters are relatively warm. The mean annual rainfall is 489 mm and the mean annual temperature is 21.7 °C (Mucina and Rutherford 2006). The soil type is deep sands and shallow sand lithosoils (Mucina and Rutherford 2006).

Scarabaeus goryi was collected at Stonehenge Farm outside Tlakgameng village, Vryburg, North West Province during November 2015. The vegetation of the area is Molopo Bushveld (Mucina and Rutherford 2006). This is a summer rainfall area with very hot summers and the winters are dry and cold. The mean annual rainfall is 333 mm and the mean annual temperature is 19.1 °C (Mucina and Rutherford 2006). The soil type is red Aeolian sand and sand (Mucina and Rutherford 2006).

Dung beetles were picked up from dung pats of cattle on the roadside in the rangelands of the village at Thengwe Village and on dung pats at Stonehenge Farm. Cattle graze on pasture throughout the year and sometimes on maize stalks after harvesting. After collection, beetles were placed in 10 litre containers with firmly packed soil and fed cow dung collected from the same location.

1.5 Dung beetle cultures

Dung was collected from Cluny Farm (25° 57’ 30” S, 28° 03’ 57”E) in Midrand, Gauteng Province. Cows on the farm are pasture fed with supplement. The dung was collected in 4 litre plastic bags, and then stored in a freezer. Dung was collected about three times a year. Dung, thawed at 20 °C was added in 150 ml aliquots to the containers.

After field collection, the dung beetles were taken to the insectary in the Oppenheimer Life Sciences building at the University of the Witwatersrand. The *E. intermedius* beetles were placed in plastic pots of 15 x 13 x 13 cm. Each pot was filled with 1.75 litres of moist sandy loam soil which was gently compacted.

Pairs of *E. intermedius* were added to each pot. The pots were reset every week and the dung beetles fed twice per week with freshly thawed dung. Fresh soil which had been sterilised by heating in an oven at 60°C for at least three days was used for the substrate. The culture was kept at a photoperiod of 12 hours light/dark and at 25°C.

During resetting, the soil contents of the pots were sieved, brood balls were placed individually into CELLSTAR® Well Cell Culture Plates (Greiner Bio-One, Frickenhausen, Germany), which were closed then placed into a 3 litre plastic box lined with wet tissue, to keep the interior of the container humid and the contents moist. When new beetles emerged from their brood balls they were then added to the culture, ensuring that different generations were kept separate.

Scarabaeus goryi were put in a 35 litre containers filled with moist soil which had been compacted. Fresh soil which had been sterilised as above, was used. The culture was kept at a photoperiod of 12 hours light/dark and at 25°C. The soil was replaced every 2 months. The container had 30 beetles which were fed about 500ml of dung each week. No breeding occurred in the container which held the *S. goryi* colony.

Chapter 2. Particle size manipulation by dung beetles

2.1 Introduction

2.1.1 Mouthpart structure and feeding

The structure of the mouthparts and diet of an organism has an effect on the way that organism will collect and process its food (Krenn *et al*. 2005; Blanke *et al*. 2015). Organisms that feed on liquids or nectar have mouthparts which can suck up liquids, while those which feed on solid food have mouthparts that can chew or grind food (Krenn *et al*. 2005).

Some organisms have developed ways of collecting and concentrating their food, one way being filter feeding, or self-selection of particles when feeding. Crustaceans like Cladocera, feed on phytoplankton and zooplankton which are very tiny and suspended in the water (Burns 1986). They suck up water and filter out the zooplankton and phytoplankton while releasing the water back to the system.

Many aquatic insects are also known to be filter feeders (Merritt and Wallace 1981; Cheer and Koehl 1987). These insects have specialised body parts that help them to filter small particles to feed on. The larval black fly has fans in the mandibles and the nymph of the mayfly from genus *Isonchyia* has tiny hairs on the seta which are short and hooked (Merritt and Wallace 1981). These mouthparts help these insects in collecting food and in making them successful in their habitats. Dung beetle mouth parts are also evolved to fit their feeding behaviour. Their mandibles, which are appendages used in grasping and chewing of food, have evolved to feed on dung.

2.1.2 Dung beetle feeding and nesting

Not all dung beetles feed and live on wet dung or carrion, some dung beetles as adults nest and feed on dry dung and detritus. Beetles in the genus *Pachysoma* feed on dry dung pellets and detritus

(Scholtz *et al*. 2004). The adult *Pachysoma* beetles have mouthparts that are adapted to crushing large fragments of dung and plant matter, and they ingest large dung fragments unlike dung beetles feeding on wet dung which are thought to feed on smaller particles (Davis *et al*. 2008; Holter *et al.* 2009). *Pachysoma* dung beetles make a nest by accumulating loose dung and detritus (Scholtz *et al*. 2004; Holter *et al.* 2009). They lay a single egg in a nest and the larvae feed and develop on this organic matter (Davis *et al*. 2008).

Because of their soft mouth parts, most adult dung beetles cannot chew dung fragments (Holter 2016). Looking at the mandibular structure of wet dung feeding dung beetles Hata and Edmonds (1983) proposed that these dung beetles select smaller dung particles to feed on which range between 2 and 200 µm. Holter (2000) estimated the maximum particles size that five *Aphodius* (Scarabaeidae: Aphodinae) species, namely: *Aphodius ater* (Degeer), *A. contaminatus* (Herbst), *A. erraticus* (Linnaeus), *A. fossor* (Linnaeus) and *A. rufipes* (Linnaeus) can ingest, by mixing the dung that dung beetles were feeding on with latex or glass balls of different sizes. He then measured the latex or glass balls which were found in the midgut and estimated the maximum particle size that can be ingested by dung beetles compared to those that could not be ingested. He estimated the particle size to range from 5 to 25 µm in *Aphodius* dung beetles. Later Holter *et al*. (2002) found that even large Scarabaeinae dung beetles select small particles and that the maximum particle size diameter of ingested particles was related to body size. With small dung beetles like *Onitis mendax* Gillet having a range between 8 to 16 µm and a bigger beetle, *Heliocopris japetus* Klug was having a range of 40 to 60 µm.

The same method of feeding synthetic particles to adult beetles was used in *Geotrupes* (Geotrupidae, Geotrupinae) and *Hydrophilids* (Hydrophilidae, Sphaeridiinae) by Holter (2004). The wet dung feeding *Geotrupes* and *Hydrophilid* dung beetles have mouthparts similar in structure to those in the genus *Aphodius* and the *Scarabaeinae*. *Geotrupes* were found to consume particles with a diameter range of 40 to 90 µm while the Hydrophilids selected 16 to 19 µm sized particles.

Current theory proposed by Holter and Scholtz (2007) suggests that the finer dung particles which pass through the filter mechanism in the mandibles, are then concentrated, probably by squeezing out the liquid, then these concentrated finer particles get ingested. It is believed that this process reduces the amount of undigested fibres which would need chewing, from the diet (Holter 2016).

With wet dung feeders, when reproducing, the maternal dung beetle packs dung into a brood ball and lays an egg inside. The larva feeds and develops on the dung which is in the brood ball (Emlen 1994). The larva has mandibles that can chew these larger particles of dung (Scholtz *et al*. 2009). The hard parts of the mouth are shed during metamorphosis into the adult stage and the new mouth parts never harden (Davis *et al*. 2008).

Byrne *et al*. (2013) microscopically compared the particle sizes of the dung used to form the brood balls and found that *E. intermedius* selects smaller dung particles ranging from 2 to $4mm^2$ to make brood balls from dung with mean particle size of 16 – $22mm^2$. The maternal gift is made of dung particles which are finer (3 to $5mm^2$) than those in the dung but similar to those of the brood ball (Byrne *et al*. 2013). He found that dung beetles used smaller dung particles in the dung to make the brood ball and maternal gift but there was no significant difference between the particle sizes of brood ball and maternal gift.

2.2 Aim and objectives

The purpose of this study is to use a different, more accurate method to test if dung beetles do select smaller particles when feeding and nesting by measuring the actual particles ingested naturally. Maximum particle size diameter of the dung fed upon was compared to that of the particles in the foregut of the beetle to test if dung beetles select smaller particles when feeding. Maximum particle size diameter of the brood ball dung and the maternal gift was also compared with that of the dung to test if dung beetles use certain particles when making a nest.

The volume weighted mean, which is the mean particle size measured in volume, was measured from the dung, and foregut and hindgut and the excreta of the dung beetles and the changes in the particle size along the gut was recorded.

2.3 Materials and Methods

2.3.1 Sample preparation

Two study species were used for dung particle size analysis, the larger, *S. goryi* for alimentary canal and excreta measurements and *E. intermedius* for brood ball and maternal gift measurements. Six *S. goryi* beetles were placed in a 3 litre container with 1 litre dung for 48 hours to allow them to enough time to feed and fill up their guts. Then these beetles were killed by immersion in boiling water for 30 seconds.

The full gut was immediately removed from the body and the contents of the foregut and the hindgut were dissected out, removed from the peritrophic membrane and put in Ethylene Glycol solution in separate 5ml *Eppendorf* tubes. Faecal samples (the excreta) were also collected from the containers where the *S. goryi* was feeding, they were then separated from the peritrophic membrane and stored separately in 5 ml *Eppendorf* tubes filled with Ethylene Glycol.

Six pairs of *E. intermedius* were placed separately in 2 litre containers filled with 1 litre of soil and 300ml of dung and left to breed for about a week. After a week, the soil was sieved and the brood balls were collected. From each pair one freshly made brood ball, which still had an egg inside, was dissected. The soil was removed from the outside of the brood ball and the maternal gift was also separated from the brood ball.

The brood ball and maternal gift samples were placed separately in an Ethylene Glycol solution in 5ml *Eppendorf* tubes. From the dung that was used to feed both dung beetles, six dung samples were collected and put separately inside 5ml *Eppendorf* tubes. All samples were sterilised by autoclave for 20 minutes at 120°C, before being sent to the Geography Science laboratories in the Department of Geography at University of Cambridge for particle size measurement.

2.3.2 Particle size measurements

Particle size analysis was done using the Malvern Mastersizer 2000 (Malvern Instruments ltd). This instrument can measure and automatically analyse small particles down to 0.4 µm in size. Particle size was analysed using laser diffraction analysis. Light at red and blue wavelengths is passed through the sample at varying angles and the light intensity of the resulting scatter pattern is then analysed to determine particle sizes within the sample.

Samples contained in 5 ml *Eppendorf* tubes were vortexed at 2000 rpm for 20 seconds before being dispersed into 25 ml of water. Tubes were refilled and the process repeated to remove any residue. Following a background measurement to eliminate the influence of particles that can contaminate the dung samples, the suspension was added to the presentation vessel of the Malvern Mastersizer which contained approximately 350 ml of water.

Small amounts of the samples were analysed using a 'Small Volume Sample Dispersion Unit' which reduced the volume of the dispersant to approximately 50 ml. Samples were run on the instrument's automated program where for each sample the result comprised an average of three measurements of the particle distribution from the same 50 ml. The particle size range which can be detected by Malvern Mastersizer is between 0.4 µm and 3000 µm. Distribution cut off was not observed indicating that the particle size of the samples fell within the working parameters of the instrument. Particle sizes range, maximum particle size and average particle size were measured.

2.3.3 Statistical analysis

All statistical analysis was done using Statistica software package version 8 (Stat Soft Inc. 2007) and R version 2.15.2 (R Foundation for Statistical Computing 2012). All the data was tested for normality first using the Shapiro-Wilk test of normality. A Kruskal-Wallis test was used to test for significant differences between in the maximum particle size diameter and the One-Way Analysis of variance (ANOVA) was used to test for significant differences in the volume weighted mean of all samples at 95% significance level. Wilcoxon Matched Pairs Test was used to test for significant differences between the maximum particle size diameters of the gut samples, the Tukey's HSD was used for post hoc tests to test differences within samples for the volume weighted mean.

2.4 Results

There was a clear difference in the distribution of particle size between different samples (Fig. 2.1 and Fig 2.2). The untreated dung had the largest diameter particle size with most particles (particle size diameter with a volume of 8% and above) ranging between 125 µm and 1000 µm.

The dung particles from the foregut had a lower range of particle size diameter with most particles ranging between 44.2 µm and 176.8 µm followed by the hindgut (between 88.4 µm and 353.6 µm) and excreta (between 125 µm and 250 µm) respectively while the dung had the largest range of particle size diameters (Fig. 2.1 and Fig. 2.2). There is selection of smaller particles in the beetle's mouth which is reflected in the foregut. The particle size diameter decreases through the gut passage from the foregut to the excreta.

For the nesting experiment, there was no clear difference in particle size distribution between the dung, brood ball and maternal gift. Most of the brood ball particle sizes diameter range between 176.8 µm and 1414 µm while the maternal gift had a smaller range of 250 µm to 707 µm. However there is a difference in the peaks, where most particles of a particular size diameter are found.

The maternal gift particle size diameter has a peak around 500 µm which suggests that female dung beetles actively select particles of that size when preparing the maternal gift or that the excreta is used to make the maternal gift. Comparing the untreated dung and the brood ball, there is no clear particles size selection. The maternal gift has a bigger particle size range than the dung, suggesting that dung beetles actively use coarser particles of dung when making the brood ball. Even though the dung beetle species used for the nesting and feeding experiment were different, the foregut sample from the larger *S.* goryi still has a smaller particle size range compared to the maternal gift made by the much smaller *E. intermedius*.

The maximum particle size diameters follow the trends that are seen in the average particle size distribution graph (Fig. 2.3 and Fig. 2.4). The maximum particle size diameters were significantly different between some samples. The untreated dung had greatest maximum particle size diameter. *Scarabaeus goryi* selected smaller particles when feeding, the maximum particle size of the foregut content was about half of the dung value (Wilcoxon Rank test, $p = 0.043$). The maximum particle size measurements of the excreta and the hindgut were omitted from this study because they were expected to be similar to those of the foregut since they are already in the gut and there will not be any further selection by the dung beetle.

The foregut contents also had a significantly lower maximum particle size compared to the brood ball (Wilcoxon Rank test, $p = 0.043$) but was not significantly different from the maternal gift (Wilcoxon Rank test, $p = 0.273$) which could suggest that the maternal gift is regurgitated material. Dung beetles use smaller particles of unknown origin to make the maternal gift, as a provision for the larva.

There was no significant difference between the mean maximum particle size of the dung and that used to make the brood ball (Multiple Comparison Test, $p > 0.05$). The maternal gift particles were found to be smaller than those used to make the brood ball (Wilcoxon Rank test, $p = 0.018$).

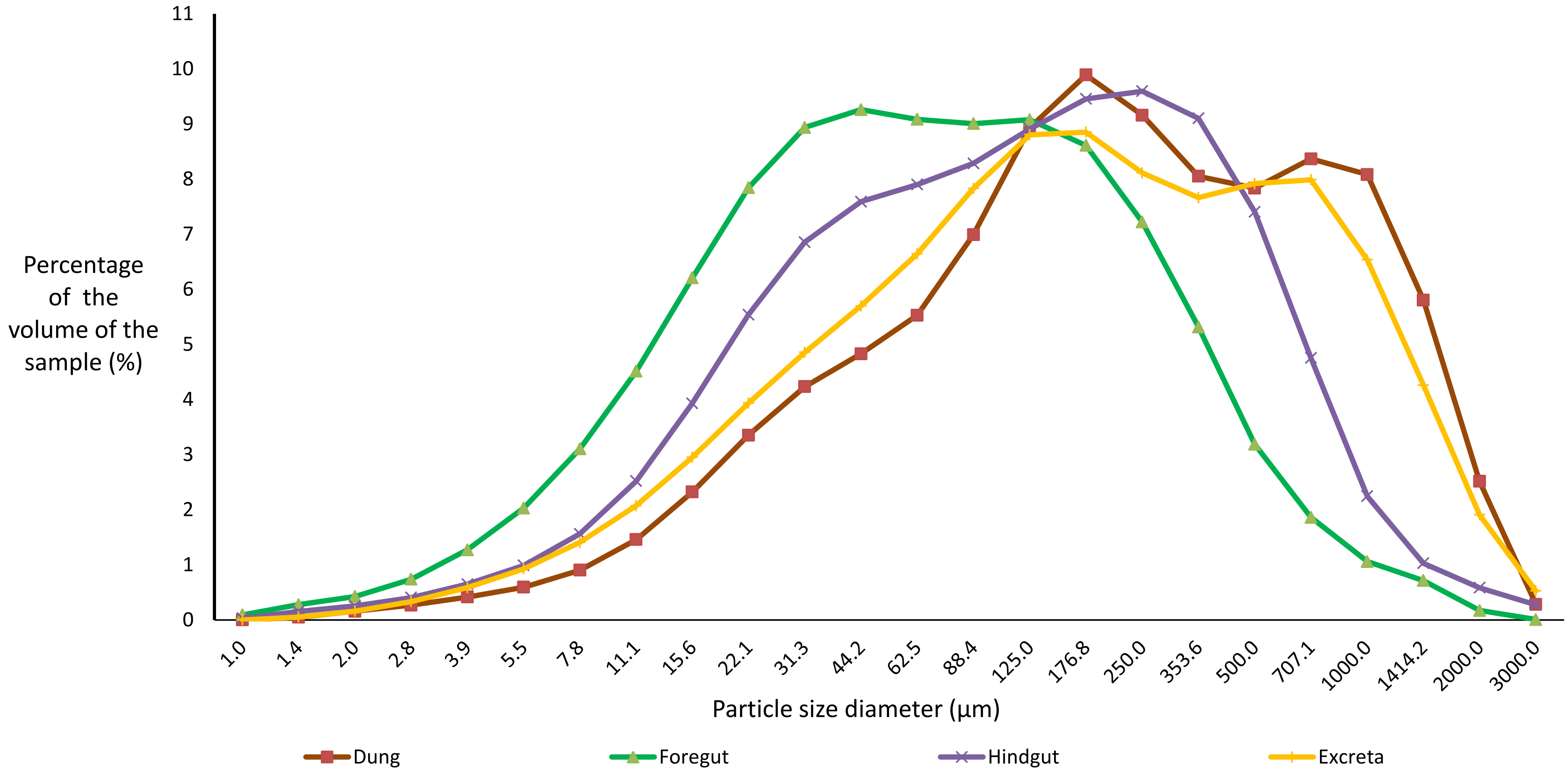

Figure 2.1. The average particle size distribution in micrometres of untreated cow dung (n = 6) and dung from the foregut (n = 6), hindgut (n = 6) and excreta (n = 5) of *Scarabaeus goryi*. The total area under the graph represents 100% volume of the samples. Note; the particle size diameter range is logarithmic.

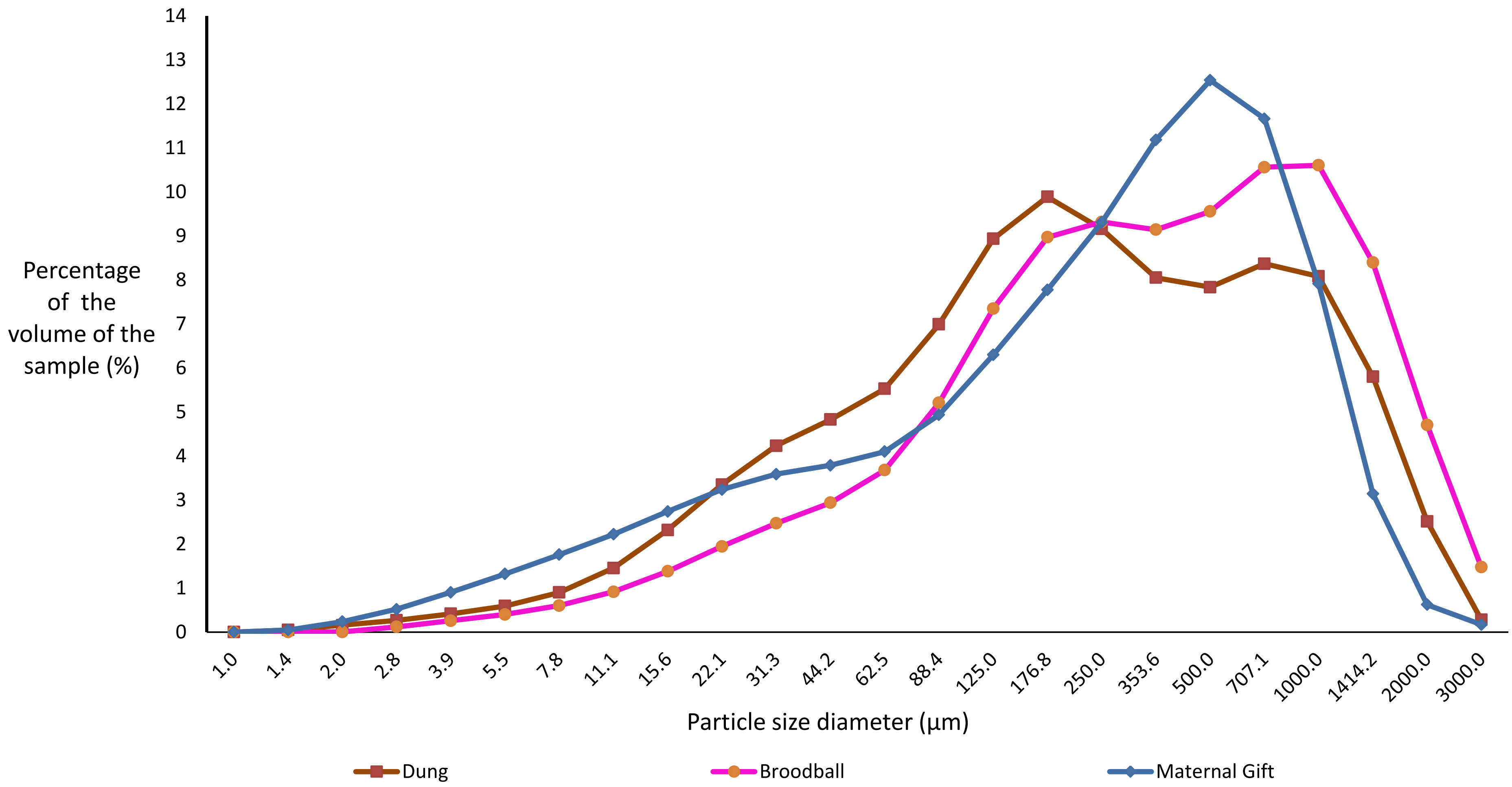

Figure 2.2. The average particle size distribution in micrometres of untreated cow dung (n = 6), the dung from the brood ball (n = 10) and maternal gift (n = 10) of *Euoniticellus intermedius*. The total area under the graph represents 100% volume of the samples. Note; the particle size diameter range is logarithmic.

The volume weighted mean diameter (Fig. 2.5 and Fig. 2.6) shows similar trends to the maximum particle size data. The dung had a larger average particle size and the size increased from the foregut to the excreta. Dung beetles manipulated the dung to select particles which were three times smaller in volume in the foregut compared to the dung when feeding ($p < 0.001$).

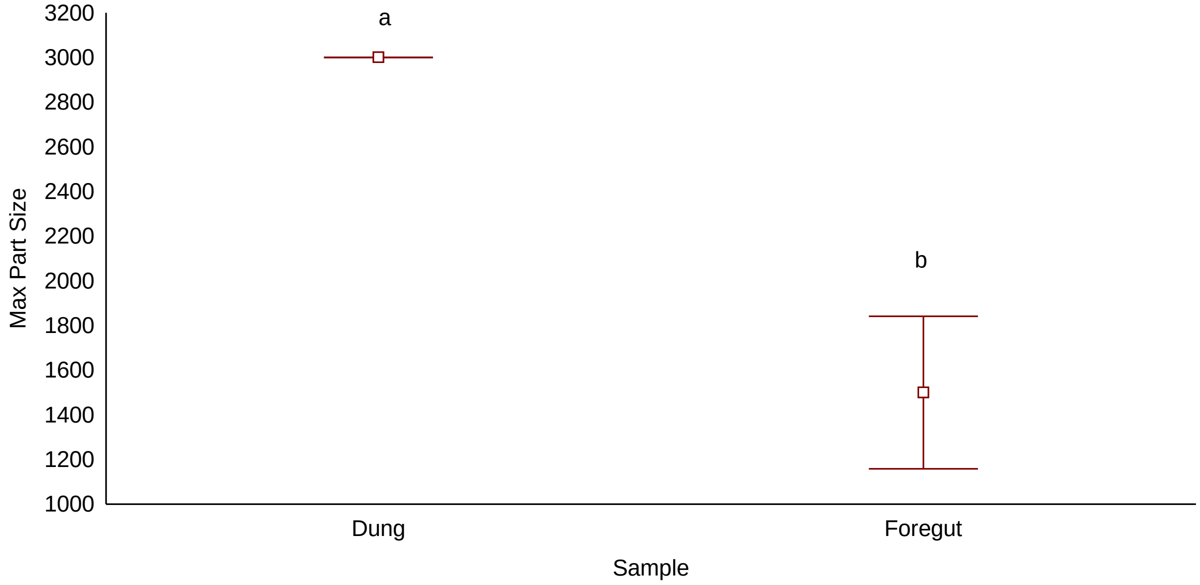

Figure 2.3. The mean maximum particle size diameter of untreated cow dung (n = 6) and dung from the foregut (n = 6) of *Scarabaeus goryi*. Bars represent standard error. Wilcoxon Rank Test, $p = 0.043$

As the dung moved along the gut passage, the mean diameter of the remaining particles increased. In the hindgut the particle size was still smaller compared to those in the dung ($p = 0.04$). At the end of the gut, the mean particle size of the excreta and the dung were not significantly different from each other ($p = 0.747$).

The smaller food particles are apparently assimilated leaving bigger particles in the gut. The particle size distribution seems to correlate with a pattern of digestion and movement of food through the gut as dung particles are moved from the foregut to the hindgut and leaving the gut as excreta.

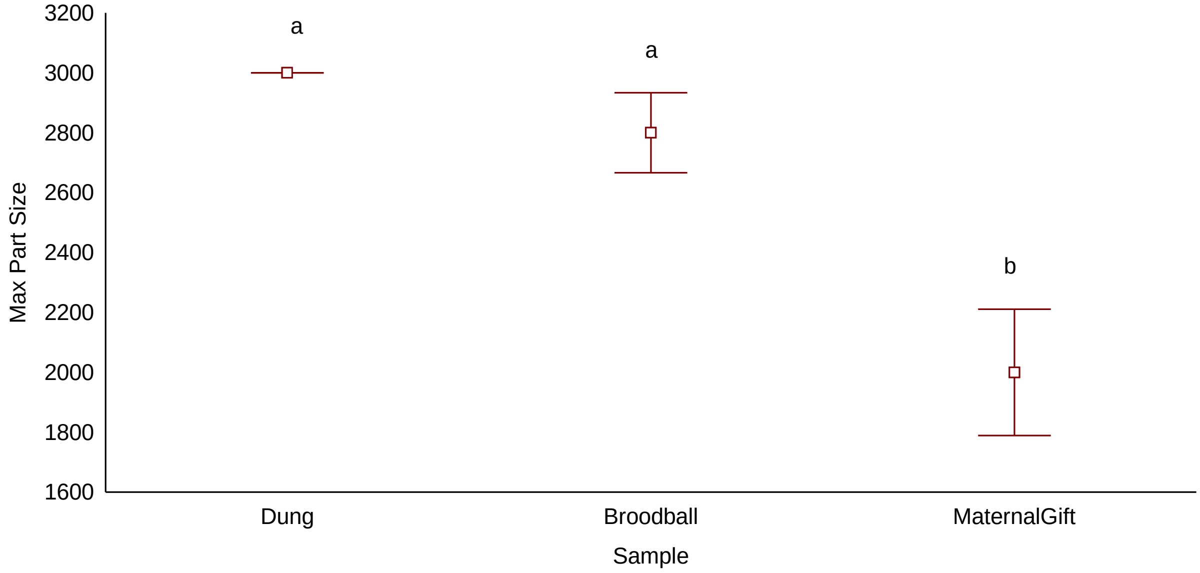

Figure 2.4. The mean maximum particle size diameter of untreated cow dung (n = 6) and, the dung from the brood ball (n = 10) and maternal gift (n = 10) of *Euoniticellus intermedius*. Bars represent standard error. Kruskal – Wallis Test. Kruskal-Wallis test: $H_{(2,26)}$ =12.296, p = 0.0021.

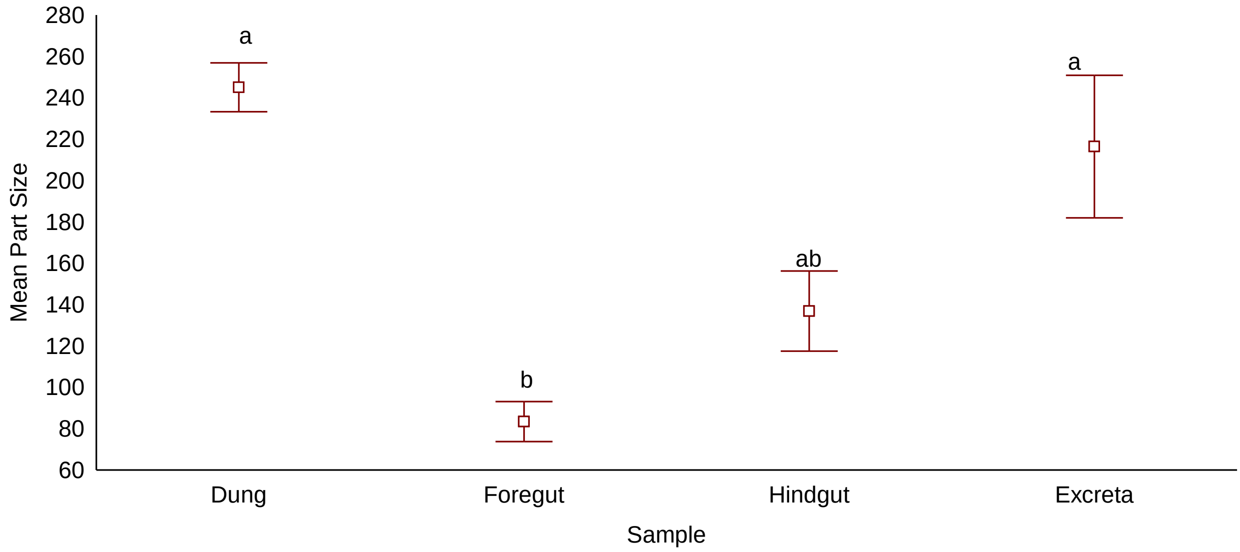

Figure 2.5. The volume weighted mean diameter of untreated cow dung (n = 6) and dung from the foregut (n = 6), hindgut (n = 6) and excreta (n = 5) of *Scarabaeus goryi*. Bars represent standard error. ANOVA; $F_{(3,19)}$ = 14.496; p < 0.001.

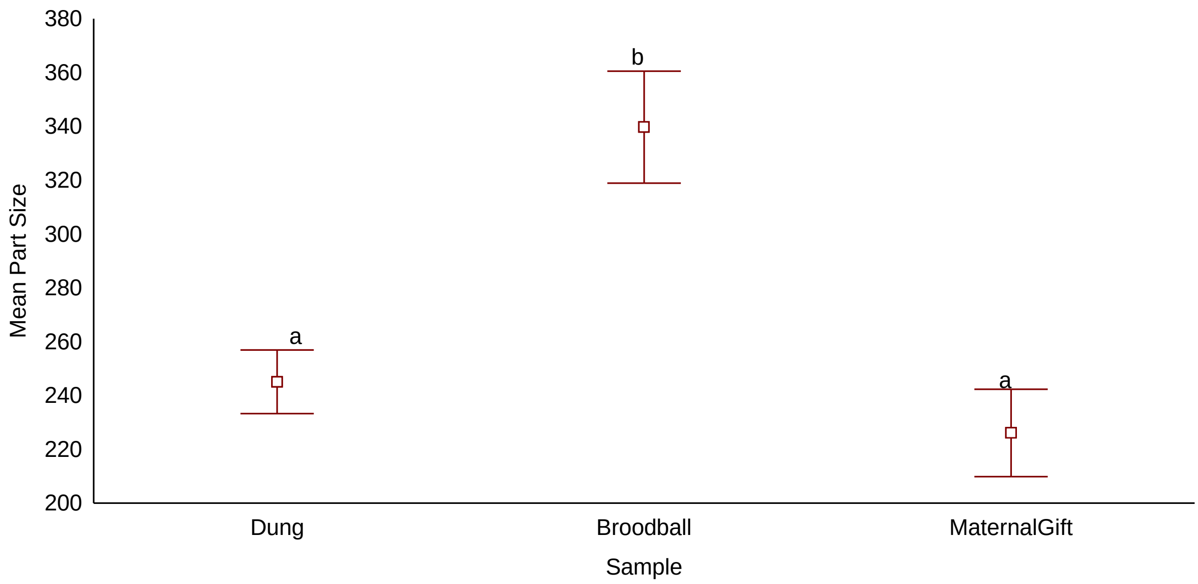

Figure 2.6. The volume weighted mean diameter of untreated cow dung (n = 6) , the dung from the brood ball (n = 10) and maternal gift (n = 10) of *Euoniticellus intermedius*. Bars represent standard error. ANOVA; $F_{(2,23)} = 12.247$; $p < 0.001$.

However, looking at the volume weighted mean there is proof that dung beetles select smaller dung particles when making the maternal gift compared to the particles used in creating the brood ball. The brood ball was on average made from coarser particles compared to those in the dung (p=0.007) and the maternal gift (p = 0.776). The sizes of the particles in the maternal gift were over two and a half times bigger than those in the foregut of *S. goryi*. The particle size of the excreta was similar to that of the maternal gift.

2.5 Discussion

Adult dung beetles which feed on wet dung, represented here by *Scarabaeus goryi*, suggest that they are not able to chew larger dung particles when feeding, they select smaller particles which they then ingest. These smaller particles are then digested and absorbed in the midgut and hindgut. By the time the dung is excreted, only relatively large particles that are left behind. *Euoniticellus* *intermedius* brood balls are made from much coarser dung particles than those ingested. Inside the brood ball, the maternal dung beetle leaves a provision for the larva to feed on after hatching which is made up of smaller particles than those in the brood ball in terms of maximum and mean particle size. However the mean particle size of the untreated dung and maternal gift are similar, suggesting that the brood ball is made up of deliberately selected larger particles, or that all of the preferred edible-sized particles have been removed or eaten.

The results of this study show that during feeding, dung beetles select particles from the dung to eat which are much bigger than those previously estimated (Hata and Edmonds 1983; Holter 2000; 2004; Holter and Scholtz 2007; Holter *et al*. 2009). The average maximum particle size diameter was found to be just over 800 µm, which is over eight times bigger than what was previously estimated for *Circellum bacchus* Fabricius (106 µm), the species with the largest ingestible particle size diameter. According to Holter and Scholtz (2007), there is a relationship between body size and the size of the maximum particle that can be ingested in Scarabaeni dung beetles. This means that *Scarabaeus goryi* which is one of the larger dung beetles, is one of the best species to test for the maximum particle size that dung beetles can ingest. And therefore previous measurements underestimated the particle size, therefore the nutrient content of the dung which is ingested.

With these huge differences in the maximum particle size diameter between this study and previous ones, we can say that the maximum particle sizes that can be ingested by other Scarabaeini dung beetles must be bigger than those estimated sizes. All wet dung feeders have similarly structured mouthparts (Hata and Edmonds 1983; Holter and Scholtz 2000) and feed in the same way by filtering smaller particles. *Geotrupes*, *Aphodius* and Hydrophilids, which also feed on wet dung, are likely to be able to ingest particles bigger than those estimated by Holter and Scholtz (2000) and Holter (2004). Therefore the theory underlying how this filtering system might work should be reinvestigated by trying to relate the physical structures of the mouthparts to the actual size of the particles actually ingested.

As the food passes through the gut, the proportion of smaller particles decreases which shows that the smaller particles are the ones that are being digested. In the gut, the food particles are presumably further broken down and then absorbed into the body, this could be the reason why the size of dung particles measured by Holter and Scholtz (2000) in the midgut area show a discrepancy between particles size estimations from latex balls and actual measurements for *Aphodius* dung beetles in that study.

Since undigested and partially digested particles in the dung are bigger in size, smaller particle selection could be an adaptive way of avoiding bigger particles. The selection of smaller particles for feeding has more benefits beyond reducing the chewing effort. Selecting smaller particles also allows collection of microbiota like bacteria and fungi which have higher nitrogen content (Holter and Scholtz 2007). Smaller particles also have a larger surface to volume ratio for digestion and absorption into the body. Holter and Scholtz (2007) has also shown that smaller particles contain a higher nitrogen content compared to larger particles, selecting smaller particles might be a way of increasing nitrogen content of the dung which dung beetles are going to feed on.

Even though the dung beetle larva does have biting mouth parts and can chew larger pieces of dung than the adults, increasing the nitrogen content of the dung forming the brood ball can help with growth and development. The larvae only feed on what is available to them inside the brood ball until pupation (Emlen 1994). After hatching of the egg, the larva feeds on the maternal gift which is also used to anchor the egg and on the inner wall of the brood ball (personal observation). Reducing the particle size of the maternal gift can presumably help speed up feeding after hatching and that could help with quicker assimilation of nutrients. Selecting smaller particles can also help by improving the diet quality if they are higher in nitrogen as Holter (2016) suggests, so providing the maternal gift will help with the larval development. What remains unknown is the origin of the maternal gift, its maximum particle size overlaps with the ingested material suggesting that it is regurgitated material, but the mean particle size suggests that it may be of faecal origin.

Underground where dung beetles lay their brood balls is moist and warm, these conditions make it possible for dung and soil microbes to be active (Gallardo and Schlesinger 1992). Selecting coarser particles when constructing the brood ball, could be a way for creating a stable structure which will withstand degradation by microbes until the larva hatches and can manage the microbes, or until the adult emerges. The use of material with particles of a certain size when building nests is very common to other organisms. *Odontotermes* nr. *pauperans* is known to build its mounds with fine grained soil particles. When presented with soil of different sized particles, workers will modify the soil texture for building certain parts of their nest (Jouguet *et al*. 2002). Manipulation of the dung particles to build the brood ball creates a suitable nest that will be the best for the offspring to develop in.

The average maximum particle size in untreated cow dung was 3000 µm which is the upper end, of the Malvern Masterizer 2000 measuring range. The average particle size of cow dung has been measured to be 3800 µm (Clauss *et al*. 2015). Dung beetles are able to select particles on average 3800 µm to about 100 µm for adults to feed on and to about 203 µm for their larva to feed on. These show how dung beetles have evolved to use their mouthparts to overcome the challenge of having to grind particles.

Filter feeding is very common in most organisms that feed on food particles that are suspended in liquids. Many crustaceans, Baleen whales and bivalves feed by filtering particles (Merritt and Wallace 1981; Burns 1968). These organisms have specialised mouthparts that select finer particles from the water and ingest them (Cheer and Koehl 1987). Some species of flamingo like *Phoenicopterus roseus* feed on the larvae of small insects in salt pans, which most of the time are mixed with other materials (Deville *et al*. 2013). Filter feeding helps organisms to separate food from inorganic substances like silt and sand, and also with selecting the part of the diet that is required.

Using the particle sizes estimated from feeding dung beetles glass or latex balls, Holter and Scholtz (2007) sieved dung falling in to those ranges found in their feeding studies to measure the nutrient content, to see if smaller particles have a higher nitrogen content. They found that when selecting dung particles up to 106 µm, the nitrogen content will increase up to 4.64%. All previous studies underestimated both the maximum and the mean particle sizes which dung beetles select when feeding or creating a nest. The difference in particles sizes from this study and previous ones have implications for the nutrient content of the dung, either eaten or used to make a nest. The next chapter will investigate the selected dung content to test if the nutrient content does change and compare this with previous studies.

Chapter 3 Nutrient manipulation by dung beetles

3. 1 Introduction

3.1.1 Importance of nutrients

Insects need amino acids, nitrogen, phosphorus, carbohydrates and other important nutrients for growth, development, reproduction, and for energy to perform activities (Chefurka 1954; Telang *et al*. 2002; 2003). Getting enough nutrients from a food source is a challenge for most insects. When it comes to optimal growth and performance, balancing nutrients is the dominant factor in self-selecting a diet (Raubenheimer and Simpson 2003; Behmer and Joern 2008). To get the best out of their food, many insects can self-select nutritional components consciously, to adjust the proportions of the required nutrients like protein and carbohydrates ratio (Waldbaeur *et al*. 1984; Waldbauer and Friendman 1991).

3.1.2 Nutrient manipulation

Some insects have found a way of getting most nutrients from the food; one way to compensate is by eating a lot (Behmer 2009). To reduce digestion effort some insects have resorted to detrivory, they eat decaying organisms or the faeces of organisms including their own (Price *et al*. 2011). Some insects use chemoreceptors to select certain particles in their food with the most protein or carbohydrate content based on their requirements (Altner and Prillinger 1980). As detrivores, dung beetles adults are capable of balancing diet this way.

Dung beetle are known to exploit specific portions of the dung that they feed on, but their nutritional requirements still remain unknown (Holter 2016). Dung beetles have shown the ability to distinguish between dung of different animals and also dung which has higher water content or nutritional value (Al-Houty and Al-Musalam 1997; Moczek 1998). A study by Moczek (1998) suggested that adult dung beetles are able to measure the resource quality of the dung when they provision brood balls for their offspring. He found that when *Onthophagus taurus* was reared on horse dung which was of higher quality (lower C:N ratio and higher nitrogen content) they used less dung in making brood balls compared to when they were reared using cow dung which was of relatively low quality. How they make this assessment is unknown, but it implies that the higher quality components are not selected out of the dung mass but less of it is required for the larva to complete development.

Paracoprid and telecoprid dung beetles provide some additional food for their offspring inside the brood ball using the maternal gift, a dung paste laid at the base of the brood ball by the maternal dung beetle. The previous chapter has shown this to be fairly large dung particles of unknown origin, which could be either oral or faecal and unknown nutrient quality. The amount and quality of the dung in the brood ball will influence offspring size which is directly related to the male reproductive success, and survival and fecundity in females (Hunt and Simmons 1997; 2000; 2001; Hunt *et al*. 2002), one would expect the adult making the ball to respond in some way to dung quality.

Because plants are composed of mainly carbohydrates, they usually have excess carbon while nitrogen content maybe low to very low, which makes it the most limiting nutrient in the diet (Mattson 1980; Chapin III *et al*. 1986, Douglas 2009). Nitrogen in plant tissue ranges between 0.03 to 7.0%, and in dung it ranges from 1.14 to 3.49% (Chown and Nicholson 2004; Holter and Scholtz 2007).

Dung beetles which feed on wet dung are thought to increase nitrogen content of the dung they ingest when feeding through selective feeding (Holter and Scholtz 2007; Holter 2016). They do this by manipulating dung particles, by selecting smaller particles (Chapter 2). Holter and Scholtz (2007) found that by selecting smaller particles, the C: N ratio decreases to less than half of the untreated dung and the nitrogen content increases twofold. When selecting dung particles sized 0-20µm from elephant bulk dung, the C: N ratio decreased from 38.9 to 15.2 and rhino dung from 34.2 to 12.7. The nitrogen content of elephant dung increased from 1.14 % nitrogen in bulk dung to 2.33 % in the dung particles sized 0-20 µm, and from 1.20 % in bulk dung to 2.51% dung particles sized 0-20 µm in rhino dung. However these experiments were done by sieving the dung, using the particle sizes estimated from feeding dung beetles with latex or glass balls. As chapter two has shown this method dramatically underestimated the actual size of dung particles consumed, and therefore affects the measurement of the nutrient content.

Dung beetles have also been shown to manipulate dung particles when nesting. Byrne *et al*. (2013) compared the particle sizes of the dung in the brood ball and maternal gift, and found that *E. intermedius* selects smaller dung particles ranging from 2 to 4 mm^2 to make brood balls from dung with average particle size of 16 – 22 mm^2. It is thought that this manipulation of dung particles is how the proportion of beneficial nutrient is increased (Byrne *et al*. 2013). However Byrne *et al*. (2013) estimated the particle size using image analysis software on wet slides of the dung. Chapter two again has shown the values of the dung, brood ball and maternal gift to be different from the ones measured by the Malvern Mastersizer which analyses each sample completely. The dung beetle nest (brood ball and maternal gift mixed) has been shown to have about half of the C: N ratio compared to the dung (G Lindsay-Smith unpublished data).

3.2 Aims and objectives

The purpose of this study was to measure the changes in the nutrient content of the dung when adult dung beetles selectively feed and also when they create a brood ball as a larval nest. Nitrogen and carbon content was measured from the dung mass, and the gut contents and excreta of the larger dung beetle, *S. goryi* to test if adult beetles can manipulate the nitrogen content of their food by particle size selection and absorb it. The dung was also compared with the brood ball and maternal gift to see if maternal dung beetles manipulate nutrient quality using nitrogen and carbon, when provisioning for their offspring. Nitrogen and carbon stable isotopes of all samples were measured to determine if dung particles that are being selected can be identified by their isotope signatures.

3.3. Materials and Methods

3.3.1 Sample preparation

Two study species were used for nutrient analysis, *S. goryi* for measurements of gut content and *E. intermedius* for brood ball and maternal gift measurements. Eight *S. goryi* dung beetles were placed in a 3 litre container one third filled with cow dung for 48 hours and then individually sacrificed by immersion in boiling water for 30 seconds. The full gut was removed from the body by dissection and contents of the foregut and the hindgut were removed from the peritrophic membrane and wrapped in tin foil. Faecal samples were also collected from the containers where *S. goryi* feeding, they were then separated from the peritrophic membrane and wrapped in tin foil.

Fifteen pairs of *E. intermedius* were placed in 3 litre containers filled with 2 litres of soil and 500ml of dung and left to breed for about a week. After a week, the soil was sieved out and brood balls were collected. From each pot one freshly laid brood ball which still had an egg inside was dissected. The soil was scraped off each brood ball and the maternal gift was also separated from the inner surface of the brood ball. The brood balls and maternal gift were individually wrapped in tin foil.

From the dung that was used in both the feeding and nesting experiments, eleven dung samples were collected and wrapped in tin foil. All samples were dried and sterilised at 60°C in an oven for 24 hours.

3.3.2 Nutrient measurements

Nutrient measurement was done at the iThemba labs, Johannesburg at the University of Witwatersrand. Nitrogen and carbon content was measured using mass spectrometry. Analyses were done on a Flash HT Plus elemental analyser coupled to a Delta V Advantage and isotope ratio mass spectrometer by a ConFloIV interface (all equipment supplied by ThermoFisher, Bremen, Germany).

3.3.3 Stable Isotope measurements

Stable Isotope analysis was done at the iThemba labs, Johannesburg at the University of Witwatersrand. All samples were wrapped in tin cups and weighed at 0.6 mg. Nitrogen and Carbon content was measured using mass spectrometry. Analyses were done on a Flash HT Plus elemental analyser coupled to a Delta V Advantage and isotope ratio mass spectrometer by a ConFloIV interface supplied by ThermoFisher, Bremen, Germany.

3.3.4 Data analysis

All statistical analysis was done using Statistica software package version 8 (Stat Soft Inc. 2007) and R version 2.15.2 (R Foundation for Statistical Computing 2012). All the data was tested for normality first using the Shapiro-Wilks test of normality. One-Way Analysis of variance (ANOVA) was used to test for significant differences between the samples for nitrogen and carbon content, carbon to nitrogen ratios and stable isotopes at 95% significance level. Tukey's HSD was used for post hoc tests to test differences between samples.

3.4 Results

Nitrogen content differed between the different dung samples from both the gut of *S. goryi* and brood balls *E. intermedius* (Fig. 3.1 and Fig. 3.2). The foregut contents from *S goryi* had 3 times more nitrogen than the untreated dung on which the dung beetles had fed ($p < 0.001$). The foregut contents N values were significantly the highest of all the samples. All foregut samples were above 4%, which is more than double the nitrogen content of the raw dung sample, some foregut samples were over 6%, . The hindgut contents had significantly lower nitrogen content compared to the foregut ($p < 0.001$), where the nitrogen content decreased from an average of 5.14% in the foregut to 3.76% in the hindgut. The excreta had the lowest compared to the foregut and the hindgut. The nitrogen content of the foregut contents therefore decreased to that of the excreta to the same level of the dung ($p < 0.001$).

The maternal gift of *E. intermedius* had a higher nitrogen content compared to the untreated dung ($p < 0.001$) and the brood ball ($p < 0.001$). The nitrogen content of the maternal gift was almost double that of the dung and the brood ball. There were no nutrient level changes between the raw dung and the brood ball created by the maternal dung beetle ($p = 0.453$). Even though the nitrogen content was significantly greater in the maternal gift, its nitrogen content was still less than half of that of the foregut.

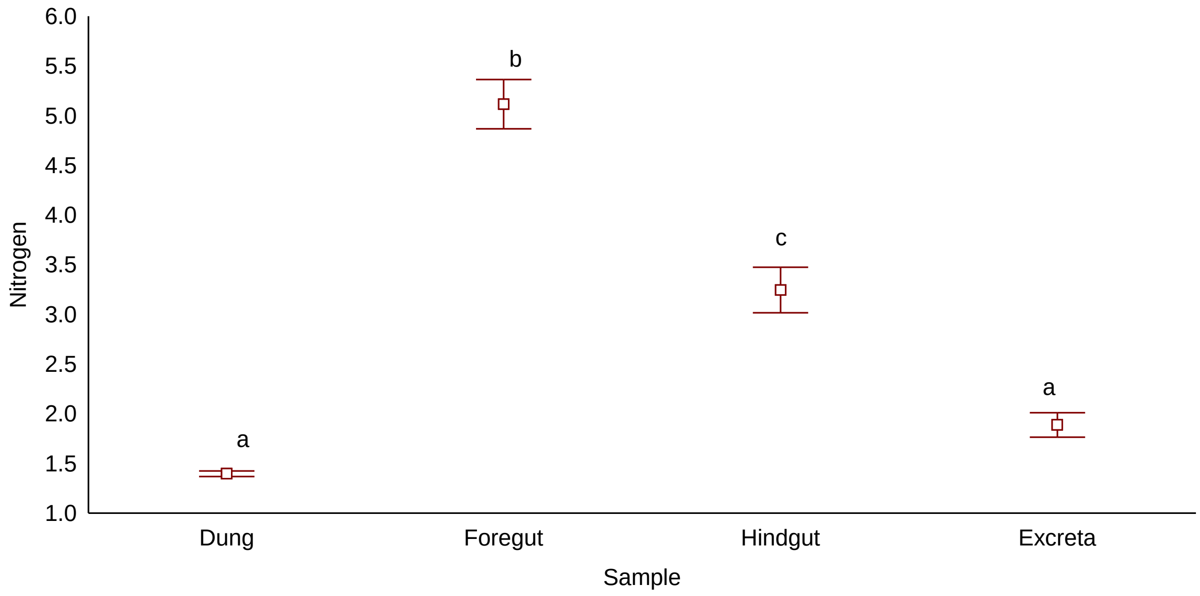

Figure 3.1. The nitrogen content of untreated cow dung (n = 11) and dung from the foregut (n = 8), hindgut (n = 7) and excreta (n = 7) of *Scarabaeus goryi*. Bars represent standard error. ANOVA, $F_{(3,28)} = 116.35$, $p < 0.001$.

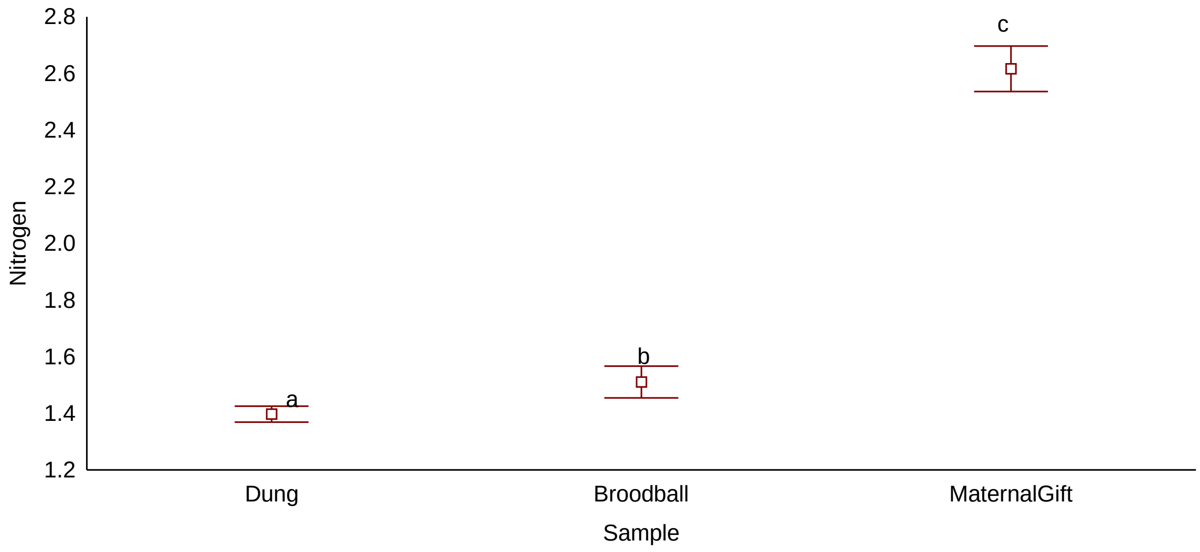

Figure 3.2. The nitrogen content of untreated cow dung (n = 11), and dung from the brood ball (n = 15) and maternal gift (n = 15) of *Euoniticellus intermedius*. Bars represent standard error. ANOVA, $F_{(3,38)} = 114.88$, $p < 0.001$.

Compared to the nitrogen content values, there is no obvious trend in the carbon content of the samples even though there are significant differences between samples (Fig. 3.3). The carbon content of the dung is not significantly different to that of the foregut (p= 0.543) and the hindgut (p=0.970), suggesting that particle size selection by the feeding dung beetle is not for the accumulation of carbon.

From the foregut contents to the excreta, on average, about 11% of the carbon content was removed. There is no difference between the carbon content of the maternal gift and the raw dung, nor the brood ball (Fig. 3.4). The brood ball had a significantly lower carbon content than the dung (p<0.001). The beetle excreta had the same carbon content as that of the maternal gift, reinforcing the conclusion that the maternal gift is primarily faecal in origin.

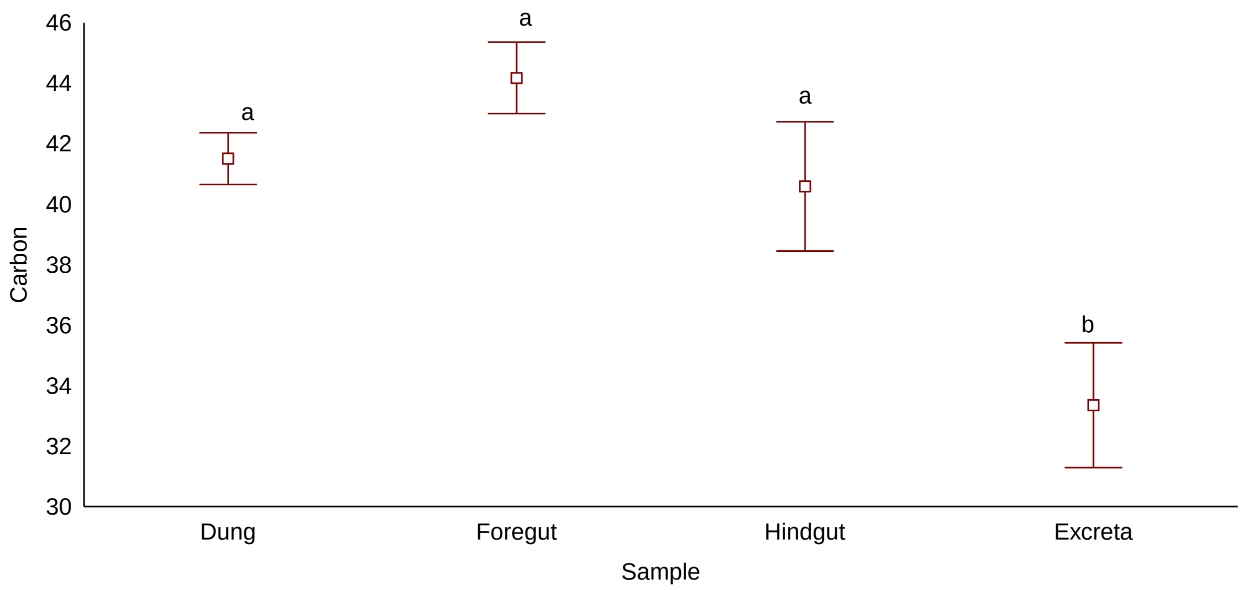

Figure 3.3. The carbon content of untreated cow dung (n = 11) and dung from the foregut (n = 8), hindgut (n = 7) and excreta (n = 7) of *Scarabaeus goryi*. Bars represent standard error. ANOVA, $F_{(3,29)}$= 8.66, p< 0.0001.

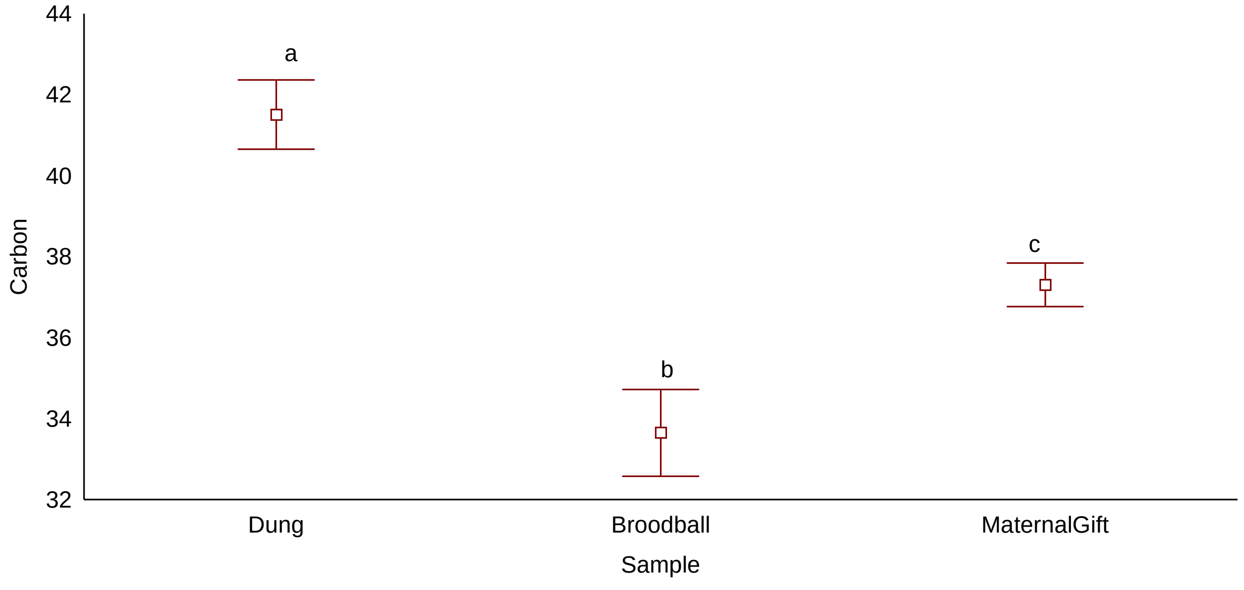

Figure 3.4. The carbon content of untreated cow dung (n = 11), and the dung from the brood ball (n = 15) and maternal gift (n = 15) of *Euoniticellus intermedius*. Bars represent standard error. ANOVA, $F_{(2,37)}$= 19.426, p< 0.0001.

The C:N ratios show a similar but reverse trend to nitrogen content (Fig. 3.5 and Fig. 3.6). The C:N ratio of the foregut contents is 3 times lower than that of the untreated dung. The C:N ratio increases as the ingested food moves through the gut passage, from the foregut to the excreta. The C: N ratio for the excreta is twice that of the foregut samples and half to that of the untreated dung ratio, indicating that even though carbon is removed from the gut contents during digestion, relatively more nitrogen is absorbed from the beetles' gut. The C:N ratios show a clearer difference between the dung and other samples compared to the nitrogen and carbon contents ($p < 0.001$).

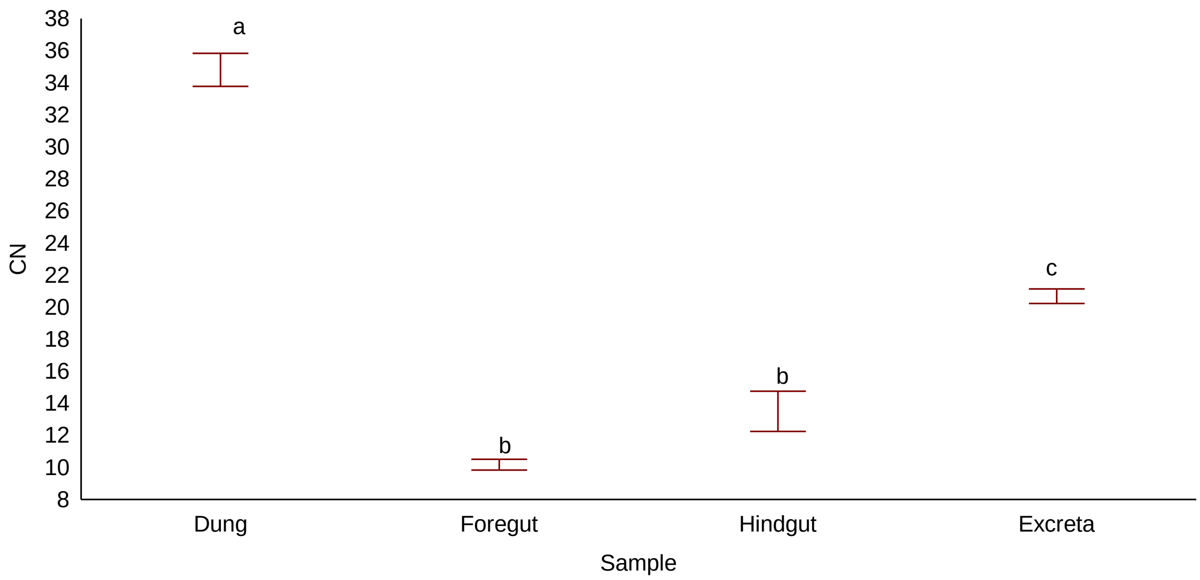

Figure 3.5. The nitrogen to carbon ratio of untreated cow dung (n = 11) and dung from the foregut (n = 8), hindgut (n = 7) and excreta (n = 7) of *Scarabaeus goryi*. Bars represent standard error. ANOVA, $F_{(3,29)} = 116.84$, $p < 0.0001$.

The difference in the $\delta^{15}N$ isotope signal between the dung and foregut contents (Fig. 3.7 and Fig. 3.8) is similar to that of the nitrogen content and the C:N ratio. Untreated dung $\delta^{15}N$ values were significantly lower compared to the foregut contents $\delta^{15}N$ values, where $\delta^{15}N$ values increased by 1‰ ($p<0.001$). There was an overall decrease in the $\delta^{15}N$ values along the gut but there was no significant difference between any of the samples from the different gut regions. The heavier nitrogen isotope was selected during feeding and later absorbed in the gut of the beetle.

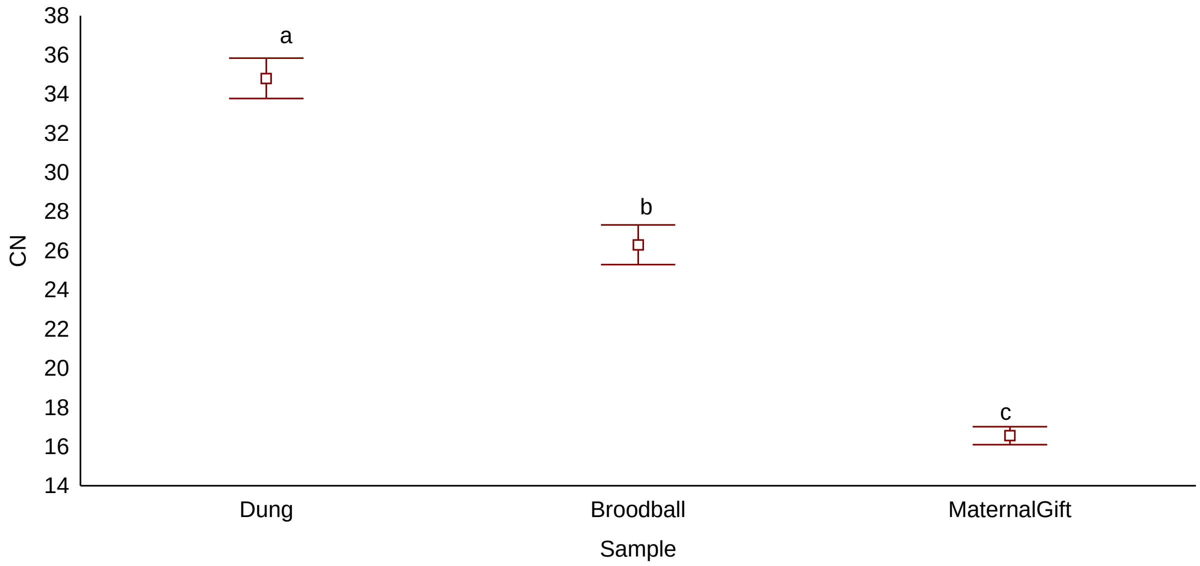

Figure 3.6. The nitrogen to carbon ratio of untreated cow dung (n = 11), and the dung from the brood ball (n = 15) and maternal gift (n = 15) of *Euoniticellus intermedius*. Bars represent standard error. ANOVA, $F_{(2,28)} = 107.91$, $p<0.0001$.

When it comes to nesting material, the maternal gift was significantly different from both the brood ball (p = 0.02) and the maternal gift (p=0.086) (Fig. 3.8). The brood ball had a higher $\delta^{15}N$ compared to the untreated dung and the maternal gift had the lowest $\delta^{15}N$ compared to both the untreated dung and the brood ball. However, the maternal gift had significantly lower $\delta^{15}N$ values compared to the brood ball (p<0.001). Yet again, the values obtained for the maternal gift were closer to those of the beetle's excreta, rather than the foregut contents, for the $\delta^{15}N$ isotope, supporting the conclusion that the maternal gift is largely maternal faeces.

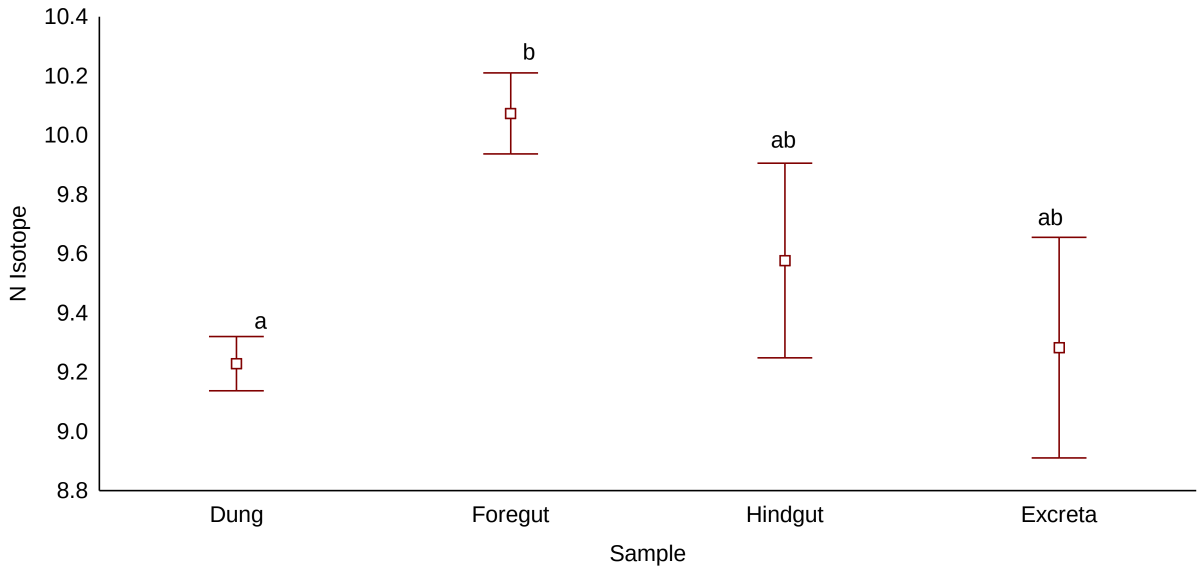

Figure 3.7. The nitrogen isotope ratio of untreated cow dung (n = 11) and dung from the foregut (n = 8), hindgut (n = 7) and excreta (n = 7) of *Scarabaeus goryi*. Bars represent standard error. ANOVA, $F_{(3,29)} = 2.979$, p= 0.047.

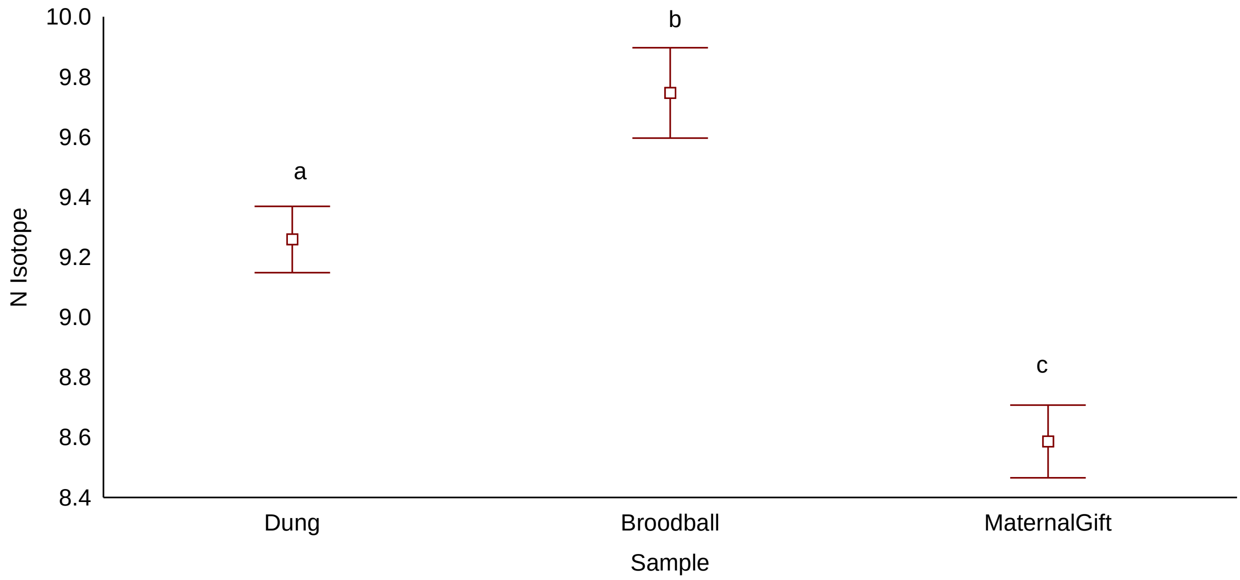

Figure 3.8. The nitrogen isotope ratio of untreated cow dung (n = 11), and the dung from the brood ball (n = 15) and maternal gift (n = 15) of *Euoniticellus intermedius*. Bars represent standard error. ANOVA, $F_{(2,38)} = 22.006$, p<0.001.

Carbon stable isotopes (Fig. 3.7) showed the same trend as the carbon content. There were significant differences between the $\delta^{13}C$ values in some of the samples. There was no significant difference between the dung and the foregut (p=0. 893) suggesting no manipulation of the carbon content, but that there was a decrease in the $\delta^{13}C$ values along the gut passage. The $\delta^{13}C$ values decreased by 1.34‰, from the foregut to the excreta (p<0.001). Like the $\delta^{15}N$ results, the lighter carbon isotope is being absorbed preferentially as dung moves through the gut.

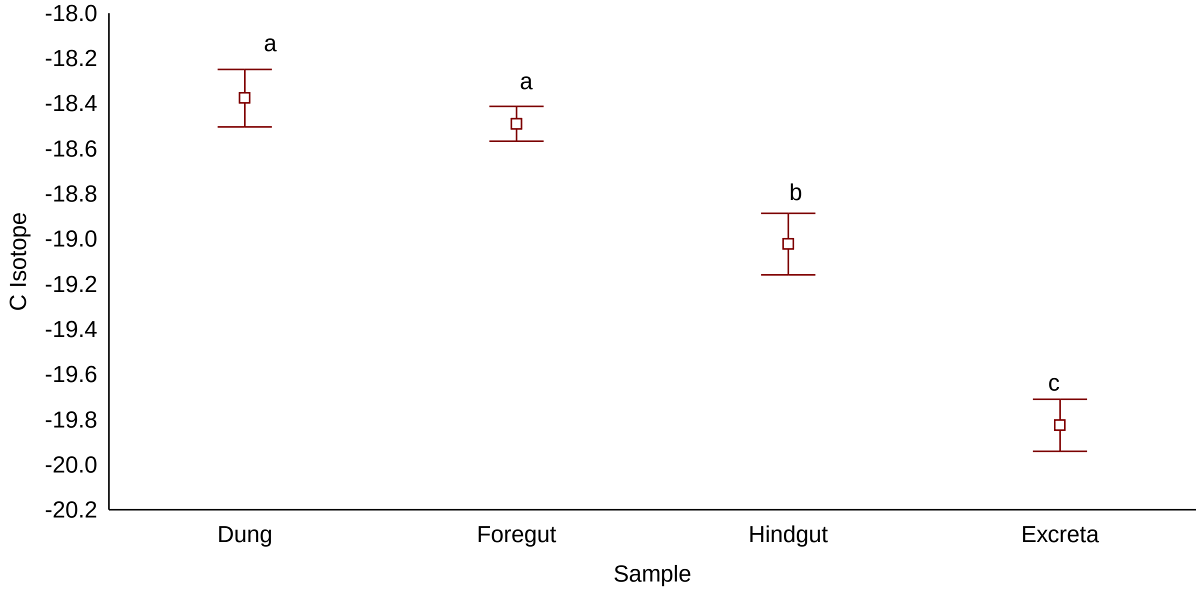

Figure 3.9. The carbon isotope ratio of the untreated cow dung (n = 11) and dung from the foregut (n = 8), hindgut (n = 7) and excreta (n = 7) of *Scarabaeus goryi*. Bars represent standard error. ANOVA, $F_{(3,29)} = 28.994$, p<0.001.

In the nesting material, the results were opposite of those of the carbon content (Fig. 3.8). There was no significant difference between the dung and the brood ball (p<0.001) again suggesting minimal selection in terms of food quality for the larva. The brood ball $\delta^{13}C$ values were 1.03‰ (significantly) higher than those of the maternal gift. There is a selection for the lighter isotope when preparing the maternal gift.

The $\delta^{13}C$ suggests that the maternal gift is of faecal origin. In both carbon and nitrogen isotope analysis the trend of isotope absorption looks similar, but with the carbon isotope, it is the lighter isotope that is being absorbed into the body.

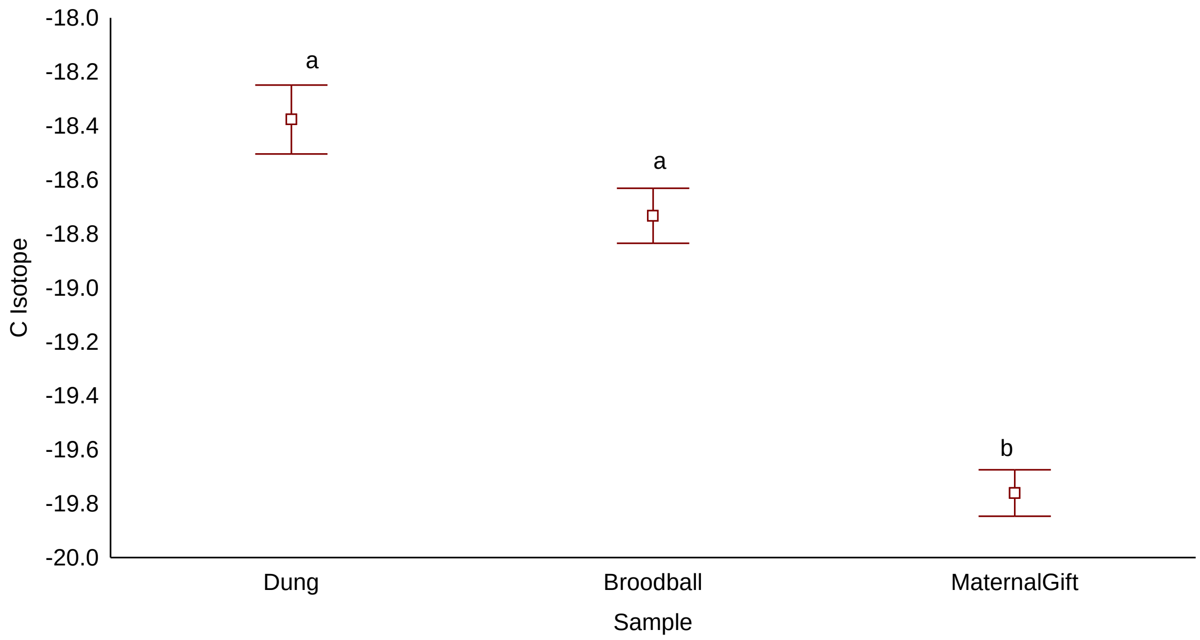

Figure 3.10. The carbon isotope ratio of the untreated cow dung (n = 11), and the dung from the brood ball (n = 15) and maternal gift (n = 15) of *Euoniticellus intermedius*. Bars represent standard error. ANOVA, $F_{(2,38)} = 48.194$, $p<0.001$.

Discussion

These results show that when dung beetles manipulate particle size in the wet dung when feeding and nesting, they can increase the nitrogen content and decrease the C:N ratio of the adult food and also in the maternal gift. The maternal gift has a reduced carbon content, but nitrogen remains the same as the brood ball content.

This confirms the works of Holter and Scholtz (2007) who stated that dung beetles select dung particles which have higher nitrogen content or a lower C: N ratio when feeding, which they predicted would increase the nitrogen content of the ingested dung between two and, two and a half times.

The results from this study showed that dung beetles actually increase the nitrogen content of ingested dung by more than three times and reduce the C: N ratio by a third compared to the raw dung. There was no difference between the carbon content of the dung and that which was ingested by dung beetles. This means that dung beetles are not carbon limited, contrary to what Holter (2016) claims, since carbon was not selected from the dung by the beetles. This is not surprising as carbon is in excess in plant matter.

Nitrogen content decreased sharply from the foregut to the hindgut and excreta suggesting that the initial increase in nitrogen content was intentional manipulation by the dung beetles. The changes from the foregut to the excreta, in both nitrogen and carbon content suggest that both nitrogen and carbon were absorbed in the gut by the dung beetles. The changes of nutrient content along the gut passage correspond with the general trend in feeding and assimilation, nutrients are absorbed as the food passes through the gut (Klowden 2007). Nitrogen was probably absorbed as protein or amino acids, and carbon as carbohydrates or sugars along the gut (Behmer 2009). The needed nutrients get assimilated into the body based on the requirements of the body.

Even though the dung beetle larva has biting mouth parts and can chew large dung particles (Scholtz *et al* 2009), this study shows that the maternal gift is probably important for feeding the newly hatched larva. The maternal gift has been shown not to be essential in transmission of microbes from the mother to the larvae (Byrne *et al*. 2009), and was suggested as larval food or to work as an anchor for the egg. The nitrogen content and the C: N ratio of the maternal gift are shown here to be almost half the C: N of the brood ball. The fitness of the larva depends on what the larva eats, which is all found inside the brood ball. This has to be sufficient for the whole larval stage, so the maternal gift is very important in the development of the larva (Emlen 1994).

Holter and Scholtz (2007) suggested that the increase in the nitrogen content increase and the C: N ratio decrease is caused by selection of very fine particles, ranging between 0 and 106 µm, the biggest particles they estimated dung beetles can ingest, while Hata and Edmonds (1983) suggested the particles range from 2 to 200µm (see Chapter 2). In this study the size of particles in the foregut exceeded 1400 µm. This shows that the changes in nitrogen cannot be due to the smaller particles alone, since the nitrogen content increases with decreasing particle sizes as stated by (Holter and

Scholtz 2007), dung beetles must be selecting particles of certain nature in the dung. In this study, the maximum particle size that dung beetles were found to ingest was over 10 times that of Holter and Scholtz (2007) measured and the nitrogen content was also greater than theirs by a percentage. This shows that the changes are not only due to smaller particles being selected as dung beetles are selecting much larger particles, but are selecting certain particles in the dung with specific nutrient value.

From the stable isotope results, it is clear that there was a selection of certain dung particles. Based on the nitrogen isotopes signature, there was a selection of the heavier isotope in the foregut which were then assimilated, which can be seen, first by the increase of the isotope ratio from the untreated dung to the foregut, and then a decrease from the foregut to the excreta where the isotope ratio went back to level of the dung. Selection of heavier isotope is common among most organisms, like chimpanzees (Phillips and Cornell 2016). Dung beetles are probably selecting gut epithelial cells from the herbivore in its dung from which have a higher isotopic signature compared to plant material, along with microbes (Schoeninger 2014). There are no studies analysing the stable isotope signatures of the gut contents of insects. This suggests that dung beetles could be detecting the particles which have higher nitrogen content and selectively consuming them.

With the carbon stable isotope ratio there is no change in the isotope ratio from the dung to the foregut as there was apparently no manipulation of carbon, but there is a difference in the excreta. With the carbon isotopes, there was an absorption of the lighter isotope. This could mean that dung beetles are selecting the C_3 content over the C_4 content. The C_3 grasses have a different photosynthetic pathway compared to the C_4 grasses, this leads to different structural and nutrient properties (Barbehenn *et al*. 2004a). In general C_3 plants tend to have more proteins, sucrose, water and biomass compared to C_4 plants (Barbehenn *et al*. 2004a). On the other hand C_4 tend to be tougher and have more fibres, this makes it difficult for insects to chew and sequester nutrients from them (Heldorn and Joern 1984; Sponheimer *et al*. 2003; Barbehenn *et al*. 2004a). Generalist grasshopper *Melanoplus sanguinipes* and caterpillar *Spodoptera frugiperda* have been observed making similar choices of choosing the lighter carbon isotope or C_3 plant matter when feeding on grasses (Barbehenn *et al*. 2004b,c). Again this confirms that dung beetles are making an informed decision when selecting their food.

When nesting, the stable isotopes confirm both nutrient content results, that there were no changes in the isotopic ratios when the brood ball was being constructed. Therefore brood balls are not selected on their food value. However there was a change in both the nitrogen and the carbon isotopes when the maternal gift was made. The maternal gift shows an isotopic signal similar to that of the dung beetle faeces. However the nitrogen content of the maternal gift is higher than that of the faeces, so some other material might be added to it by the maternal dung beetle.

Manipulation of food in insects is very common. Many insect species can detect food with higher nutrient content and some can select food with the best amount of required nutrients over other food sources instead of over feeding on food with low nutrient quality (Chapman 2003). Some insects like the grasshopper, *Locusta migratoria* have chemoreceptors which are sensitive to chemicals like amino acids and carbohydrates (Altner and Prillinger 1980; Chapman 1982; 2003). Dung beetles probably have similar structures which they use to select particles which contain higher nitrogen content.

Most studies on nutrient manipulation compare protein (or amino acids) and carbohydrates as the main nutrients (Behmer and Joern 2008; Lee *et al.* 2006; Schoonhoven *et al.* 2005). Most insects regulate intake of nutrients which cannot be synthesized like amino acids, sterols and vitamins (Chapman 2003). Other nutrients that need to be regulated are the ones that are limiting or are essential (Chapman 2003). Other insects go as far as manipulating other organisms or form a symbiotic relationship in order to increase limiting nutrients for them (Douglas 2003). These symbionts are mutualistic and depend completely on their hosts (Price *et al.* 2011). Some of the symbionts are endo-symbiotic, which are found inside insects like archaea and others are ectosymbiotic, are found outside the insects like fungi (Price *et al.* 2011). Shukla *et al*. (in press) has shown different bacterial families in the gut and body of *Euoniticellus intermedius* and its brood balls but at this moment, we cannot confirm if any of those bacterial families are symbionts or not.

Dung beetles from other families like Hydrophilidae, Geotrupidae and Aphodiinae have been shown to feed in the same way (Holter 2000 and 2004), it can be concluded that dung beetles from these families also increase the nitrogen content when they select smaller dung particles. When it comes to nesting, both paracoprids and telecoprid dung beetles create a brood ball for their larva, with the possibility of each having a maternal gift inside. Dung beetles manipulate this so that the maternal gift can have slightly higher nitrogen content than that of the untreated dung. Both carbon and nitrogen isotope signatures indicate that the maternal gift is of faecal origin.

Selecting particles which have higher nitrogen is important when feeding on a food source that has very low nutrient content. This new content is higher than what was predicted before by Holter and Scholtz (2007). Stable isotopes results show that dung beetles do make an intentional selection of particles when they select particles which they feed on. The particles selected are then ingested and the nitrogen content and isotopic signature goes back to the level of the untreated dung, again suggesting that the increase was intentional. Carbon is not manipulated before feeding but does get consumed in the gut. This is how dung beetles manipulate the low nutrient content dung and turn into a high nutrient food source for themselves and their offspring.

Chapter 4. General Discussion

4.1 Discussion

This study has made an important contribution to understanding of wet dung feeding by adult dung beetles with a simple undifferentiated gut tube without a fermentation chamber. Manipulation of dung particles by adult dung beetles had been a theory since 1960s, which was supported by experiments which gave a rough estimate of particle size and nutrient content of dung fed upon by the beetles. The brood ball was later examined in this regard in this study.

The findings of this study address firstly, the manipulation of particles by adult dung beetles when feeding and nesting. Previously, Miller (1961) noticed the structure of dung beetles mouthparts and pointed out that dung beetles could actively select smaller particles when feeding. Through studying the mouth parts of adult dung beetles, Hata and Edmonds (1983) estimated the particle sizes that dung beetles could ingest.

Holter (2000) was the first to test if dung beetle mouth parts could select smaller particles and to bring an estimation of the maximum particle size of dung that could be ingested by dung adult dung beetles. He fed adult dung beetles with latex balls of different diameters and then measured the size of the balls which were found in the gut. From those measurements the maximum particle size was estimated. Holter *et al*. (2002) and Holter and Scholtz (2004) followed the same process and estimated maximum particle size that can be ingested by other species of dung beetles. This current study is the first one to directly measure the particles that adult dung beetles can ingest. This study went further and measured the actual sizes of particles in the hindgut and the dung beetle faeces.

From those estimated sizes, Holter and Scholtz. (2007) measured the nitrogen content of the particles that are ingested by adult dung beetles. In this study, the nitrogen and carbon content of the particles which dung beetles selected in the mouth were measured and also what was digested through the gut passage. This is the first real evidence that adult dung beetles do manipulate dung particles to increase the nitrogen content when feeding.

Nitrogen content in plants is very low, and in the dung on which dung beetles feed on, it is even lower, this makes nitrogen the limiting nutrient for dung beetles (Mattson 1980). Dung beetles need nitrogen for growth and development in both larval and adult stage (Hunt and Simmons 2000; 2001; Hunt *et al*. 2002). With the ability to select particles which have higher nitrogen content, dung beetles should be able to meet the shortfall of limited nitrogen in their diet.

Manipulation of particles to improve nitrogen content is not only limited to adult feeding, adult dung beetles also improve the nitrogen content of the larval food (fig. 3.2). Maternal dung beetles, after making a brood ball nest, lay a dung paste known as the maternal gift to anchor the egg and also for the larva to feed on after hatching. Building on Byrne *et al*. (2014), particle size of brood ball and maternal gift were measured and compared with those of the dung. We found that the brood ball was made up of coarser material compared to the dung and that the maternal gift had very small dung particles with higher nitrogen content than the brood ball. Since the larva feeds on the contents of the brood ball until it emerges as an adult (Emlen 1994), smaller particles will be the best starter food for the larva with small jaws and a higher nitrogen could help improve body growth and fitness.

From both the feeding and nesting experiment it can be seen that nitrogen is the driver of change in the nutrients, carbon hardly had an effect. From the stable isotope results, one can tell that the increase in the nitrogen content is not only due to smaller particles being selected. Dung beetles are selecting certain particles in the dung and this is leading to increases in the nitrogen content. These are used in feeding and in nesting. With carbon isotopes, dung beetles do not seem to be manipulating the carbon content when they are feeding or nesting but they do absorb it along with nitrogen.

The gut nitrogen content includes the nitrogen from gut secretions of herbivores and dung beetles like digestive enzymes, bacteria, fungi, cellulose and animal gut cells (Byrne *et al*. 2013; Shukla et al. in press). The same can be said about the maternal gift. There are differences in the bacterial gut communities of dung beetles, the male and the female dung beetles contain different bacterial communities (Shukla *et al*. in press). The larva had higher similarity of bacterial community to the female gut, brood ball and the maternal gift compared to the untreated dung and the least similarity with the male gut (Shukla *et al*. in press). Untreated dung had higher abundance of micro-organisms containing enzymes that could break down cellulose, xylan and pectin, and enzymes which could fix nitrogen (Shukla *et al*. in press). Estes et al. (2013) and Shukla *et al*. (in press) have shown different bacterial families in the gut and body of dung beetles but at this moment, we cannot confirm if any of those are symbionts or not. The microbiota in the maternal gift was proven to be not necessary for larval development (Byrne *et al*. 2013). The nutritional requirements of dung beetles still remain unknown but now we know that nitrogen is a big part of it (Holter 2016).

4.2 Conclusion

To cover for the shortfall on limited nitrogen from their diet, dung beetles select finer dung particles when feeding as adults and for the larva to feed on. The selection of finer particles increases the nitrogen content of the dung. There is no increase of the carbon content but carbon is absorbed along with nitrogen. The results suggest that the maternal gift is of faecal origin. However, the nutritional requirements of dung beetles remain unknown.

4.3 Future Research

Understanding of nutrient manipulation through particle selection by dung beetles is not yet complete. The dung beetle gut secretions can be removed from the gut contents to determine the nitrogen content of the plant material being selected by acid cleaning. According to Holter and Scholtz (2007) there is a relationship between body size and maximum particle size that can be ingested, more research can be done to investigate the relationship between body size and the amount of nitrogen content in the dung which is being ingested. And also whether the dung containing different nitrogen levels will be manipulated to the same nitrogen level when being ingested by dung beetles.

5. Reference List

Al-Houty, W., and Al-Musalam, F. 1997. Dung preference of the dung beetle *Scarabaeus cristatus* Fab (Coleoptera-Scarabaeidae) from Kuwait. *Journal of Arid Environments* 35: 511-516

Altner, H., L. Prillinger. 1980. Ultrastructure of invertebrate chemo- thermo- and hygroreceptors and its functional significance. *International Review of Cytology* 67: 69–139

Bang, H.S., Lee, J.H., Kwon, O.S., Na, Y.E., Jang, Y.S. and Kim, W.H. 2005. Effects of paracoprid dung beetles (Coleoptera: Scarabaeidae) on the growth of pasture herbage and on the underlying soil. *Applied Soil Ecology* 29: 165-171

Barbehenn, R. V., Chen, Z., Karowe, D. N., & Spickard, A. 2004a. C3 grasses have higher nutritional quality than C4 grasses under ambient and elevated atmospheric CO2. *Global Change Biology* 10: 1565-1575

Barbehenn, R. V., Karowe, D. N., & Chen, Z. 2004b. Performance of a generalist grasshopper on a C3 and a C4 grass: compensation for the effects of elevated CO2 on plant nutritional quality. *Oecologia* 140: 96-103

Barbehenn, R. V., Karowe, D. N., & Spickard, A. 2004c. Effects of elevated atmospheric CO2 on the nutritional ecology of C3 and C4 grass-feeding caterpillars. *Oecologia* 140:, 86-95

Batra, L. R. 1963. Ecology of ambrosia fungi and their dissemination by beetles. *Transactions of the Kansas Academy of Science (1903-)* 66:213-236

Behmer, S. T. 2009. Insect herbivore nutrient regulation. *Annual Review of Entomology* 54: 165-187

Behmer, S. T., and Joern, A. 2008. Coexisting generalist herbivores occupy unique nutritional feeding niches. *Proceedings of the National Academy of Sciences* 105: 1977-1982

Bertone, M. 2004. Dung beetles (Coleoptera: Scarabaeidae and Geotrupidae) in North Carolina Pasture Ecosystems. MSc thesis. North Carolina University, Raleigh, North Carolina, USA

Bishop, A.I., H.J. McKenzie, L.J. Spohr and I.M. Barchie 2005. Interactions between dung beetles (Coleoptera: Scarabaeidae) and the arbovirus vector *Culicoides brevitarsis* Kieffer (Diptera: Ceratopogonidae). *Australian Journal of Entomology* 44: 89-96

Blanke, A., Rühr, P.T., Mokso, R., Villanueva, P., Wilde, F., Stampanoni, M., Uesugi, K., Machida, R. and Misof, B., 2015. Structural mouthpart interaction evolved already in the earliest lineages of insects. *Proceedings of the National Academy of Sciences* 282: 1033

Brown, J., Scholtz, C. H., Janeau, J. L., Grellier, S., and Podwojewski, P. 2010. Dung beetles (Coleoptera: Scarabaeidae) can improve soil hydrological properties. Applied Soil Ecology 46: 9-16

Burns, C. W. 1968. The relationship between body size of filter-feeding Cladocera and the maximum size of particle ingested. *Limnology and Oceanograhy* 13: 675-678

Byrne, M. J., Watkins, B. and Bouwer, G. 2013. Do dung beetle larvae need microbial symbionts from their parents to feed on dung? *Ecological Entomology* 38: 250-257

Chapin III, F. S., Vitousek, P. M., and Van Cleve, K. 1986. The nature of nutrient limitation in plant communities. *American naturalist* 1:48-58

Chapman, R.F. 1982. Chemoreception: the significance of receptor numbers. *Advances in Insect Physiology* 16: 247–356

Chapman, R.F., 2003. Contact chemoreception in feeding by phytophagous insects. *Annual Review of Entomology* 48: 455-484

Cheer, A. Y. L., and Koehl, M. A. R. 1987. Fluid flow through filtering appendages of insects. *Mathematical Medicine and Biology* 4: 185-199

Chefurka, W. 1954. Oxidative metabolism of carbohydrates in insects. I. Glycolysis in the housefly *Musca domestica* L. *Enzymologia* 17:73-89

Chown, S.L. and S.W. Nicolson 2004. *Insect Physiological Ecology – Mechanisms and Patterns*. Oxford University Press, Oxford, UK

Clauss, M., Steuer, P., Erlinghagen-Lückerath, K., Kaandorp, J., Fritz, J., Südekum, K. H. and Hummel, J. 2015. Faecal particle size: digestive physiology meets herbivore diversity. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology* 179: 182-191

Davis, A.L.V. 1977. The endocoprid dung beetles of Southern Africa (Coleoptera: Scarabaeidae). Unpublished MSc thesis, Rhodes University, Grahamstown, South Africa

Davis, A.L.V., A.V. Frolov and C.H. Scholtz 2008. *The African dung beetle genera* Protea Book Publishers, Pretoria, South Africa

De Bruyne, M., C.G. and Warr. 2006. Molecular and cellular organization of insect chemosensory neurons. *BioEssays* 28: 23-34

Deville, A. S., Grémillet, D., Gauthier-Clerc, M., Guillemain, M., Houwald, F., Gardelli, B., and Béchet, A. 2013. Non-linear feeding functional responses in the Greater Flamingo (*Phoenicopterus roseus*) predict immediate negative impact of wetland degradation on this flagship species. *Ecology and evolution* 3: 1413-1425.

Douglas, A. E. 2003. Buchnera bacteria and other symbionts of aphids. Pp 23-38 in Bourtzis, K., and Miller, T. A. editors. *Insect Symbiosis* (Vol. 1). CRC, Florida, USA.

Edwards, P. B. 1988. Contribution of the female parent to survival of laboratory-reared offspring in the dung beetle *Kheper nigroaeneus* (Boheman) (Coleoptera: Scarabaeidae). *Journal of Australian Entomological Society* 27: 233-237

Edwards, P. B., and Aschenborn, H. H. 1989. Maternal care of a single offspring in the dung beetle Kheper nigroaeneus: the consequences of extreme parental investment. Journal of Natural History 23: 17-27

Edwards, P., and Aschenborn, H. 1987. Patterns of Nesting and Dung Burial in *Onitis* Dung Beetles: Implications for Pasture Productivity and Fly Control. *Journal of Applied Ecology* 24: 837-851

Emlen, D. J. 1994. Environmental control of horn length dimorphism in the beetle *Onthophagous acuminatus* (Coleptera: Scarabaeidea). *Proceedings of the royal society B: Biological Sciences* 256: 131- 136

Endrody-Younga, S. 1982. *Annotated checklist of dung-associated beetles of the Savanna Ecosystem Project study area, Nylsvley*. National Scientific Programmes Unit: CSIR, Pretoria, South Africa

Engel, P., & Moran, N. A. 2013. The gut microbiota of insects–diversity in structure and function. *FEMS Microbiology Reviews 37*: 699-735.

Estes AM, Hearn DJ, Snell-Rood EC, Feindler M, Feeser K, Abebe T, et al. 2013. Brood Ball-Mediated Transmission of Microbiome Members in the Dung Beetle,*Onthophagus taurus* (Coleoptera: Scarabaeidae). PLoS ONE 8: 1-15

Fincher, G.T., Monson W.G. and Burton G.W. 1981. Effects of cattle faeces rapidly buried by dung beetles on yield and quality of coastal Bermuda grass. *Agronomy Journal* 73: 775-779

Gallardo, A. and Schlesinger, W. H. 1992. Carbon and nitrogen limitations of soil microbial biomass in desert ecosystem. *Biogeography* 18: 1-17

Gullan, P. J. and Cranston, P. 2010. *The insects: an outline of entomology* 4th ed. Wiley-Blackwell, Oxford, UK

Halffter, G. and Edmonds, W.D .1982. *The nesting behavior of dung beetles (Scarabaeinae). An evolutive approach* . Instituto de Biologia, Mexico City, Mexico

Halffter, G. and Matthews, E . 1966. The natural history of dung beetles of the subfamily Scarabaeinae . *Folia Entomologia Mexicana* 12-14, 3-312

Halffter, G., Huerta, C., and Lopez-Portillo, J. 1996. Parental care and offspring survival in *Copris incertus* Say, a sub-social beetle. *Animal behaviour*, 52: 133-139

Hanski, I. and Cambefort, Y. 1991. *Dung Beetle Ecology* . Princeton University Press , Princeton, New Jersey

Hata, K., and Edmonds, W. D. 1983. Structure and function of the mandibles of adult dung beetles (Coleoptera: Scarabaeidae). *International Journal of Insect Morphology and Embryology* 12: 1-12

Haufe, W. 1987. Host-parasite interaction of blood feeding dipterans in health and productivity of mammals. *International Journal of Parasitology* 17: 607-614

Heidorn, T., and Joern, A. 1984. Differential herbivory on C3 versus C4 grasses by the grasshopper *Ageneotettix deorum* (Orthoptera: Acrididae).*Oecologia*, 65: 19-25

Holter, P. 1974. Food utilization of dung-eating *Aphodius* larvae (Scarabaeidae). *Oikos* 25:71-79

Holter, P. 2000. Particle feeding in *Aphodius* dung beetles (Scarabaeidae): old hypotheses and new experimental evidence. *Functional Ecology* 14: 631-637

Holter, P. 2004. Dung feeding in hydrophilid, geotrupid and scarabaeid beetles: Examples of parallel evolution. *European Journal of Entomology* 101: 365-372

Holter, P. 2016. Herbivore dung as food for dung beetles: elementary coprology for entomologists. *Ecological Entomology* 41: 367-377

Holter, P. and Scholtz, C.H. 2007. What do dung beetles eat? *Ecological Entomology* 32: 690-697

Holter, P. and Scholtz, C. H. 2013. Elongated hindguts in desert-living dung beetles (Scarabaeidae: Scarabaeinae) feeding on dry dung pellets or plant litter. *Journal of morphology* 274: 657-662

Holter, P., Scholtz, C. H., and Stenseng, L. 2009. Desert detritivory: Nutritional ecology of a dung beetle (*Pachysoma glentoni*) subsisting on plant litter in arid South African sand dunes. *Journal of arid environments* 73: 1090-1094

Holter, P., Scholtz, C.H. and Wardhaugh, K.G. 2002. Dung feeding in adult scarabaeines (tunnellers and endocoprids): even large dung beetles eat small particles. *Ecological Entomology* 27: 169-176

Hunt, J. and House, C. 2011. The Evolution of Parental Care in the Onthophagine Dung Beetles. Pp 152-176 *in* Simmons L.W. and Ridsdill-Smith, T.J. *Ecology and Evolution of Dung Beetles*. John Wiley and Sons, Chichester, UK

Hunt, J. and L.W. Simmons 1997. Patterns of fluctuating asymmetry in beetle horns: an experimental examination of the honest signalling hypothesis. *Behavioural Ecology Sociobiology* 41: 109-114

Hunt, J. and Simmons, L. W. 2000. Maternal and paternal effects on offspring phenotype in the dung beetle Onthophagus taurus. *Evolution* 54: 936-941

Hunt, J. and Simmons, L. W. 2001. Status-dependent selection in the dimorphic beetle *Onthophagus taurus*. *Proceedings of the Royal Society of London. Series B: Biological Sciences* 268: 2409-2414

Hunt, J. and Simmons, L.W. 2004. The genetics of maternal care: direct and indirect genetic effects on phenotype in the dung beetle *Onthophagus taurus. Proceedings of the National Academy of Sciences* 99: 6828–6832

Hunt, J., Simmons, L. W. and Kotiaho, J. S. 2002. A cost of maternal care in the dung beetle *Onthophagus taurus*?. *Journal of Evolutionary Biology* 15: 57-64

Klowden, M. J. 2007. *Physiological systems in insects*. Academic press, USA

Krenn, H. W., Plant, J. D. and Szucsich, N. U. 2005. Mouthparts of flower-visiting insects. *Arthropod Structure and Development* 34: 1-40

Lastro, E. 2006. Dung beetles (Coleotpera: Scarabaeidae and Geotrupidae) in North Carolina pasture ecosystems. MSc thesis. North Carolina State University, Raleigh, North Carolina, USA

Lee, K. P., Cory, J. S., Wilson, K., Raubenheimer, D. and Simpson, S. J. 2006. Flexible diet choice offsets protein costs of pathogen resistance in a caterpillar. *Proceedings of the Royal Society of London B: Biological Sciences* 273: 823-829

Mattson, William J. 1980. "Herbivory in relation to plant nitrogen content." *Annual review of ecology and systematics* 11: 119-161

Merritt, R. W., and Wallace, J. B. 1981. Filter-feeding insects. Scientific American 244: 132-144

Michener, R. H. and Schell, D. M. 1994. Stable Isotope Ratios and Tracers in Marine Aquatic Food Web. Pp. 138- 157 *in* Lajtha, K. and Michener, R. H. editors. *Stable isotopes in ecology and environmental science*. Blackwell Scientific, Oxford, UK

Miller, A. 1961. The mouth parts and digestive tract of adult dung beetles (Coleoptera: Scarabaeidae), with reference to the ingestion of helminth eggs. *The Journal of parasitology* 47: 735-744

Miranda, C. B., Santos, J. D., and Bianchin, I. 2000. The role of *Digitonthophagus gazella* in pasture cleaning and production as a result of burial of cattle dung. *Pasturas Tropicales* 22: 14-18

Moczek, A.P. 1998. Horn polyphenism in the beetle *Onthophagus taurus*: larval diet quality and plasticity in parental investment determine adult body size and male horn morphology. *Behavioural Ecology* 9: 636–641

Mucina, L., and Rutherford, M. C. 2006. *The vegetation of South Africa, Lesotho and Swaziland*. South African National Biodiversity Institute, Pretoria, South Africa

Mueller, U. G., and Gerardo, N. 2002. Fungus-farming insects: multiple origins and diverse evolutionary histories. *Proceedings of the National Academy of Sciences* 99: 15247-15249

Mueller, U. G., Schultz, T. R., Currie, C. R., Adams, R. M., and Malloch, D. 2001. The origin of the attine ant-fungus mutualism. *Quarterly Review of Biology* 72 :169-197

Nation, J. L. 2008. *Insect physiology and biochemistry*. CRC Press, Florida, USA

Nichols, E. and Gómez, A. 2013. Dung beetles and fecal helminth transmission: patterns, mechanisms and questions. *Parasitology* 141: 614-623

Paetel, C. 2002. Ecological aspects of the radiation of coprophagous Scarabaeoidea as "follow up" evolution of the evolutionary differentiation of ungulates. PhD thesis, Humboldt-Universitat zu Berlin, Berlin, Germany

Phillips, C. A. and O'Connell, T. C. 2016. Fecal carbon and nitrogen isotopic analysis as an indicator of diet in Kanyawara chimpanzees, Kibale National Park, Uganda. *American Journal of Physical Anthropology* 161: 685–697

Pomfret, J.C. and Knell, R.J. 2006. Sexual selection and horn allometry in the dung beetle *Euoniticellus intermedius*. *Animal Behaviour* 71: 567-576

Price, P. W., Denno, R. F., Eubanks, M. D., and Finke, D. L., and Kaplan, I. 2011. *Insect ecology: behavior, populations and communities*. Cambridge University Press, UK.

Raubenheimer, D. and Simpson, S.J. 2003. Nutrient balancing in grasshoppers: behavioural and physiological correlates of dietary breadth. *Journal of Experimental Biology*, 206: 1669-1681

Ridsdill-Smith, T.J. and Hayles, L. 1987. Mortality of eggs and larvae of the bush fly (*Musca vetustissima* Walker (Diptera: Muscidae)), caused by Scarabaeine dung beetles (Coleoptera: Scarabaeidae) in favourable cattle dung. *Bulletin of Entomological Research* 77: 731-736

Ridsdill-Smith, T.J. and Hayles, L. 1990. Stages of bush fly, *Musca vetustissima* (Diptera: Muscidae) killed by scarabaeine dung beetles (Coleoptera: Scarabaeidae) in unfavourable cattle dung. *Bulletin of Entomological Research* 80: 473-478

Schoeninger, M. J. 2014. Stable isotope analyses and the evolution of human diets. *Annual Review of Anthropology* 43: 413-430

Scholtz, C. H., Harrison, J., and Grebennikov, V. V. 2004. Dung beetle (*Scarabaeus* (*Pachysoma*)) biology and immature stages: reversal to ancestral states under desert conditions (Coleoptera: Scarabaeidae)?. *Biological Journal of the Linnean Society* 83: 453-460

Schoonhoven, L. M., Van Loon, J. J., and Dicke, M. 2005. *Insect-plant biology* 2nd edition. Oxford University Press, UK

Shukla, S., Sanders, J. G., Byrne, M. and Pierce, N. E. In press. Gut microbiota of dung beetles correspond to dietary specializations of adults and larvae. *Molecular ecology*

Simmons, L. W. and Ridsdill-Smith, T. J. 2011. Reproductive competition and its impacts on the evolution and ecology of dung beetles. Pp 1-20 *in* Simmons L.W. and Ridsdill-Smith, T.J. editors. *Ecology and Evolution of Dung Beetles*. John Wiley and Sons, Chichester, UK

Sitters, J., Maechler, M. J., Edwards, P. J., Suter, W., and Olde Venterink, H. 2013. Interactions between C: N: P stoichiometry and soil macrofauna control dung decomposition of savanna herbivores. *Functional Ecology* 28: 776-786.

Sowig, P. 1996. Brood care in the dung beetle *Onthophagus vacca* (Coleoptera: Scarabaeidae): the effect of soil moisture on time budget, nest structure, and reproductive success. *Ecography* 19: 254-258

Telang, A., Buck, N. A., and Wheeler, D. E. 2002. Response of storage protein levels to variation in dietary protein levels. *Journal of Insect Physiology*, 48: 1021-1029

Telang, A., Buck, N. A., Chapman, R. F., and Wheeler, D. E. 2003. Sexual differences in postingestive processing of dietary protein and carbohydrate in caterpillars of two species. *Physiological and Biochemical Zoology* 76: 247-255

Tyndale-Biscoe, M. 1984. Adaptative significance of brood care of *Copris diversus* Waterhouse (Coleoptera: Scarabaeidae). *Bulletin of entomological Research* 74: 455-461

Waldbauer, G. P., Cohen, R. W., and Friedman, S. 1984. Self-selection of an optimal nutrient mix from defined diets by larvae of the corn earworm, *Heliothis zea* (Boddie). *Physiological Zoology* 57: 590-597

Waldbauer, G.P. and Friedman, S. 1991. Self-selection of optimal diets by insects. *Annual review of entomology* 36: 43-63

Yamada, D., Imura, O., Shi, K. and Shibuya, T. 2007. Effect of tunneler dung beetles on cattle dung decomposition, soil nutrients and herbage growth. *Grassland Science* 53: 121-129