Constitutive chemical defense of *Eucalyptus* against herbivory by *Gonipterus* sp. n. 2

By

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MAGISTER SCIENTIAE

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DECLARATION OF ORIGINALITY

I, Johannes Christoffel Joubert, declare that the dissertation, which I hereby submit for the degree MSc Zoology and Entomology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institute

Signature.....

Date.....

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Preface:

The *Gonipterus scutellatus* species complex Gyllenhaal, 1833 (Curculionidae, Coleoptera) are *Eucalyptus* defoliators indigenous to South East Australia and Tasmania. This species complex contains 8 cryptic species, 3 of these, *G. platensis*, *G. pulverulentus* and *Gonipterus* sp. n. 2, are invasive pests, with South Africa containing only *Gonipterus* sp. n. 2. *Gonipterus* sp. n. 2 was first discovered in South Africa in 1916, were it caused widespread defoliation of *Eucalyptus* trees, preferentially feeding on young leaves and new shoots. These beetles were initially controlled through the use of a biocontrol agent *Anaphes nitens* Girault, 1928 (Hymenoptera: Mymaridae), which was discovered and implemented in 1926. By 1950 mass releases of the biocontrol agent were discontinued as the beetle was considered under economic control. However, over the last two decades, massive beetle populations were recorded in South African *Eucalyptus* plantations, and widespread damage is once again occurring. Therefore, alternative management options should be investigated. One potential option that has never been studied in detail is the use of the plant's inherent resistance mechanisms against feeding by *Gonipterus* sp. n. 2. *Gonipterus* sp. n. 2 shows a very distinct host selection behavior, preferably feeding on certain *Eucalyptus* species while ignoring others. Therefore, understanding the differences in *Eucalyptus* chemistry between susceptible and resistant hosts is a key step towards optimizing the use of plant resistance to help alleviate some of the damages caused by these beetles. Therefore, we conducted this in-depth study on identifying the constitutive *Eucalyptus* defense compounds which affect the host preference of *Gonipterus* sp. n. 2.

The first chapter of this thesis consists of a comprehensive review of the literature pertaining to *Gonipterus* sp. n. 2, specifically focusing on its history, biology, life-cycle and control, as well as discussing plant resistance and *Eucalyptus* chemistry as a method for mitigating the damage caused by *Gonipterus* sp. n. 2. The second chapter focuses on identifying the constitutive *Eucalyptus* defense compounds which mediate the host preference of the beetle. We identified resistant and susceptible genotypes of *Eucalyptus*, then selected genotypes of species which showed large intraspecific variation in feeding for chemical analysis. We identified compounds which show correlation between feeding and compound concentration then tested them for their ability to affect the behavior of *Gonipterus* sp. n. 2 using an artificial diet amended with pure compounds. Using this approach, we identified three compounds which clearly stimulated beetle feeding as well as two deterrent compounds. This information could now be implemented in breeding *Eucalyptus* genotypes with higher resistance to feeding by *Gonipterus* sp. n. 2.

Chapter 1:
A short overview of the *Gonipterus scutellatus* species complex, and *Eucalyptus* resistance to herbivory

1.1 - Abstract:

The *Gonipterus scutellatus* species complex Gyllenhaal, 1833 (Curculionidae, Coleoptera) is comprised of 8 cryptic species native to East Australia and Tasmania. Of these 8 species only *G. platensis, G. pulverulentus* and *Gonipterus* sp. n. 2 are invasive pests. *Gonipterus* sp. n. 2 was first discovered outside of its native range in 1916 in South Africa, and caused widespread defoliation of *Eucalyptus* trees, preferentially feeding on young leaves and new shoots. In 1926 *Anaphes nitens* Girault, 1928 (Hymenoptera: Mymaridae), an egg parasitoid, was discovered and released in South Africa. *Anaphes nitens* was historically very effective until the early 2000's when large outbreaks of the beetle were occurring with increasing frequency. Therefore, investigations for alternative management options were initiated. Both biopesticides and pesticides were tested. However, biopesticides showed little reduction in beetle populations and pesticides are expensive and under legislative control, thus lowering accessibility and hampering large scale use, which results in inefficient control of the beetle. However, *Gonipterus* sp. n. 2 shows a very distinct host selection behavior, preferentially feeding on certain *Eucalyptus* species and not at all on others. Several studies have indicated Eucalyptus defense compounds, (such as 1,8-cineole) which may be able to repel Gonipterus sp. n. 2 and could partially explain this selective feeding behavior. Therefore, understanding these compounds which potentially mediate the feeding behavior of Gonipterus sp. n. 2 can offer an alternative control strategy for these beetles. However, this feeding preference has not been studied in depth and more research is needed to understand how these compounds can be developed into a control measure for commercial *Eucalyptus* plantations. Therefore, the inherent resistance mechanisms of the plant against feeding by *Gonipterus* sp. n. 2 should further investigated.

1.2 - Introduction:

The *Gonipterus scutellatus* species complex Gyllenhaal, 1833 (Curculionidae, Coleoptera) is indigenous to South East Australia and Tasmania, where *Eucalyptus* trees grow endemically (Clarke et al., 1998). These beetles cause limited damage to *Eucalyptus* trees within their native habitats, due to their small population sizes (Tooke, 1955). It is believed that the population size of these beetles is controlled by the sparse distribution of suitable *Eucalyptus* host trees in their native habitat, coupled with the presence of their natural predators (Tooke, 1955). However, commercial plantation forestry utilizes *Eucalyptus* trees as monocultures resulting in an overabundance of food for the beetle, which has drastically changed its ecology and population dynamics (Tooke, 1955).

South African commercial plantation forestry first introduced *Eucalyptus* trees during the 1850's, due to their vigorous growth and yield (Bennett, 2010). In November of 1916 an unknown insect folivore was discovered feeding on *Eucalyptus* hedges. In 1921 this unknown insect was identified as *Gonipterus scutellatus* (Mally, 1924), a name that was recently revised to *Gonipterus* sp. n. 2 (Mapondera et al., 2012). *Gonipterus* sp. n. 2 became a devastating commercial pest shortly after it was first identified (Tooke, 1955). Fortunately, an effective egg parasitoid *Anaphes nitens* Girault, 1928 (Hymenoptera: Mymaridae) was discovered and in 1926 introduced into South Africa (Tooke, 1955). This wasp proved very successful and within 5 years reduced the size of *Gonipterus* sp. n. 2 populations to below economically significant levels in many affected areas (Tooke, 1955). However, in recent years there have been many large-scale outbreaks of *Gonipterus* sp. n. 2 (Huber and Prinsloo 1990; Loch 2008; Loch and Floyd 2001; Reis et al. 2012; Valente et al. 2017; Valente et al. 2004) that also show lower levels of parasitism than those historically recorded by Tooke 1955 (Michelle Schröder, personal communication; https://forestry.co.za/*Gonipterus*-scutellatus/, accessed: 2019-08-04).

Gonipterus sp. n. 2 populations have been increasing in size over the last two decades and are causing widespread damage (Huber and Prinsloo 1990; Loch 2008; Loch and Floyd 2001; Reis et al. 2012; Valente et al. 2017; Valente et al. 2004). This could stem from the potential loss of *A. nitens* as an effective biocontrol agent. Therefore, alternative management options need to be investigated. One alternative is the use of pesticides. However, the broad scale, high cost, negative environmental impact and restrictions imposed by certification bodies, pose severe limitations on their use (Loch, 2005; Santolamazza-Carbone and Fernández, 2004; Mapondera et al., 2012). Another option is the use of plant resistance, since studies have shown large variation in susceptibility of different host eucalypts (Clarke et al.,1998; Lanfranco and Dungey, 2001; Newete et al., 2011; Santolamazza-Carbone and Fernández, 2004; Tooke, 1955). Therefore, understanding the differences in *Eucalyptus* chemistry between susceptible and resistant hosts is a key step towards improving the application of plant resistance against commercial pests. This review will focus on the history of the beetle's introduction into the country, its biology, life-cycle and control. Furthermore, we will also discuss plant resistance and *Eucalyptus* chemistry as a method for mitigating *Gonipterus* sp. n. 2 damage.

1.3 – History of *Gonipterus* sp. n. 2 in South Africa:

The *Gonipterus scutellatus* species complex originated from South East Australia and Tasmania (Clarke et al., 1998) from where it was introduced into nearly every continent, likely because of human activities, such as international trade (Tooke, 1955). Countries affected include Brazil (Fenilli, 1982), Chile (Lanfranco and Dungey, 2001), Mauritius (Williams et al., 1951), New Zealand (Clark, 1938), Spain (Rivera et al., 1999), South Africa (Mally, 1924), and USA (Hanks et al., 2000). South Africa was one of the first countries into which the insect was introduced (Mally, 1924) and *G. scutellatus* was the second introduced pest of *Eucalyptus* trees in the country. The first record of this insect in South Africa was documented in November of 1916, where at the time, an unknown slug-like larva was found to be feeding on hedges of *Eucalyptus cornuta* in Wupperthal, Newlands (Suburb of Cape Town) (Mally, 1924). South Africa immediately began an inquiry into these larvae to identify whether they were indigenous insects expanding their host range to *Eucalyptus* or an invasive species (Mally, 1924). Upon closer inspection of the site, some of the largest larvae were found to be burrowing into the soil, forming cells, yet no pupae were found (Mally, 1924). These burrowing larvae were collected and were sent to the Entomological Experiment Station, Rosebank, and entrusted to Mr. S. H. Skaife for rearing. On the 15th of December 1916 several beetles emerged at the Entomological Experiment Station. The larvae and adult beetles where then taken to the South African Museum for identification, however the beetle was not found within the museum's collection (Mally, 1924).

The search for the identity of this beetle was of the utmost importance, as this would reveal the origin of the invasive species and give insight into its natural predators (Tooke, 1955). The first scientist to attempt this was Dr. L. Peringuey who identified it as *Gonipterus reticulatus, Boisd.*, an Australian species of snout beetle (*Coleoptera*, *Curculionidae*). Dr. CW Mally was not convinced by Peringuey's identification and therefore sent samples to the Commonwealth Institute of Entomology in London and to Mr. W. W. Froggatt in Sydney, Australia for identification. In 1921 the samples were identified as *Gonipterus scutellatus*, (Gyllenhal), both by the Commonwealth Institute of Entomology and Mr. Froggatt (Mally, 1924).

During the 5-year period in which the identity of this beetle was unknown, the insect had begun to spread throughout South Africa and was causing major damage to *Eucalyptus* plantations. The damage caused by these beetles was seldom fatal to the trees, but due to selective feeding on young leaves, growth of the trees was often stunted. This resulted in malformations and lowered both the quality and quantity of the wood produced (Mally, 1924; Tooke, 1955). At this time,

large losses were reported in many South African *Eucalyptus* plantations. This prompted the South African government to enlist the services of an entomologist, Dr. F.G.C Tooke, and in 1926 sent him to Australia to search for natural enemies of *G. scutellatus* (Tooke, 1955).

In Penola, Australia, Dr. Tooke discovered a small black wasp with elongated and stalked hind wings and with short hairs fringing both pairs of wings (Mally, 1924). This wasp was named *Anaphes nitens* (Girault) (Huber and Prinsloo, 1990). *Anaphes nitens* is a parasitoid chalcid wasp (family Mymaridae) that oviposit eggs within the egg masses of *G. scutellatus.* One to two days after oviposition, the eggs hatch and the *A. nitens* larvae begin to feed on the yolk of the *G. scutellatus* eggs. In 1926 (in the same year in which it was discovered), *A. nitens* egg capsules where shipped to South Africa and employed as a biocontrol agent (Tooke, 1955). Until 1950 *A. nitens* was released throughout South Africa. This wasp proved very successful and could within 3 years, in certain areas, reduce the beetle populations to levels where their damage was deemed insignificant (Tooke, 1955). After the success seen in South Africa, many other countries such as Spain (Rivera et al., 1999), USA (Hanks et al., 2000) and Portugal (Reis et al., 2012) started to use *A. nitens* with varying levels of success.

In 2012 a study analyzing the mitochondrial cytochrome oxidase I genes and the male genitalia of a large collection of *Eucalyptus* snout beetles from various geographic localities, revealed several cryptic species within *Gonipterus scutellatus* (Mapondera et al., 2012). These new findings allowed the reclassification of *G. scutellatus* into a species complex with 8 cryptic species and 2 closely related species. Within this classification system all South African *Gonipterus* beetles are now known as *Gonipterus* sp. n. 2 (Mapondera et al., 2012).

1.4 - An overview of the life cycle of the *G. scutellatus* species complex:

Adult females of the *G. scutellatus* species complex locate suitable *Eucalyptus* trees for feeding and oviposition. The mechanism of how the beetles detect susceptible species are largely unknown. Bouwer (2013) showed that *Gonipterus* sp. n. 2 is able to detect volatile compounds from *Eucalyptus* leaves and that susceptible tree species elicit larger responses in electroantennograms than tolerant species. Therefore, since these beetles are more sensitive to compounds from susceptible trees, it is possible that they could use these compounds to differentiate between hosts. However, as no

behavioral trials have been conducted using volatiles, it is not clear which volatiles are attractive and which are repellent. Once a suitable plant is chosen, the female beetle will, over the course of its life oviposit between 20 to 30 ± 3mm brownish-black egg capsules (Figure 2, D) on the adaxial surfaces of the leaves, with each capsule containing, on average, nine eggs (Mally, 1924; Tooke, 1955).

Two to three weeks after oviposition, the larvae (Figure 2, C) emerge from the egg capsule by eating through the leaf, leaving the egg mass intact. These larvae are yellowish green, possess a black stipe along the sides, are studded with black dots, and are often coated in a layer of green slime (Tooke, 1955). The larvae also carry a tail/tendril of black excrement on their back that may be a defense mechanism, and together with their striking coloration may indicate they are not palatable (Hanks et al., 2000). Once larvae emerge, they feed primarily on the mesophyll and epidermis of young leaves. This feeding behavior results in tracks on the surface of young leaves caused by the larvae feeding on the mesophyll and leaving the fibrous tissue behind (Mally, 1924; Tooke, 1955). During the next 24 to 40 days the larvae undergo four instars and reach an approximate length of 1.5 cm (Tooke, 1955). After the fourth instar is complete the larvae relinquish their grip on the leaves and fall to the ground. They then proceed to burrow into the soil, forming an oblong cell held together by larval secretions, in which they pupate (Tooke, 1955).

Thirty to forty days after entering the pupal life stage, adult beetles (Figure 2, B) emerge. The young beetles have a rusty red color and a well-defined X shaped mark on their elytra (hardened forewing). They also possess a white mark which forms a median prothoracic (foremost segment of the thorax) stripe which extends to the head (Tooke, 1955). The color and markings of the beetles fade over time due to the rubbing of its scales, leaving the beetles nearly uniformly brown (Tooke, 1955).

The female beetles are often larger than the males and occur in larger numbers, with the new beetles usually emerging with a male to female ratio of 2:3 (Tooke, 1955). Other than the size difference, males and females are nearly indistinguishable, however when the beetles are cleaned with potassium hydroxide and viewed under 40 times magnification there are minor differences in the sternite (ventral portion of a segment of the thorax or abdomen). Female beetles possess a penultimate (second last) sternite that has a round edge on the border with the final sternite, and the final sternite is densely covered in hair (Santolamazza-Carbone and Rivera, 1998). The male beetles possess a straight posterior margin that is covered by dark hairs. However, the hairs do not mask the color of the sternite (Santolamazza-Carbone and Rivera, 1998).

The adult beetles search for susceptible *Eucalyptus* trees. This search is aided by the beetle's strong ability to fly. Once in the air, beetles are also carried by wind which aids in dispersal across treeless areas (Mally, 1924). Once a suitable *Eucalyptus* tree is found, the beetles feed on its leaves, and unlike the 1st and 2nd instar larvae, can consume the entire leaf. The adult beetles also demonstrate a form of thanatosis as a defensive behavior when the *Eucalyptus* tree is disturbed: the beetles will relinquish their grip on the plant and fall to the ground, where they tightly grasp twigs or leaves, and due to the beetle's similar color to the surrounding detritus, renders them difficult to detect (Mally, 1924).

Once the adult beetles emerge, they begin to mate, employing a polygynandry mating system. Males show a clear courtship behavior with a complex copulation behavior that has multiple phases. Mating can last between 0.7 hour to 55 hours (Santolamazza-Carbone and Rivera, 1998). The reason for the variability and long duration of copulation is unknown. Santolamazza-Carbone and Rivera (1998) tested many different hypotheses that could explain the adaptive significance of the prolonged copulations. They showed that during mating the male's genitals are not long enough to reach the interior of the spermatheca (organ that stores sperm from previous matings), therefore the prolonged mating does not enable the beetles to remove the sperm of rival males (Santolamazza-Carbone and Rivera, 1998). Furthermore, they showed that copulation lasts far longer than necessary to fill the spermatheca and that there was no correlation between the duration of copulation and the volume of sperm ejaculated (Santolamazza-Carbone and Rivera, 1998). They concluded that the most likely explanation is that the copulation behavior evolved under sexual selection by cryptic female choice. This type of selection operates by the female utilizing physical or chemical means to control the success of mating, thus allowing females to only utilize sperm of the males with the highest fitness. The females are capable of ovipositing two days after mating and continue ovipositing two to three months after copulation. Beetles can have two to three generations per year depending on climate and availability of food sources. During winter, adult beetles hibernate beneath bark and leaves, and do not oviposit during and leading up to the winter months (Mally, 1924; Tooke, 1955). Therefore, no eggs or larvae are present during the winter months.

1.5 - Control of *Gonipterus* sp. n. 2:

Anaphes nitens is seen as one of the world's most effective examples of biological control in the forestry industry (Hanks et al., 2000; Lanfranco and Dungey, 2001; Rivera et al., 1999). The success of this wasp has been attributed to its rapid dispersal and strong ability to fly (Tooke, 1955; Hanks et al., 2000). Furthermore, these wasps tend to parasitize all eggs within a *Gonipterus* egg capsule (Tooke, 1955; Hanks et al., 2000). This allows the wasps to spread quickly after introduction and rapidly reduce the beetle's population (< 33 weeks in warm areas) (Tooke, 1955). The biocontrol is also aided by *Gonipterus* which oviposit on the adaxial surface of leaves, thus making the egg capsules easily accessible to these parasitoids (Tooke, 1955; Hanks et al., 2000).

Although *Anaphes nitens* has generally been shown to be an effective biocontrol agent, there are some problems associated with using this wasp. For example, in South Africa, *A. nitens* was only effective in reducing the number of *Gonipterus* sp. n. 2 in plantations with a similar climate to its natural habitat (Penola, Australia) (Tooke, 1955). In Penola, winters (June to August) have an average temperature ranging from 4°C to 19°C with high average rainfall ranging from 90 to 109 mm per month. Furthermore, this high rainfall triggers an increase in new flushing foliage of *Eucalyptus* trees which is required by *Gonipterus* to trigger reproduction (Loch, 2008). During the winter period *Gonipterus* sp. n. 2 continues to oviposit which provides a food source for *A. nitens* to persist year-round (Tooke, 1955). South African winter rainfall regions possess a similar climate to Penola and therefore the *A. nitens* populations are maintained throughout the entire year resulting in effective biocontrol. However, South Africa also has summer rainfall areas, usually at higher altitudes (above 1219 meters). In these areas *Gonipterus* sp. n. 2 hibernates during the cold dry winters (Tooke, 1955) without laying egg capsules for the wasps, thus rendering them unable to reproduce during winter (Figure 1). Therefore, once spring arrives the *A. nitens* population is no longer present in sufficient numbers to significantly reduce the *Gonipterus* sp. n. 2 population, which results in higher levels of feeding damage and can cause wood malformation, stunted growth and in extreme cases even tree death (Tooke, 1955).

While *A. nitens* historically had been incredibly effective in controlling *Gonipterus* sp. n. 2, in the past two decades massive beetle outbreaks have occurred and these beetles are once again causing severe defoliation in *Eucalyptus* plantations (Loch 2005; Loch and Floyd 2001; Reis et al. 2012; Rivera et al. 1999; Valente et al. 2017; Valente et al. 2004). This is often attributed to a loss in the biocontrol's efficacy (Rivera et al., 1999). The reason for this loss is currently unknown, but I believe it may be linked to global climate change. Therefore, it is important to consider alternative control strategies to control *Gonipterus* sp, 2. The most common alternative to biocontrol is the use of pesticides like azadirachtin,

flufenoxuron and etofenprox (Santolamazza-Carbone and Fernández, 2004). Even though these pesticides are effective, there are numerous drawbacks to using them as they are expensive, harmful to the environment, can further reduce the parasitism rates of *A. nitens*, and their use is limited by restrictions imposed by certification bodies (Santolamazza-Carbone and Fernández, 2004). *Bacillus thuringiensis* was proposed as a biopesticide against *Gonipterus* sp. n. 2, but only provided small reductions in the *Gonipterus* populations (Loch, 2005).

Selecting for host resistance is another method that can be used to manage populations of *Gonipterus* sp. n. 2. When insects feed on different genotypes of *Eucalyptus*, they often show a large variation in their infestation levels on individual plants. This is thought to be attributed to the presence of some form of plant resistance, present in some genotypes and absent in others (Rhoades, 1985). While plant resistance plays a major role in plant-pest interactions, there are several disadvantages to using this approach in pest management. One of the major drawbacks is the manner in which plant resistance traits are tested. Often bioassays under controlled conditions are used to find these traits, but when these results are extrapolated to field conditions, other factors may have much larger effects (Henery et al., 2009). Another major disadvantage is the variation in defensive traits, since over the course of a plant's life it will need to defend itself from many different types of herbivores and pathogens. Each of these different agents may prefer certain traits and be deterred by others. Therefore, selecting resistance traits for one attacker may render the plant susceptible to another (Henery, 2011). Even though there exist many disagvantages to plant resistance, there are still many studies (Kulkarni, 2010; Prabhu, 2010; Quang Thu et al., 2009; Agrawal et al., 1999) which have successfully studied and utilized plant resistance to reduce pest damage. A good example of this is *Leptocybe invasa* (*Hymenoptera: Eulophidae*), which is a gall forming wasp of *Eucalyptus*. The widespread damage caused by this wasp prompted extensive research into the host plant's resistance (Kulkarni, 2010; Prabhu, 2010; Quang Thu et Al., 2009). This research was then used to make informed decisions of which genotype to plant to reduce damage caused by the wasp. Due to the value of this research it was added to the general information sheets on *L. invasa*, and provided to foresters to help alleviate damage caused by this insect species (https://www.forestry.co.za/information-on-leptocybe-invasa/).

Plant resistance has limitations and can be difficult to study, however these types of studies can provide crucial information to help alleviate pest damage (Kulkarni, 2010; Prabhu, 2010; Quang Thu et al., 2009; Agrawal et al., 1999). Furthermore, the available methods of controlling *Gonipterus* sp. n. 2 are limited, making the development of new control strategies of utmost importance. Therefore, understanding resistance in *Eucalyptus* can lead to much-needed alternative management options for *Gonipterus* sp. n. 2.

1.6 – *Eucalyptus* resistance to herbivory:

Plant resistance can be partitioned into three main components: antibiosis (plant characteristics which reduce insect survival (e.g. slower growth)), preference (plant characteristics which alter insect behavior), and tolerance (plant characteristics which allow plants to compensate for the detrimental effects of herbivory) (Rechcigl and Rechcigl, 1999).

1.6.1 - Antibiosis:

Antibiosis occurs when one organism adversely affects another. This includes the detrimental effects suffered by insects when feeding on a resistant plant, such as reduced fecundity, decreased size, abnormal lifespan, and increased mortality (Rhoades, 1985). This form of resistance is often considered by plant breeders to be the most important trait as it directly reduces insect feeding and population size (Rhoades, 1985). Antibiosis against herbivores occurs as a result of the plant's physical and chemical defense mechanisms. Physical defenses include structural modifications to deter feeding, such as trichomes and high fiber content (Rhoades, 1985; Pérez-Estrada et al., 2000; Stackpole et al., 2011). Chemical defenses include secondary metabolites and various proteinacious toxins which are produced by the plant to deter feeding. Plants produce a large variety of secondary metabolites for defense, which protect them by, for example, attracting natural predators through volatile compounds, reducing nutrient content via tannins or even directly interfering with vital insect metabolic processes. Therefore, chemical defenses, especially secondary metabolites, are the primary cause of antibiosis with physical defense only playing a minor role (Rechcigl and Rechcigl, 1999).

1.6.1.1 – *Eucalyptus* antibiosis:

Eucalyptus trees mainly employ toxic compounds to achieve antibiosis. One of the most important compounds are tannins. Tannins occur in two forms, condensed tannins and hydrolysable tannins. Condensed tannins (proanthocyanidins) are polymers and oligomers of polyhydroxyflavan-3-ols linked through carbon-carbon bonds between flavan-3-ol subunits (Schofield et al., 2001). These molecules possess a high number of phenolic hydroxyl groups which can interact with proteins to form indigestible complexes (Schofield et al., 2001). These complexes reduce the nutritional value of leaves. Furthermore, condensed tannins can also act directly as toxins by causing oxidative stress when consumed (Marsh et al., 2017; Barbehenn and Constabel, 2011). Donaldson and Lindroth (2004) showed that increasing tannin concentration between first year micropropagated ramets of five aspen clones (*Populus tremuloides* Michx.) resulted in a 30% decrease in larval growth rates of cottonwood leaf beetles, *Chrysomela scripta.*

Hydrolysable tannins are sub-divided into two groups, galloyl glucoses and ellagitannins. Hydrolysable tannins are composed of a central polyol core with galloyl groups attached via ester bonds. (Barbehenn and Constabel, 2011). This monomer can be oligomerized into larger structures, and when the structure reaches a size large enough to precipitate proteins it is considered a galloyl glucose. (Barbehenn and Constabel, 2011). Ellagitannins are synthesized from galloyl glucose by oxidative coupling of galloyl groups, usually to form hexahydroxydiphenoyl moieties (Barbehenn and Constabel, 2011). Hydrolysable tannins also possess the ability to precipitate proteins, and at high pH they show very strong oxidative activities (Barbehenn et al., 2006).

Another major component of *Eucalyptus* antibiosis are cyanogenic glycosides, *β*-glycosides of *α*-hydroxynitriles (Gleadow and Woodrow, 2000). Cyanogenic glycosides, upon hydrolyzation by *β*-glycosidases, release hydrogen cyanide, which imparts a bitter taste to plant tissue and inhibits cellular respiration (Després et al., 2007; Wittstock and Gershenzon, 2002). Ballhorn et al. (2009) tested the effects of cyanogenic potential to the feeding damage seen on wild lima bean (*Phaseolus lunatus L.*) by *Gynandrobrotica guerreroensis* (Coleoptera, Chrysomelidae) and *Cerotoma ruficornis* (Coleoptera, Chrysomelidae). This study showed that there was a significant negative correlation between leaf damage by both insects and the cyanogenic potential of the host.

1.6.2 – Preference:

Insect preference for a host is determined by the characteristics of the plant which rendering it a favorable or unfavorable food source (Rhoades, 1985). Preference can be influenced by many factors such as plant stimuli recognized by the insect to trigger feeding, the absence or presence of compounds that can harm or repel insects and the absence or presence of physical defenses (Rhoades, 1985). Preference and antibiosis are both determined by the plant defense systems. However, a non-preferred host can be distinguished from a host employing antibiosis since the non-preferred host prevents feeding by the insects prior to ingestion of the plant tissue (e.g. avoiding detection, producing unpalatable leaves etc.), while antibiosis harms the insects once they are already feeding on the plant (e.g. toxic compounds (cyanogenic glycosides), or compounds that reduce feeding (tannins) etc.) (Rechcigl and Rechcigl, 1999).

1.6.2.1 – *Eucalyptus* Preference:

Eucalyptus preference is influenced by both physical and chemical defenses. *Eucalyptus* trees possess thick bark, hard leaves and cuticular waxes. These physical defenses render the trees less accessible or less palatable and thereby affect herbivore preference. A vital components of the *Eucalyptus* chemical defense influencing herbivore preference is essential

oils. *Eucalyptus* essential oils are a mixture of different types of monoterpenes, sesquiterpenes, aromatic phenols, oxides, ethers, alcohols, esters, aldehydes and ketones (Batish et al., 2008). A major component of *Eucalyptus* essential oil is *1,8*-cineole (eucalyptol), which in certain *Eucalyptus* species can comprise over 55% of the essential oil. *1,8*-Cineole can act as both an attractant of beneficial insects and specialist herbivores, however, it can also act as a repellent of insect pests (Batish et al., 2008). Other important components of *Eucalyptus* essential oil include α-pinene (which mediates multiple plant-insect communication systems), γ-terpinene (inhibitory effects on various bacteria (Oussalah et al., 2007)), linalool, α-terpineol and limonene (insect repellent activity (Nerio et al., 2010)).

1.6.3 – Tolerance:

Tolerance differs from preference and antibiosis as it has not evolved due to any specific insect-plant interaction, but rather represents a suite of general mechanisms plants use to cope with environmental and biotic stress, such as insect damage (Rechcigl and Rechcigl, 1999; Strauss and Agrawal, 1999). It is thought that "tolerant plants can outgrow an insect infestation or recover and add new growth after the destruction or removal of damaged tissues" (Rechcigl and Rechcigl, 1999; Strauss and Agrawal, 1999). Tolerance is a highly variable trait as it is influenced by multiple plant factors (age, size, etc.) as well as environmental conditions and insect populations. Therefore, this trait is very difficult to study and to implement in farming since, as the environment changes, so too does the level of tolerance (Rechcigl and Rechcigl, 1999).

1.7 - *Eucalyptus* chemistry and its effects on *Gonipterus* sp. n. 2:

Eucalyptus resistance to herbivory has been extensively studied but is still not well-understood (Gleadow and Woodrow, 2000; O'Reilly-Wapstra et al., 2007; Troncoso et al., 2011). This is complicated by specialist feeders which develop unique strategies to overcome their hosts defenses, which leads to trees with a myriad of different defense compounds and yet only a small subset of these having any deterrent effect on the pest. Furthermore, these specialist feeders can overcome these host defenses and use them to benefit the pest such as the larvae of the Australian sawfly (*Perga affinis*) which sequester oils from host *Eucalyptus* trees for protection against predators like ants, birds, and mice (Morrow et al.,

1976). It is therefore critical to gain a detailed understanding of which components of plant resistance are effective against specific pests (Gatehouse, 2002; Ali, 2012).

Information on the mechanism underlying host preference of *Gonipterus* sp. n. 2 is limited (Schröder et al. 2019). Tooke (1955) grouped the different *Eucalyptus* species known in 1927 into four groups, based on their level of susceptibility to feeding by *Gonipterus* sp. n. 2. He hypothesized that there must be a botanical or a chemical reason for the observed susceptibility of *Eucalyptus* species to *Gonipterus* sp. n. 2. He tested this hypothesis by comparing his list of susceptible species to a study conducted by Baker and Smith (1920), were they sorted *Eucalyptus* species into eight groups based on the chemical composition of their essential oils (Table 1). Tooke hoped to find an essential oil profile that correlated with the observed host susceptibility. However, he finally concluded that "it is apparent that the classification of the eucalypts according to their essential oils throws little light on the snout beetle's partiality for certain species" (Tooke, 1955).

Despite not finding a strong correlation between essential oil composition and susceptibility, the analysis by Tooke (1955) did reveal that low levels of cineole oils decrease the palatability of *Eucalyptus* for the snout beetle. This can be seen in Class 1, 7B and 8 (low 1,8-cineole levels) where only *E. robusta* is severely attacked (Table 1). Due to this data, Tooke (1955) stated that he "is convinced that the attraction of the insect to its host plant is primarily of an olfactory nature, hence a chemical one".Edwards et al. (1993) compared feeding preference of the Christmas beetle (*Anoplognathus pallidicollis*) on *Eucalyptus* species with differing chemical compositions. When comparing the data shown by Tooke (1955) (Table 1) to the feeding preference of Christmas beetles the opposite results were observed, where higher concentrations of cineole oils make the leaves less palatable (Edwards et al., 1993). This result was confirmed by Matsuki et al. (2011) who found that Christmas beetles prefer *Eucalyptus* species with low levels of sideroxylonals or 1,8-cineole and lower concentrations of α-pinene, limonene, and terpineol but higher concentrations of p-cymene and α-phellandrene.

Ever since Tooke's studies in the early 1900s, few studies (Santolamazza-Carbone and Rivera, 1999; Dungey and Potts, 2003; Newete et al., 2011; Bouwer, 2013) have expanded on his research into the host preference of *Gonipterus* sp. n. 2, Furthermore, the recent reclassification of *G. scutellatus* into a species complex by Mapondera et al., (2012) calls into question some of these studies (Santolamazza-Carbone and Rivera, 1999) as we are uncertain which species was tested. These studies that do exist often focus mainly on ranking the species present in that country. One such study by Newete et al. (2011) tested the host range of *Gonipterus* sp. n. 2. This study compared the feeding and survival of both *Gonipterus* adults and larvae on 15 different species of *Eucalyptus* present in South Africa, under both laboratory and field conditions. While such studies are invaluable to the forestry industry, they offer little insight into why a specific host preference

exists. Even fewer studies (Bouwer, 2013) focused on the mechanisms that underlie host preference. In one such study, Bouwer (2013) demonstrated that *Gonipterus* sp. n. 2 females could detect *Eucalyptus* volatiles and that these females show greater responses to host plants (*E. globulus*) than non-host plants (*E. citriodora).* These experiments showed that host susceptibility is linked to the chemical composition of the essential oils produced by *Eucalyptus* species. However, no recent studies have been conducted that provide an in-depth look into the effects of chemical composition on the susceptibility of *Eucalyptus* genotypes to herbivory by *Gonipterus* sp. n. 2.

The limited understanding of *Eucalyptus* chemical defenses to *Gonipterus* sp. n. 2 is a major knowledge gap, impeding breeding efforts for more resistant tree varieties. For example, two resistant species might derive resistance from high levels of the same compound. Crossing these species will thus likely not increase resistance. However, if a mildly resistant species containing a novel mechanism of resistance is crossed with a resistant species, this would likely create a hybrid with multiple mechanisms of resistance and could greatly reduce its susceptibility (O'Reilly-Wapstra et al., 2005).

1.8 - Conclusion:

Gonipterus sp. n. 2 is one of the earliest introduced pests in South Africa. With the loss in control by *A. nitens* and the lack of environmentally safe and cost-effective chemical control strategies, *Gonipterus* sp. n. 2 has again become a very damaging pest in *Eucalyptus* plantations. The use of plant resistance offers an alternative approach to manage *Gonipterus* sp. n. 2 populations. However, even though *Eucalyptus* chemistry is well understood there is little research on how it affects *Gonipterus* sp. n. 2. This limits the potential to use resistance breeding programs for the management of this beetle. A greater understanding of what drives host preference in *Gonipterus* sp. n. 2 is needed to further explore plant resistance as a management strategy for this important pest of *Eucalyptus* plantation monocultures.

1.9 – Tables:

Table 1: Baker and Smith 1920 classified *Eucalyptus* based on composition of essential oils. Color codes are based on Tooke 1953 grouping of *Eucalyptus* species based on their level of susceptibility. Where Red = severely attacked species (not recommended for further plantation), yellow = susceptible in certain locations and tolerant in other locations, green = "slightly attacked species with little risk of planting them" (Tooke, 1953) and blue = species mostly immune to beetle attacks.

Class	Essential oil composition	*Eucalyptus* Species									
1	Contains pinene without phellandrene and cineole	*E. diversicolor*	*E. corymbosa*	*E. botryoides*	*E. robusta*	*E. saligna*	*E. nova-angelica*	*E. alphina*			
2	Contains pinene and cineole oils. Cineole oils never exceeding 40%. Phellandrene is absent and aldehydes rarely occur	*E. eugeniodes*	*E. microcorys*	*E. redunca*	*E. lehmanni*	*E. leucoxylon*	*E. maculata*	*E. paniculata*	*E. cornuta*	*E. bosistoana*	
3A	Contains pinene and cineole oils. Cineole oils: 40-55%. Phellandrene is absent and aldehydes rarely occur.	*E. polyanthemos*	*E. stuartiana*	*E. bicolor*	*E. longifolia*	*E. camphora*	*E. maideni*	*E. cinerea*	*E. resinifera*	*E. urnigera*	*E. dalrympleana*
3B	Contains pinene and cineole oils. Cineole oils exceeding 55%. Phellandrene is absent and aldehydes rarely occur	*E. sideroxylon*	*E. smithii*	*E. bridgesiana*	*E. populifolia*	*E. pulverulenta*	*E. longicornis*	*E. muculosa*	*E. goniocalyx*	*E. globulus*	
4A	Contains pinene and cineole oils. Cineole oils exceeding 40%. Lower levels of pinene than class 3. Phellandrene is absent but aldehydes are present.	*E. punctata*	*E. stricta*								
4B	Contains phellandrene and cineole oils. Cineole oils not exceeding 40%.	*E. melliodora*	*E. risdoni*	*E. linearis*							

Table 1: continued

Class	Essential oil composition	*Eucalyptus* Species										
5	Contains pinene, aromatic aldehydes and cineole oils. Cineole oils not exceeding 40%. Phellandrene is absent	*E. tereticornis*	*E. rostrata*	*E. propinqua*	*E. deanei*	*E. occidentalis*	*E. marginata*	*E. albens*	*E. hemiphloia*			
6	Contains pinene, phellandrene and cineole oils. Cineole oils not exceeding 40%.	*E. viminalis*	*E. rubida*	*E. harmostoma*	*E. microtheca*	*E. fastigata*	*E. macrorrhyncha*	*E. capitellata*	*E. acmeniodes*	*E. pilularis*	*E. obliqua*	*E. crebra*
7A	Contains phellandrene, piperitone oils and cineole oils. Cineole oils not exceeding 40%.	*E. piperita*	*E. coccifera*									
7B	Contains phellandrene and piperitone oils. Cineole oils are mostly absent	*E. coriacea*	*E. siberiana*	*E. regnans*	*E. gomphocephala*	*E. dives*	*E. radiata*					
8	Previously unclassified oils. Cineole oils are mostly absent	*E. stellulata*	*E. macarthuri*	*E. aggregate*	*E. citriodora*							

1.10 – Figures

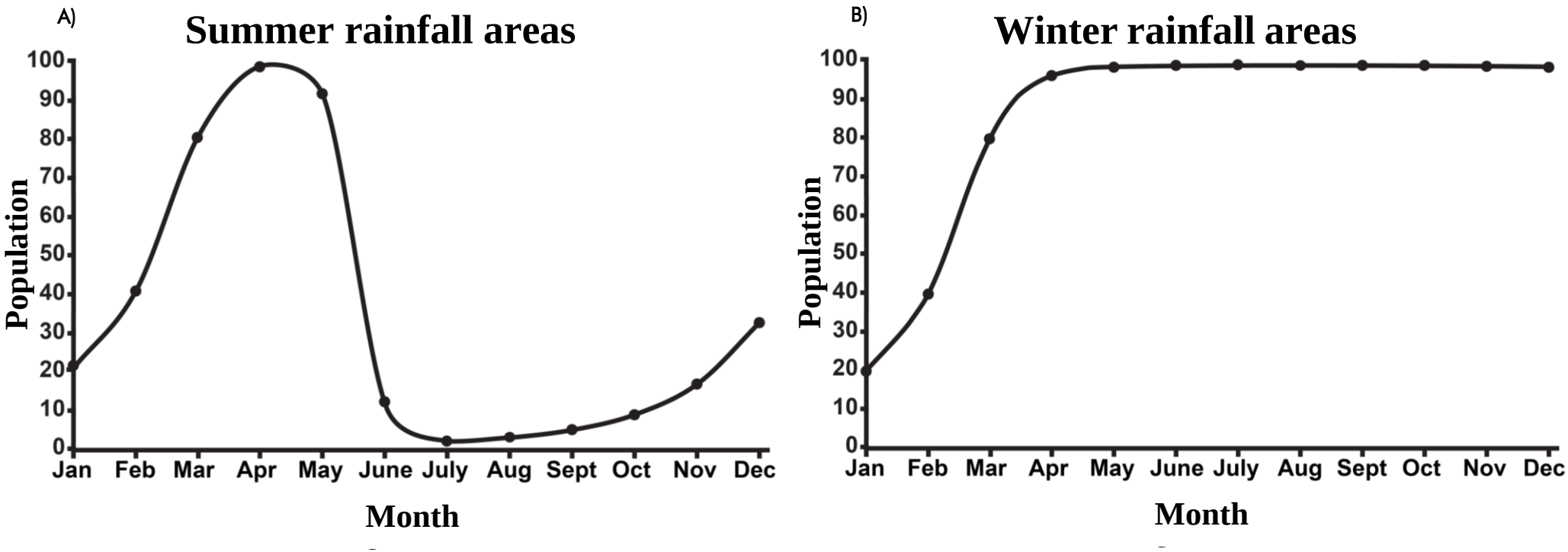

Figure 1: A) Theoretical growth curve of A. *nitens* population after introduction into a summer rainfall area depicting the reduction in population size due to hibernation of *Gonipterus* sp. n. 2. **B)** Theoretical growth curve of A. *nitens* population after introduction into a winter rainfall area depicting a persistent population size.

Figure 2: A) Images of *Eucalyptus* trees damaged by *Gonipterus* sp. n. 2 (Photographed in Richards Bay, KZN, South Africa). **B)** Images of *Gonipterus* sp. n. 2 adults. **C)** Images of *Gonipterus* sp. n. 2 larvae. **D)** Image of *Gonipterus* sp. n 2 egg capsule.

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Chapter 2:
Bioassay and mass spectrometry-guided identification of constitutive plant compounds associated with the feeding preference of the *Eucalyptus* snout beetle, *Gonipterus* sp. n. 2.

2.1 – Abstract:

Gonipterus sp. n. 2 Gyllenhaal, 1833 (Curculionidae, Coleoptera) is a devastating commercial pest, which displays a strong feeding preference for certain *Eucalyptus* genotypes. However, this feeding preference and the chemistry underlying it is poorly understood. We assessed the relative levels of susceptibility of 59 *Eucalyptus* genotypes from 22 species to feeding by *Gonipterus* sp. n. 2 using a laboratory choice assay. This revealed large intraspecific variation in susceptibility to beetle feeding, which for certain species, exceeded the interspecific variation. We selected 27 genotypes from *E. dunnii*, *E. grandis x urophylla* hybrids, and *E. nitens* for a detailed metabolite profile analysis. Using linear regressions, where feeding behavior was plotted against metabolite abundance, we identified ten compounds that correlated significantly with feeding preference. Eight of these compounds were tested for their ability to alter the behavior of *Gonipterus sp. 2* using a newly developed standardized artificial diet in an *in vitro* feeding preference assay. This revealed three phagostimulants (1,8-cineole, oxalic acid, and sucrose) and two repellent compounds (shikimic acid and palmitic acid) for *Gonipterus* sp. n. 2. Results from this study have the potential of being used as quantitative traits for resistance to *Gonipterus* sp. n. 2 in commercial *Eucalyptus* breeding programs.

2.2 – Introduction:

The *Eucalyptus* snout beetle (*Gonipterus* sp. n. 2 Gyllenhaal, 1833 (Curculionidae, Coleoptera)) is a devastating pest of *Eucalyptus*, which has been introduced over the last 100 years from South East Australia and Tasmania into every continent where *Eucalyptus* trees are planted (Paine et al. 2011; Withers 2001). These beetles primarily feed on young leaves of *Eucalyptus* trees and occasionally on the soft bark of young branches (Tooke 1955). Due to this selective feeding pattern, *Gonipterus* sp. n. 2 outbreaks are usually sub-lethal to trees. However, prolonged beetle feeding can severely stunt tree growth, which can cause malformations and lowers both the quality and quantity of the wood produced (Mally, 1924; Tooke, 1955). Severe *Gonipterus* sp. n. 2 outbreaks, where around 50% of the tree's crown is consumed annually, are thought to result in a loss of over 85% in wood production over a 10-year growth period (Reis et al. 2012).

Gonipterus sp. n. 2 can be controlled by *Anaphes nitens* Girault, 1928 (Hymenoptera: Mymaridae), a parasitoid wasp which was specifically introduced as a biocontrol agent into countries where this beetle causes significant damage, such as South Africa (Mally, 1924), Brazil (Fenilli, 1982), Chile (Lanfranco and Dungey, 2001), Spain (Rivera et al., 1999), and USA (Hanks et al., 2000). While historically *A. nitens* was very effective in controlling *Gonipterus.* sp. n. 2, in South

Africa, many large-scale beetle outbreaks have been recorded during the last two decades' (Verleur, 2012; Schröder et al., 2019). The reason for the increased levels of *Gonipterus* sp. n. 2 damage is not known, but some evidence has emerged that the parasitism rates of *A. nitens* has decreased (Schröder et al., 2019). Due to the large-scale damage caused by the beetle, alternative management options are being considered.

While many management options have been tested, such as chemical control (Santolamazza-Carbone and Fernández de Ana-Magán, 2004) and biopesticides (Loch, 2005), the inherent resistance mechanisms of *Eucalyptus* against feeding by *Gonipterus* sp. n. 2 have not been studied in detail. *Gonipterus* sp. n. 2 displays a very strong feeding preference and specifically targets several *Eucalyptus* species, including *E. globulus* (Mally, 1924; Tooke, 1955), *E. viminalis* (Mally, 1924; Tooke, 1955; Newete et al, 2011) and *E. smithii* (Newete et al. 2011), while avoiding species like *E. citriodora*, and *E. saligna* (Mally, 1924; Tooke, 1955; Newete et al, 2011). Tooke (1955) attempted to explain this preference by grouping the different *Eucalyptus* species known in 1927 into four groups, based on their level of susceptibility. He then compared this list, to Baker and Smith's (1920) list of *Eucalyptus* species sorted into eight groups based on the chemical composition of their essential oils. Although no clear patterns emerged, this comparison did show that most of the species which contained high levels of 1,8-cineole were susceptible to *Gonipterus* sp. n. 2 (Tooke 1955). A study by Bouwer (2013) tried to link this host preference to volatiles released by the tree. The authors discovered that *Gonipterus* sp. n. 2 females could detect *Eucalyptus* volatiles and that these females showed greater electrophysiological responses to *Eucalyptus* hosts (*E. globulus*) than non-hosts (*E. citriodora).*

The positive electrophysiological responses of *Gonipterus* sp. n. 2 to volatile essential oils of susceptible *Eucalyptus* trees indicate that the chemical composition of *Eucalyptus* trees is likely linked to the beetles' feeding preference. However, little is known about the specific compounds responsible for the beetle's preference. In this study we therefore determined the feeding preference of *Gonipterus* sp. n. 2 for different *Eucalyptus* genotypes and conducted an in-depth analysis of the chemistry of preferred and less preferred genotypes. Our analysis revealed several compounds that correlated strongly with feeding preference. The beetles' behavior towards these compounds of interest was assessed using a newly developed artificial medium amended with the pure compounds.

2.3 – Methods:

2.3.1 - Feeding preference trial:

Young leaves of 59 *Eucalyptus* genotypes were harvested from the distal end of branches (leaf position (LP) 3-6) from the experimental farm of the University of Pretoria (25.7472° S, 28.2588° E) and on Tom Jenkins plantation (25°44'07.7"S 28°14'17.9"E) in Pretoria (Table 1). Several leaves of each genotype were frozen, immediately after collecting, and stored at -80 °C for chemical analysis. The remaining leaves were used a maximum of 1 day after harvesting for bioassays, to ensure freshness. *Eucalyptus* snout beetles (*Gonipterus* sp. n. 2*)* were field-collected from KwaZulu Natal (Richard's Bay, Zululand, Midlands and New Hanover) and Mpumalanga (Piet Retief) provinces in South Africa. For each experiment, beetles were starved for 3 days while being stored in a Memmert IN110 incubator (Memmert, Germany) at a temperature of 22 °C and with a 14:10 H day:night cycle under 80% humidity.

The choice arena for the feeding preference trials was a 65 mm diameter x 14.5 mm height Petri-dish (Jplast, South Africa) modified by inserting four thumbtacks equidistantly through the bottom, 1 cm from the edge, in a circular arrangement (Figure 1). Moist filter paper was then placed in the Petri-dish allowing the exposed tacks to pierce through the paper, thereby holding it in place. Leaf disks were cut from freshly harvested leaves using a straight edged cork borer with a diameter of 1.5 cm. The choice arena was set up by alternately fastening two leaf disks of the genotype of interest and two leaf discs of the control species (*Eucalyptus dunnii*, Dun00*)* to the tacks. Dun00 was selected as the control, as it is a known susceptible genotype and has been used by others at the University of Pretoria to feed/maintain captive *Gonipterus* sp. n. 2 populations (Dr. Michelle Schröder, personal communication). The initial leaf surface area measurements were recorded by photographing each assay plate from a height of 25 cm using a Sony Xperia XA1 smartphone. Following this, eight beetles, of unknown gender and unknown age, were placed into the center of the choice arena and the arena was stored in a Memmert IN110 incubator at a temperature of 22 °C and with a 14:10 H day:night cycle under 80% humidity. Eight beetles were selected, as this was the minimum number of beetles which could produce a measurable level of feeding. The beetles were allowed to feed for 2 days, before removing them. The plates were again photographed to record the final leaf surface area measurement. Each preference assay was performed with three replicates that ran concurrently.

The number of colored pixels (leaf surface area) in each leaf disk before and after feeding were quantified using Abode Photoshop CC 2015 software. Because the data could not be transformed to a normal distribution, the software R (R Core Team, 2017) was used to perform a Kruskal–Wallis Chi^2 test to determine if the differences in the level of feeding between

the test genotype and the control (Dun00) were significant. These results were used to rank the genotypes by their feeding preference relative to the *E. dunnii* control genotype, Dun00 (Table 1).

Trials were also conducted, using the above protocol, where all the genotypes determined resistant or mildly resistant in the above test were assayed against each other (e.g. leaf disks of genotype A compared to leaf disks of genotype B in one assay (with three replicates) followed by genotype B vs genotype C, then genotype C vs genotype D, and lastly genotype D vs A) to determine the relative preference of the beetle for each genotype, allowing for ranking of genotypes by their feeding preference. The same trial was also conducted comparing all the genotypes that were susceptible or mildly susceptible in the previous assay.

2.3.2 – Leaf selection for chemical analysis:

Twenty-eight genotypes, representing ten *E. dunnii*, fourteen *E. grandis x urophylla*, and four *E. nitens* genotypes were selected for further chemical analysis (Table 1). These genotypes were selected as they represented a large range of resistance to feeding by *Gonipterus* sp. n. 2 (Table 2).

2.3.3 - Liquid Chromatography – Mass Spectrometry:

Leaves from four trees/saplings of each of the 28 genotypes were sampled when the bioassay was conducted and stored at – 80 °C. The samples from the genotypes selected for chemical analysis were frozen in liquid nitrogen and hand ground using a mortar and pestle. Subsamples of the ground leaves were freeze dried using a Virtis adVantage lyophilizer (SP Scientific, USA) for 24 H at a pressure of 13,33 kPa and then stored at room temperature. Each sample (26 mg) was weighted and extracted with 1.8 mL absolute methanol (Sigma, USA) for 4 H and centrifuged at maximum speed. The supernatants (1.2 mL) were transferred to glass vials. Ten µL of this extract was used for liquid chromatography-mass spectrometry (LCMS) analysis using a Bruker Daltronics Esquire 3000 ion trap mass spectrometer (Bruker Daltronics, Germany) coupled to an Agilent 1100 high pressure liquid chromatograph following the methods outlined in Hammerbacher et al. (2014).

The ProteoWizard (http://proteowizard.sourceforge.net/) software was used to convert standard LC-MS result .d file type to .mzXML file type for ease of use. The chromatograms were processed using a Multigroup statistical analysis on XCMS online (https://xcmsonline.scripps.edu/), where the chromatograms were grouped by genotype. This software corrects

chromatographic retention time deviation and compares ion intensity between groups by pairwise statistical analysis. Mean peak height, mass and retention time were extracted from the results table. Following this, the software R (R Core Team, 2017) was used to conduct a Shapiro-Wilk test of normality to ensure the data was normally distributed. A linear regression of peak height to level of feeding was then conducted and masses showing significant correlation were selected for further identification and analysis.

2.3.4 - Gas Chromatography – Mass Spectrometry:

2.3.4.1 - Polar Analysis:

The samples analysed by LCMS were dried at ambient temperature using nitrogen airflow and resuspended in 100 µL pyridine (Sigma, Germany) containing 20 mg mL^{-1} methoxamine HCl (Sigma, Switzerland). This solution was incubated at 30 °C for 90 min, then centrifuged at 1200 rpm for 20 min. Thirty µL of the supernatant was transferred into a glass insert in a glass vial. Thirty µL MS-TFA (Sigma, USA) was added to the supernatant and incubated at 37 °C for 30 min. One µL of the supernatant was analysed on an Aglient 7890 gas chromatograph-mass spectrometer (Agilent, USA) (GC-MS) using a HP5 column with a linear temperature program starting at 70 °C increasing at a rate of 5 °C min^{-1} until a maximum temperature of 300 °C then held for 2 min. The parameters of the GC-MS were a solvent delay of 6 min, split inlet with a split ratio of 100:1 and a flow rate of 120 mg mL^{-1} leading to a 1.2 mL min^{-1} flow rate on the column. The mass spectrometer was set to scan mode with a low mass of 40 m z^{-1} and a high mass of 650 m z^{-1} and the ion source was maintained at 70 eV.

Solutions of 39 mg mL^{-1} fructose, 40.5 mg mL^{-1} oxalic acid, 7.5 mg mL^{-1} palmitic acid, 4.5 mg mL^{-1} shikimic acid, and 26 mg mL^{-1} sucrose were made in 100 µL pyridine (Sigma, Germany) containing 20 mg mL^{-1} methoxamine HCl and derivatized as above. One hundred µL of each solution were added to 900 µL methanol (Sigma, USA). Following this, a series of dilutions were made (10^{-1}, 10^{-2} and 10^{-3}) for each solution. One µL of each solution was analysed as described above, to confirm the identity and concentration of the compounds of interest.

2.3.4.2 - Essential Oil Analysis:

Each ground leaf sample (41.1 mg fresh tissue) was weighted and extracted with 1 mL hexane (Sigma, Germany). This solution was incubated at 200 rpm at 24 °C for 1 H and then centrifuged at 1200 rpm for 20 min. The supernatant (0.8 mL) was transferred into a glass vial. One µL of the supernatant was analyzed on an Aglient 7890 gas chromatograph-mass spectrometer (Agilent, USA) (GC-MS) using a HP5 column with a linear temperature program starting at 40 °C

increasing at a rate of 4 °C min^{-1} until a maximum temperature of 200 °C then held for 2 min. The parameters of the GC-MS were a solvent delay of 3.5 min, split-less inlet and a flow rate of 1.2 mL min^{-1}. The mass spectrometer was set to scan mode with a low mass of 40 m z^{-1} and a high mass of 450 m z^{-1} and the ion source was maintained at 70 eV.

One ml solutions of 0.92 mg mL^{-1} 1,8-cineole (Sigma, USA), 0.858 mg mL^{-1} α-pinene (Sigma, USA), 0.85 mg mL^{-1} γ-terpinene (Sigma, USA), 0.8 mg mL^{-1} *trans-β*-ocimene (Sigma, USA) were made in n-hexane (Sigma, Germany), after which a series of dilutions were made (10^{-1}, 10^{-2} and 10^{-3}). One µL of each dilution step was analysed on an Aglient 7890 gas chromatograph-mass spectrometer as described above, to confirm the identity and concentration of the compounds of interest.

2.3.4.4 – Analysis of GC-MS results:

MassHunter Unknowns Analysis software was used to process the chromatograms. This software integrates and deconvolutes peaks, then tentatively identifies the compounds utilizing the 2017 NIST library. The parameters for the deconvolution were as follows: left m z^{-1} delta of 0.3, right m z^{-1} delta of 0.7, sharpness threshold of 25%. For the library search, a minimum match factor of 30 was used and no peak filters were applied to maximize peak detection. The compound name, retention time, mass and peak area was exported. Following this, the software R (R Core Team, 2017) was used to conduct a Shapiro-Wilk test of normality to ensure the data was normally distributed. The data was reformatted and uploaded to https://www.metaboanalyst.ca/ to generate principle component analysis plots and heat maps. Following this, a linear regression of peak area to level of feeding was conducted using the software R (R Core Team, 2017). The compounds showing a statistically significant correlation were selected for further identification and testing.

2.3.5 – Artificial Diet:

An artificial diet for *Gonipterus* was developed using the protocol described by Wheeler and Zahniser (2001) as a basis. The protocol was modified by using *Eucalyptus* foliage instead of *Melaleuca* leaves. The final artificial diet was composed of two mixtures:

Mixture 1: 5.285 g Agar (Merck, Germany), 11.6 g of alpha cellulose (Bio-Serv, USA), 8.6 g Casein (Bio-Serv, USA), 0.15 g cholesterol (Bio-Serv, USA), 3.5 g corn starch (Robertsons, South Africa), 0.25 g Lecithin (MP Biomedicals, USA), 100 µL linseed oil (Lemcke, South Africa), 0.16 g methyl paraben (Bio-Serv, USA), 0.775 g Wesson salt (MP Biomedicals, USA), and 20 g of freeze dried ground *Eucalyptus* leaves.

Mixture 2: 1.5 g Vanderzant Vitamins (Sigma, USA), 0.16 g ascorbic acid (Bio-Serv, USA), 0.16 g sorbic acid (Bio-Serv, USA) and 3 g white sugar (sucrose) (Huletts, South Africa).

Mixture 1 was added to 200 ml water, then autoclaved at 121 °C for 15 min. Mixture 2 was added to 40 ml water and filter sterilized using a 0.2 µm pore size. The two solutions were mixed and the content of 1 capsule of Dis-Chem Gold Lecithin 1200 Softgel was removed via a hypodermic needle and added to the solution. This solution was then poured into 65 mm diameter x 14.5 mm height Petri-dishes (Jplast, South Africa) and stored at 4 °C.

2.3.6 – Behavioral Assay:

2.3.6.1 - *Eucalyptus* Leaf Extract:

Eucalyptus dunnii (Dun00) leaves were harvested and stored at -20 °C. The leaves were frozen in liquid nitrogen and hand ground using a mortar and pestle. The ground leaves were added to 150 mL hexane (Sigma, Germany) until the leaves reached the 75 mL mark on a measuring cylinder and incubated for 60 min. This mixture was then poured through filter paper. This extract was stored at 4 °C.

2.3.6.2 – Behavioral Assay – Essential Oils:

The choice arena for the behavioral assay was composed of a 65 mm diameter x 14.5 mm height Petri-dish (Jplast, South Africa) modified as described for the assays using leaf disks. Five µl *Eucalyptus* leaf extract was added to the center of four filter paper circles (2.5 cm diameter) which were then fastened to the exposed tacks (Figure 2). Leaf extract was added to simulate the volatiles emitted from susceptible *Eucalyptus* leaves, allowing the beetles to choose between 2 "leaves" differing only by the concentration of the tested volatile. Three variants of this trial were conducted to test the different concentrations of each compound of the diluted series used in section 2.3.4.3 (Standard Curve – Volatiles), namely 10^{0}, 10^{-1} and 10^{-2}. Five µl of the compound was added to the center of 2 oppositely fastened 2.5 cm filter paper circles (containing leaf extract). Artificial diet disks were cut from the artificial diet plates using a straight edged cork borer with a diameter of 1.5 cm, weighed and fastened to all tacks, covering the filter paper circles. The choice assay was conducted in quadruplicate, as described above for the leaf disks (2.3.1 - Feeding preference trial). The diet disks were again weighed after beetles had been allowed to feed on the diet for 48 H. The software R (R Core Team, 2017) was used to conduct a pairwise t-test to determine if the mean weight change between groups was significant.

2.3.6.3 – Behavioral Assay – Polar compounds:

To simulate the different levels of a compound detected by the beetle within the different *Eucalyptus* genotypes included in this study, the behavior of *Gonipterus* sp. n. 2 towards different concentrations of the same compound was assayed reciprocally. In this trial, compounds were added to 2 mL water at a high, medium and low concentration (conc.): 1000 mg, 550 mg and 250 mg fructose (Sigma, USA), 1000 mg, 550 mg and 250 mg white sugar (sucrose) (Huletts, South Africa), 294 mg, 168 mg and 42 mg oxalic acid dehydrate (Sigma, Japan), 11.25 mg 7.5 mg and 3.75 mg palmitic acid (Sigma, Malaysia), 0.75 mg, 0.375 mg and 0.15 mg shikimic acid (Sigma, USA). Each of the 15 solutions was filter sterilized and added separately to a 65 mm diameter x 14.5 mm height Petri-dish (Jplast, South Africa). A variant artificial diet lacking sucrose was made and poured into each of the plates containing the prepared concentration of sucrose or fructose, mixed and then stored at 4 °C. The standard artificial diet was made and poured into the remaining plates. Artificial diet disks were cut from the diet plates and the choice arena was set up by alternatively fastening: 1) two high conc. artificial diet disks and two medium conc. artificial diet disks to the tacks, 2) two high conc. artificial diet disks and two low conc. artificial diet disks to the tacks, 3) two low conc. artificial diet disks and two medium conc. artificial diet disks to the tacks. Following this, the same methodology was followed as in the behavioral assay with essential oils.

2.4 – Results:

2.4.1 – Feeding preference trial:

In the first round of the feeding preference trial (2.3.1 - Feeding preference trial), the differences in beetle feeding between leaf disks of a genotype of interest and the control, Dun00, was tested. This allowed us to sort the genotypes into 5 categories (Table 3), high resistance (*p-value* < *0.05;* Kruskal-Wallace test and a positive Δ feeding (%) value (more feeding on the control than on the genotype of interest; Table 2)), mild resistance (*p-value* > *0.05*, with Δ feeding (%) over 7%; Table 2), similar to control (*p-value* > *0.05* and -7% < Δ feeding (%)< 7%; Table 2), mild susceptibility (*p-value* > *0.05 and* Δ feeding (%)under -7%; Table 2), and high susceptibility (*p-value* < *0.05* and a negative Δ feeding (%) value; Table 2). Of the 59 genotypes tested, 16 fell under the high resistance category, these included four *E. dunnii*, one *E. grandis x camaldulensis*, one *E. goniocalyx*, *two E. grandis*, one *E. grandis* × *urophylla*, three *E. nitens*, one *E. obliqua*, one *E. ovata*, one *E. propinqua* and one *E. sideroxlon* genotype. Thirteen genotypes fell under the mild resistance

category, these included one *E. botryoides*, one *E. citriodora*, three *E. dunnii*, two *E. grandis x camaldulensis*, four *E. grandis* × *urophylla*, one *E. macarthurii*, and one *E. nitens*. Twenty genotypes fell under the category that was similar to the control. These included one *E. dorriengoenis*, five *E. dunnii*, seven *E. grandis* × *urophylla*, one *E. macarthurii*, one *E. microcorys*, three *E. nitens*, one *E. paniculata*, and one *E. saligna* genotypes. Nine genotypes fell under the mild susceptibility category. These include one *E. benthamii*, three *E. dunnii*, one *E. grandis* × *urophylla*, two *E. nitens*, one *E. pilularis*, and one *E. saligna x urophylla*. One genotype (*E. grandis* × *urophylla*) fell under the high susceptibility category.

In the second round of the feeding preference trial the species were ranked by their level of resistance to feeding by *Gonipterus* sp. n. 2 relative to each other (Table 6). The three most resistant genotypes were an *E. grandis x urophylla* genotype, an *E. nitens* genotype and an *E. dunnii* genotype. The three most susceptible genotypes were an *E. saligna x urophylla*, an *E. nitens* and an *E. dunnii* genotype.

2.4.2 – Chemical Analysis:

Principle component analysis (PCA) plots (Figure 3 & 4) were generated to provide a general overview of the data. Sample grouping by PCA correlated strongly with genotype as indicated by well-defined cluster regions and in some cases with susceptibility. Interestingly, these plots illustrate the high variability in the chemical composition of the genotypes included in this study. However, due to the large number of variables considered in this analysis, it provided limited information on the specific effect of individual compounds on feeding preference.

To provide an overview of differences in metabolite contents between different genotypes, heat maps were generated. The heat maps (Figure 5-8) showed, that among the primary metabolites, sucrose, shikimic acid and D-allofuranose were the most abundant in the *E. grandis* x *E. urophylla* hybrids (Figure 5). The most abundant primary metabolites in the *E. dunnii* genotypes also included sucrose and shikimic acid as well as fructose and *myo*-inositol (Figure 6). Both species contained high levels of gallic acid, but low levels of polyphenols, such as catechin and chlorogenic acid (Figures 5 and 6). Among the non-polar metabolites, *E. dunnii* contained very high levels of monoterpenes, especially eucalyptol (1,8-cineole), α-phellandrene and γ-terpinene (Figure 7). The *E. grandis* x *E. urophylla* hybrids, on the other hand, contained high levels of α-pinene, γ-terpinene and the alkane dodecane (Figure 8).

Interestingly, the heat map analysis showed that changing concentration of several compounds (*γ*-terpinene, 1,8 cineole, gallic acid, fructose, shikimic acid, and sucrose) mirrored changing levels of susceptibility to *Gonipterus* sp. n. 2. While the heat maps revealed trends related to compound concentration and resistance, they did not conclusively show statistical correlation. Therefore, linear regressions (Figure 9 & 10) were conducted. This revealed 10 compounds (Table 7) with statistically significant ($p\text{-}value < 0.05$) correlations between the level of feeding and compound peak area. From these 10 compounds, 8 (1,8-cineole, *γ*-terpinene, lapachone, oxalic acid, an unknown saturated fatty acid (likely palmitic acid), sucrose, terpinen-4-ol, and trans-*β*-ocimene) showed a significant positive correlation and 2 (*α*-pinene and shikimic acid) showed a significant negative correlation. Genotypes of *E. grandis x urophylla* showed strong linear correlations ($r^2 value \leq 0.6$) for *α*-pinene, 1,8-cineole, oxalic acid, palmitic acid, sucrose, terpinen-4-ol, and trans-*β*-ocimene, while *γ*-terpinene and lapachone showed mild correlations ($0.4 > r^2 value > 0.6$). Genotypes of *E. dunnii* showed strong linear correlations (r^2value ≤ 0.6) between feeding levels and *α*-pinene, 1,8-cineole, *γ*-terpinene, lapachone, oxalic acid, sucrose, terpinen-4-ol, and trans-*β*-ocimene, while 1,8-cineole, an unknown saturated fatty acid (likely palmitic acid) and shikimic acid showed mild correlations ($0.4 > r^2 value > 0.6$).

The data from the LC-MS analysis did not reveal any significant correlations between mildly polar compounds and feeding.

2.4.3 – Behavioral Assays:

A bioassay was conducted where beetles were offered the choice between an artificial diet with different concentrations of 1,8–cineole, *α*-pinene, *γ*-terpinene, and trans-*β*-ocimene (the compounds that showed significant correlations to beetle feeding on leaf disks) and a non-amended control diet. Beetles chose the diet with high, medium and low concentrations of 1,8 – cineole (Figure 11) above the control diet ($p\text{-}value < 0.05$). No behavioral differences were observed between different concentrations of *α*-pinene, *γ*-terpinene, and trans-*β*-ocimene. However, the control medium showed significantly ($p\text{-}value < 0.05$) higher levels of feeding compared to the diet amended with the lowest concentration of *α*-pinene and *γ*-terpinene.

Feeding trials were conducted using diets amended with polar compounds that showed significant correlations to beetle feeding on leaf disks. The beetles' responses to a low concentration versus a medium and a high concentration were recorded. Oxalic acid and sucrose were attractive to the beetles (Figure 12). Higher concentrations of both compounds stimulated in all cases significantly ($p\text{-}value < 0.05$) higher amounts of feeding. Shikimic acid, on the other hand, inhibited

beetle feeding and higher concentrations of the compound in all cases showed significantly *(p-value < 0.05*) lower amounts of feeding. Different concentrations of D-(-)-fructose did not stimulate differences in feeding behavior, except at the lowest concentration where significantly (*p-value < 0.05*) higher levels of feeding were observed when compared to the medium concentration. Finally, the highest concentration of palmitic acid was deterrent to the beetles (*p-value < 0.05*).

2.5 – Discussion:

2.5.1 - Eucalyptus genotypes show high intraspecific variation, which severely affects the feeding preference of *Gonipterus sp. n.* 2:

Gonipterus sp. n. 2 is a devastating commercial pest, which displays a preference for certain *Eucalyptus* genotypes. Due to these behavioral trends observed under field conditions, several studies have tried to identify the host range and feeding preference of these beetles. These studies indicated that species such as *E. globulus* (Mally, 1924; Tooke, 1955), *E. viminalis* (Mally, 1924; Tooke, 1955; Newete et al., 2011), *E. smithii, E. goniocalyx, E. urophylla, E. grandis, E. scoparia,* and *E. dorrigoensis* (Newete et al., 2011) were highly susceptible to feeding by *Gonipterus* sp. n. 2. These studies also showed that *E. obliqua*, (Mally, 1924), *E. botryoides, E. paniculata, E. pilularis* (Mally, 1924; Tooke, 1955), *E. citriodora,* and *E. saligna* (Mally, 1924; Tooke, 1955; Newete et al., 2011) were resistant to feeding. However, in some cases these studies reported conflicting results. For example, Mally (1924) identified *E. punctata* as highly susceptible, whereas Newete et al. (2011) reported that it was resistant in plantation trials, while Tooke (1955) stated that it was susceptible in certain locations and tolerant in others. Furthermore, *E. propinqua,* was identified by Tooke (1955) as susceptible, while Mally (1924) and Newete et al. (2011) reported it to be a resistant species.

In our study a laboratory assay was conducted, where beetles were offered a choice between leaf disks of different *Eucalyptus* genotypes. While our data is in agreement to previous reports for *E. citriodora, E. propinqua, E. pilularis* and *E. obliqua* (Mally, 1924; Tooke, 1955; Newete, 2011), our results for *E. goniocalyx, E. pilularis, and E. saligna* contradict these previous reports. Furthermore, *E. dorriengoenis* and *E. paniculata* showed similar levels of resistance compared to a susceptible *E. dunnii* control used in our experiments (Dun00), thus matching previous reports (Mally, 1924; Tooke, 1955; Newete et al., 2011).

In contrast to previous studies, our study included multiple genotypes from the major *Eucalyptus* tree species planted commercially in South Africa. These included *E. grandis* x *urophylla* and *E. grandis* x *camaldulensis* hybrids, *E dunnii* and *E. nitens.* Interestingly, the beetle's preference to different genotypes from the same species varied widely among the genotypes. For example, in a range of pure *E. grandis* genotypes, we identified both attractive and repellent genotypes. Similar results have been observed for other plant species. For example, a study, by Hemming and Lindroth (1995) showed that the intraspecific variation in foliar chemistry of aspen trees could significantly affect the feeding behavior of both *Lymantria dispar L.* (*Erebidae*) and *Malacosoma disstria* Hbn. (Lasiocampidae). Furthermore, these authors theorized that the intraspecific variation in chemical composition may account for differential defoliation of aspen by these two insect species. The intraspecific variation observed in our study may account for the conflicting reports by previous studies, as these studies used single genotypes to represent species (Mally, 1924; Tooke, 1955; Newete, 2011). Taken together, our results show that the within species variation of leaf area consumed by *Gonipterus* sp. n. 2 is often larger than the variation among different species. However, our results are solely based on laboratory assays, which do not account for other variables in natural settings, such as climate, which might influence the beetle's behavior.

2.5.2 – Chemical composition of host plants play a crucial role in *Gonipterus* sp. n. 2 host choice and feeding:

To study the mechanism underlying the large intra-specific variation in feeding preference of *Gonipterus* sp. n. 2, we conducted a detailed chemical analysis using multiple genotypes from *E. grandis* x *urophylla, E. dunnii* and *E. nitens*. Our analysis revealed 10 compounds with significant positive or negative correlations to feeding preference by the beetle. These included the monoterpenes *α*-pinene, 1,8-cineole, *γ*-terpinene, terpinen-4-ol, and *trans-β*-ocimene, an *ortho*-naphthoquinone, lapachone, two organic acids, oxalic acid and shikimic acid, one sugar, sucrose, and one saturated fatty acid, palmitic acid. These compounds were generally the most abundant compounds detected in the non-polar and polar fractions of the *Eucalyptus* extracts. Of these, eight were tested for their ability to alter the behavior of *Gonipterus* sp. n. 2 (lapachone and terpinen-4-ol were excluded) using a standardized artificial diet in an *in vitro* feeding preference assay.

The monoterpenes *α*-pinene, 1,8-cineole, *γ*-terpinene, and *trans-β*-ocimene are known to be the major constituents of *Eucalyptus* essential oils. In some *Eucalyptus* species, such as *Eucalyptus polybractea*, 1,8-cineole can form up to 79% of the total foliar terpene contents (King et al., 2004). This compound was significantly attractive to *Gonipterus* sp. n. 2 in our study. This result is also supported by Tooke (1955) who found higher levels of 1,8-cineole to be an attractant of

Gonipterus sp. n. 2. and by Bouwer (2013) who found 1,8-cineole in higher concentrations in susceptible species. 1,8-Cineole appears to be a defensive compound, as many studies show it can repel certain pests of *Eucalyptus* (e.g. *Anoplognathus* spp. (Matsuki et al., 2011)) and be toxic to other insects (e.g. *Aedes aegypti* (Lucia et al., 2007)). However, specialist insects often evolve to utilize defensive compounds of their host as cues to find their preferred host species and to act as stimulants to trigger feeding (Fraenkel, 1953). Furthermore, some specialist insects sequester plant defense compounds as chemical defenses against predations. For example, larvae of the Australian sawfly (*Perga affinis*) sequester oils from host *Eucalyptus* trees for protection against predators like ants, birds, and mice (Morrow et al., 1976). In other cases, insects require host metabolites as precursors for the production of semiochemicals. For example, the mountain pine beetle (*Dendroctonus ponderosae*) can utilize the host defense metabolite, *α*-pinene, to augment the production of its aggregation pheromone, *trans*-verbenol (Chiu et al. 2018, 2019). This may also be the case for *Gonipterus* sp. n. 2, as a study by Branco et al. (2019) identified several potential pheromones for *Gonipterus platensis* (2-*α*-hydroxy-1.8-cineole and 2-oxo-1.8-cineole) which could potentially be derivatives of 1,8-cineole. The authors also identified *cis*-verbenol, *trans*-verbenol and verbenone, which are derived from *α*-pinene. However, neither this study nor Tooke (1955) found any evidence that this compound significantly affects the feeding behavior of *Gonipterus* sp. n. 2. Similarly, *γ*-terpinene and trans-*β* -ocimene which can be detected by female *Gonipterus* sp. n. 2 (Bouwer,2013) did not significantly affect their feeding behavior in our assays. These volatiles were shown to aid host-plant detection/attraction in other systems (Vrkočová et al., 2000; Farré-Armengol et al., 2017), but might act only as synergists for 1,8-cineole in *Gonipterus* sp. n. 2.

Sucrose is an important disaccharide in plants, formed through the coupling of two monosaccharides, D-(-)- glucose and D-(-)-fructose. Sugar content, particularly sucrose, has been shown to be an important phagostimulant for many beetles, such as the sweetclover weevil (*Sitona cylindricollis*) (Akfson et al., 1970) and the cereal leaf beetle (*Oulema melanopus*) (Panella et al., 1974). We found that sucrose acted as a phagostimulant for *Gonipterus* sp. n. 2. Glucose and fructose are produced by plants in their leaves via photosynthesis (source tissue), however they primarily require these sugars to their growing areas or in storage tissue (sink tissue). Therefore, plants need to transport these sugars, in the form of sucrose, via their vascular tissue. This results in high sucrose concentrations in vascular tissue, which might explain why these beetles are often found feeding on soft bark of branches of *Eucalyptus* trees (Tooke 1955).

Oxalic acid is the simplest dicarboxylic acid and one of the strongest organic acids in plants (Prasad and Shivay, 2017). Oxalic acid can be highly toxic and can form calcium oxalate crystals. A study by Hudgins et al. (2003) showed that calcium oxalate crystals in addition to fiber rows can be an effective barrier in conifer trees against bark-boring beetles.

While many studies show oxalic acid to be a defensive compound, little is known about oxalic acid as an attractant or feeding stimulant in beetles. This study showed that oxalic acid acts as a phagostimulant of *Gonipterus* sp. n. 2. Young leaves often have higher levels of calcium oxalate crystals than mature leaves (Finley 1999). For example, the number of crystals in *Cyclanthus subpalmata, Pandanus leram, Crinum amabile, Heliconia longiflora* and *Guzmania zahnii* were inversely correlated to leaf age and toughness (Finley, 1999). It is thought that oxalic acid levels may be higher in young leaves to protect them from insects before they can fully mature into tougher leaves (Finley, 1999). Thus *Gonipterus* sp. n. 2 might detect younger leaves, on which they preferentially feed, by their oxalic acid content.

In contrast to oxalic acid, another organic acid, shikimic acid, acts as a deterrent of *Gonipterus* sp. n. 2. Shikimic acid is a precursor in the formation of the amino acids, tryptophan, phenylalanine and tyrosine (Bennett, 1994). These amino acids are further transformed by plants into important defense compounds including cyanogenic glucosides, flavonoids, anthocyanins, sideroxylonals and tannins (Bennett, 1994). Another derivative of the shikimic acid pathway is salicylic acid, a common signal molecule in plants used to induce expression of multiple plant defense-related genes (Bennett, 1994). As shikimic acid is a precursor of these inducible plant defense compounds, higher base levels of shikimic acid will likely result in the formation of higher levels of these toxic derivatives in response to beetle feeding or oviposition, which might explain the beetles' aversion.

Palmitic acid is a 16 carbon, saturated long-chain fatty acid. Palmitic acid is a very common fatty acid present in animals, plants and microorganisms. Palmitic acid has been shown to act as attractant and phagostimulant of beetles such as the granary beetles (Yinon et al., 1971) and flour beetle (Starratt and Loschiavo, 1971). Alternatively, Farag et al. (2011) showed that a mixture of fatty acids including palmitic acid possess repellent and insecticidal activity against the cotton leafworm (*Spodoptera littoralis*). Our study identified palmitic acid as a repellent of *Gonipterus* sp. n. 2. Older leaves of *Luffa cylindrica* (*Cucurbitales*), *Luffa acutangular* (*Cucurbitales*) *and Mimosa scabrella* (*Fabaceae*) contained higher concentrations of both overall fatty acids and palmitic acid. As *Gonipterus* sp. n. 2 prefers to feed on younger leaves, a similar ontogenic pattern in palmitic acid can be expected in *Eucalyptus.*

2.6 – Conclusion:

Our results provide evidence that the feeding behavior of *Gonipterus* sp. n 2 is influenced by the concentration of a few select *Eucalyptus* metabolites. Furthermore, there exists a large intraspecific variation in the concentration of these metabolites and this results in substantial variation in the pest's preference for a single species. Furthermore, we demonstrated that novel insights into chemical defenses can be gained by studying variation across multiple genotypes within the same species. Using this approach, we identified five compounds which significantly altered the feeding behavior of *Gonipterus* sp. n. 2 under laboratory conditions. Three of these compounds were attractants and phagostimulants (1,8-cineole, sucrose, and oxalic acid), while two (shikimic acid and palmitic acid) were repellent of *Gonipterus* sp. n. 2. Understanding the effect of chemical defenses on host preference and implementing the knowledge in tree breeding programs, provides an opportunity to reduce damage caused by these beetles.

2.7 – Tables:

Table 1: *Eucalyptus* genotypes used in this study (control genotype indicated in yellow).

Source	Species	New Code	Estimated height (m)	
Experimental farm (SAPPI)	*E. benthamii*	BEN01	0.61	
Tom Jenkins plantation	*E. botryoides*	BOT01	2.68	
Tom Jenkins plantation	*E. citriodora*	CIT01	13.18	
Tom Jenkins plantation	*E. dorriengoenis*	DOR01	19.21	
Experimental farm (SAPPI)	*E. dunnii*	DUN01	0.62	*
Experimental farm (SAPPI)	*E. dunnii*	DUN02	0.64	*
Experimental farm (SAPPI)	*E. dunnii*	DUN03	0.59	*
Experimental farm (SAPPI)	*E. dunnii*	DUN04	0.61	*
Experimental farm (SAPPI)	*E. dunnii*	DUN05	0.60	*
Experimental farm (SAPPI)	*E. dunnii*	DUN06	0.58	*
Experimental farm (SAPPI)	*E. dunnii*	DUN07	0.63	*
Experimental farm (SAPPI)	*E. dunnii*	DUN08	0.61	*
Experimental farm (SAPPI)	*E. dunnii*	DUN09	0.61	*
Experimental farm	*E. dunnii*	DUN00	4.5	*
Experimental farm	*E. dunnii*	DUN10	0.58	
Experimental farm	*E. dunnii*	DUN11	1.52	
Experimental farm	*E. dunnii*	DUN12	1.51	
Experimental farm	*E. dunnii*	DUN13	1.53	
Experimental farm	*E. dunnii*	DUN14	1.50	
Experimental farm	*E. dunnii*	DUN15	1.54	
Tom Jenkins plantation	*E. goniocalyx*	GON01	13.15	
Experimental farm (SAPPI)	*E. grandis*	GRA01	0.59	
Experimental farm	*E. grandis*	GRA02	1.48	
Experimental farm	*E. grandis*	GRA03	1.53	

*indicates genotypes selected for chemical analysis

Table 1: continued.

Source	Species	New Code	Estimated height (m)	
Experimental farm (SAPPI)	*E. grandis* × *urophylla*	GU01	0.61	*
Experimental farm (SAPPI)	*E. grandis* × *urophylla*	GU02	0.60	*
Experimental farm (SAPPI)	*E. grandis* × *urophylla*	GU03	0.58	*
Experimental farm (SAPPI)	*E. grandis* × *urophylla*	GU04	0.63	*
Experimental farm (SAPPI)	*E. grandis* × *urophylla*	GU05	0.61	*
Experimental farm (SAPPI)	*E. grandis* × *urophylla*	GU06	0.59	*
Experimental farm (SAPPI)	*E. grandis* × *urophylla*	GU07	0.60	*
Experimental farm (SAPPI)	*E. grandis* × *urophylla*	GU08	0.62	*
Experimental farm (SAPPI)	*E. grandis* × *urophylla*	GU09	0.63	*
Experimental farm (SAPPI)	*E. grandis* × *urophylla*	GU10	0.65	*
Experimental farm (SAPPI)	*E. grandis* × *urophylla*	GU11	0.71	
Experimental farm (SAPPI)	*E. grandis* × *urophylla*	GU12	0.57	*
Experimental farm (SAPPI)	*E. grandis* × *urophylla*	GU13	0.59	*
Experimental farm (SAPPI)	*E. grandis* × *urophylla*	GU14	0.62	*
Experimental farm	*E. grandis x camaldulensis*	GC01	1.51	
Experimental farm	*E. grandis x camaldulensis*	GC02	1.48	
Experimental farm	*E. grandis x camaldulensis*	GC03	1.52	
Experimental farm	*E. macarthurii*	MAC01	1.50	
Experimental farm (SAPPI)	*E. macarthurii*	MAC02	0.62	
Tom Jenkins plantation	*E. maculata*	MAC01	17.34	
Tom Jenkins plantation	*E. microcorys*	MIC01	19.18	
Experimental farm	*E. nitens*	NIT01	1.51	
Experimental farm	*E. nitens*	NIT02	1.54	
Experimental farm (SAPPI)	*E. nitens*	NIT03	0.58	*
Experimental farm (SAPPI)	*E. nitens*	NIT04	0.61	*
Experimental farm	*E. nitens*	NIT05	1.47	
Experimental farm	*E. nitens*	NIT06	1.51	
Experimental farm (SAPPI)	*E. nitens*	NIT07	0.64	
Experimental farm (SAPPI)	*E. nitens*	NIT08	0.61	*
Experimental farm (SAPPI)	*E. nitens*	NIT09	0.62	*
Tom Jenkins plantation	*E. obliqua*	OBL01	2.72	
Tom Jenkins plantation	*E. ovata*	OVA01	2.71	
Tom Jenkins plantation	*E. paniculata*	PAN01	2.72	
Tom Jenkins plantation	*E. pilularis*	PIL01	17.31	
Tom Jenkins plantation	*E. propinqua*	PRO01	17.33	
Tom Jenkins plantation	*E. saligna*	SAL01	13.17	
Tom Jenkins plantation	*E. sideroxlon*	SID01	13.21	
Experimental farm (SAPPI)	*E. smithii*	SMI01	0.60	
Experimental farm	*E. saligna x urophylla*	SU01	1.57	

*indicates genotypes selected for chemical analysis

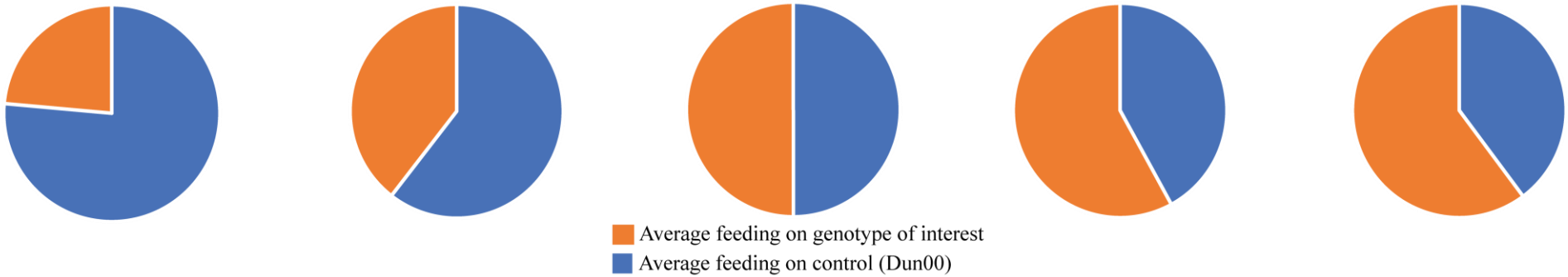
Table 2: Leaf surface area (mm^2) lost by feeding of *Gonipterus* sp. n. 2 (Feeding represents the percentage of pixels lost of the average of the 2 similar leaf disks between the photographs taken on day 1 and 3). SD = standard deviation; SE = standard error.

Genotype Code	Feeding on Dun00 (%)	SD on Dun00	SE on Dun00	Feeding on genotype (%)	SD on genotype	SE on genotype	Δ feeding (%)
BEN01	33.97	15.70	5.55	47.96	20.62	7.29	-13.98
BOT01	41.28	9.19	3.25	34.46	12.51	4.42	6.83
CIT01	34.50	16.20	5.73	18.91	7.19	2.54	15.58
DOR01	28.25	20.61	7.29	17.70	25.11	8.88	10.55
DUN01	26.27	16.45	5.82	23.08	18.30	6.47	3.19
DUN02	23.27	19.04	6.73	26.84	26.56	9.39	-3.57
DUN03	22.26	14.68	5.19	16.29	14.57	5.15	5.97
DUN04	44.61	12.41	4.39	10.94	21.03	7.43	33.67
DUN05	39.54	21.00	7.42	20.22	12.97	4.59	19.32
DUN06	33.26	26.05	9.21	38.96	35.59	12.58	-5.70
DUN07	59.06	27.96	9.89	25.67	17.73	6.27	33.39
DUN08	32.59	20.79	7.35	39.96	31.10	11.00	-7.36
DUN09	22.09	11.35	4.01	37.32	21.59	7.63	-15.23
DUN10	51.05	28.59	10.11	36.87	24.69	8.73	14.18
DUN11	66.30	32.20	11.38	42.74	31.74	11.22	23.56
DUN12	51.67	13.42	4.74	50.40	22.38	7.91	1.27
DUN13	48.85	27.17	9.61	15.36	11.40	4.03	33.50
DUN14	28.74	17.67	6.25	31.98	10.25	3.62	-3.24
DUN15	53.94	31.58	11.17	7.73	5.93	2.10	46.21
GC01	39.47	19.99	7.07	24.81	15.98	5.65	14.66
GC02	64.04	21.74	7.69	42.96	15.10	5.34	21.08
GC03	65.40	15.71	5.56	8.92	6.68	2.36	56.48
GON01	37.98	18.63	6.58	15.87	4.17	1.47	22.10
GRA01	76.88	26.03	9.20	8.14	9.09	3.22	68.73
GRA02	37.07	16.03	5.67	17.41	7.89	2.79	19.66
GU01	55.49	24.23	8.57	45.24	21.61	7.64	10.26
GU02	74.05	18.96	6.71	50.43	31.23	11.04	23.62
GU03	45.26	23.28	8.23	37.75	21.01	7.43	7.51
GU04	40.94	18.69	6.61	42.65	7.04	2.49	-1.71

Table 2: continued.

Genotype Code	Feeding on Dun00 (%)	SD on Dun00	SE on Dun00	Feeding on genotype (%)	SD on genotype	SE on genotype	Δ feeding (%)
GU05	40.23	28.97	10.24	24.97	20.10	7.11	15.26
GU06	35.50	17.66	6.24	28.74	11.29	3.99	6.76
GU07	24.65	16.07	5.68	16.15	14.72	5.20	8.50
GU08	44.95	21.81	7.71	46.69	24.91	8.81	-1.74
GU09	36.60	22.83	8.07	10.34	6.20	2.19	26.26
GU10	32.68	21.40	7.57	23.07	21.09	7.46	9.61
GU11	17.52	8.77	3.10	7.76	4.94	1.75	9.76
GU12	20.29	9.26	3.27	30.66	13.22	4.68	-10.38
GU13	18.40	9.68	3.42	17.13	14.02	4.96	1.27
GU14	33.62	20.01	7.07	37.70	24.83	8.78	-4.08
MAC01	43.38	18.45	6.52	26.93	17.38	6.15	16.44
MAC02	37.96	26.73	9.45	40.90	11.78	4.16	-2.95
MIC01	5.62	6.25	2.21	21.94	23.06	8.15	-16.32
NIT01	62.06	27.89	9.86	41.64	11.36	4.02	20.41
NIT02	38.66	6.03	2.13	37.09	14.44	5.11	1.56
NIT03	54.81	20.10	7.10	13.18	8.92	3.15	41.62
NIT04	20.04	15.58	5.51	4.68	9.54	3.37	15.37
NIT05	18.36	12.68	4.48	22.53	9.19	3.25	-4.17
NIT06	23.87	18.37	6.49	28.80	20.92	7.40	-4.92
NIT07	41.13	12.63	4.46	24.16	8.31	2.94	16.97
NIT08	10.77	6.50	2.30	11.45	5.21	1.84	-0.67
NIT09	23.84	13.97	4.94	29.20	10.88	3.85	-5.35
OBL01	26.96	15.63	5.53	4.87	4.41	1.56	22.08
OVA01	26.51	16.80	5.94	11.80	5.30	1.87	14.71
PAN01	30.62	17.82	6.30	33.42	18.21	6.44	-2.81
PIL01	21.80	21.93	7.75	45.07	21.80	7.71	-23.27
PRO01	66.72	9.28	3.28	19.61	7.39	2.61	47.12
SAL01	42.76	25.59	9.05	33.21	11.50	4.07	9.55
SID01	43.25	16.69	5.90	8.68	7.93	2.81	34.58
SU01	23.51	19.77	8.07	38.83	13.48	5.50	-15.32

Table 3: Genotypes separated into five columns based on their resistance relative to *E. dunnii* (Dun00) with *p-value* (Kruskal–Wallis Chi^2 test).



High resistance			Mild resistance			Similar to control			Mild susceptibility			High susceptibility		
Species	**χ^2**	**P value**	**Species**	**χ^2**	**P value**	**Species**	**χ^2**	**P value**	**Species**	**χ^2**	**P value**	**Species**	**χ^2**	**P value**
DUN15	5.33	0.02	DUN05	3	0.08	GU06	0.33	0.56	PIL01	2.08	0.15	GU12	5.33	0.02
GC03	5.33	0.02	CIT01	3	0.08	GU04	0.33	0.56	BEN01	1.33	0.25			
NIT03	5.33	0.02	GU02	2.08	0.15	NIT05	0.33	0.56	NIT09	1.33	0.25			
GRA03	5.33	0.02	GC02	2.08	0.15	NIT06	0.33	0.56	SU01	0.75	0.39			
GON01	5.33	0.02	GC01	2.08	0.15	MAC02	0.33	0.56	GU07	0.75	0.39			
OBL01	5.33	0.02	NIT01	2.08	0.15	DOR01	0.33	0.56	DUN14	0.75	0.39			
OVA01	5.33	0.02	DUN10	0.75	0.39	NIT02	0.08	0.77	NIT08	0.75	0.39			
PRO01	5.33	0.02	DUN11	0.75	0.39	DUN03	0.08	0.77	DUN08	0.75	0.39			
SID01	5.33	0.03	GU05	0.75	0.39	GU08	0.08	0.77						
DUN13	4.08	0.04	GU10	0.75	0.39	GU14	0.08	0.77						
NIT04	4.08	0.04	BOT01	0.75	0.39	DUN12	0	1						
DUN07	4.08	0.04	MAC01	0.75	0.39	GU03	0	1						
GU09	4.08	0.04				DUN02	0	1						
DUN04	4.08	0.04				GU13	0	1						
NIT07	4.08	0.04				DUN06	0	1						
						DUN08	0	1						
						SAL01	0	1						
						MIC01	0.33	0.56						
						PAN01	0.08	0.77						

Table 4: Leaf surface area (mm^2) lost by feeding of *Gonipterus* sp. n. 2 for the comparisons between all genotypes considered resistant in Table 3 (Feeding represents the percentage of pixels lost of the average of the 2 similar leaf disks between the photographs taken on day 1 and 3). SD = standard deviation.

Resistant Genotypes							
Genotype A	**Genotype B**	**Feeding on genotype A (%)**	**SD on genotype A**	**Feeding on genotype B (%)**	**SD on genotype B**	**Δ feeding (%)**	**P value**
GC01	NIT04	81.17	7.97	8.98	24.99	72.19	0.02
GC01	GU10	58.46	23.71	6.94	6.61	51.52	0.02
DUN11	DUN06	61.10	29.40	10.71	4.68	50.39	0.02
PAN01	CIT01	51.79	21.56	7.67	4.76	44.12	0.02
DUN10	NIT07	47.05	7.43	11.65	14.10	35.41	0.02
NIT04	DUN05	70.92	15.74	37.17	15.18	33.75	0.02
GRA01	GU11	56.66	16.58	30.45	11.72	26.21	0.02
GU09	GU11	65.17	35.93	33.83	17.42	31.35	0.04
MAC01	SID01	74.24	14.62	46.39	27.24	27.85	0.04
GU05	DUN15	84.76	6.68	72.77	7.33	11.99	0.04
GU01	GC02	58.41	21.17	23.68	20.10	34.73	0.08
NIT03	GC03	78.50	15.61	49.09	21.22	29.42	0.08
NIT07	GRA02	47.85	14.22	24.88	20.27	22.97	0.08
GU09	GRA03	54.77	14.55	32.10	18.48	22.67	0.08
OVA01	GON01	77.14	15.74	61.10	9.33	16.04	0.08
OBL01	GON01	75.37	9.63	64.71	10.49	10.66	0.15
CIT01	OBL01	56.60	8.37	41.81	23.66	14.79	0.25
DUN10	GU01	24.22	13.16	13.60	17.86	10.62	0.25
GRA02	NIT03	61.93	12.94	52.57	18.67	9.35	0.25
BOT01	MAC01	69.20	19.54	54.71	19.67	14.49	0.39
DUN06	DUN05	83.59	8.22	75.05	23.50	8.54	0.39
DUN11	GC02	43.13	33.28	34.79	12.63	8.34	0.39
OVA01	SID01	69.90	15.78	63.09	19.82	6.82	0.39
GU10	GRA01	55.44	10.16	50.02	14.47	5.42	0.39
BOT01	GRA02	63.09	8.66	60.19	12.88	2.89	0.56
DUN13	DUN15	76.59	18.20	73.70	9.86	2.88	0.56
NIT03	DUN13	64.83	25.78	65.22	15.14	0.40	1.00

Table 5: Leaf surface area (mm^2) lost by feeding of *Gonipterus* sp. n. 2 for the comparisons between all genotypes considered susceptible in Table 3(Feeding represents the percentage of pixels lost of the average of the 2 similar leaf disks between the photographs taken on day 1 and 3). SD = standard deviation.

Susceptible Genotypes							
Genotype A	**Genotype B**	**Feeding on genotype A (%)**	**SD on genotype A**	**Feeding on genotype B (%)**	**SD on genotype B**	**Δ feeding (%)**	**P value**
NIT08	GU07	66.33	7.28	37.83	12.73	28.50	0.02
SU01	DUN08	74.69	24.40	33.71	28.56	40.98	0.04
DUN08	DUN09	81.35	30.35	48.93	16.33	32.41	0.04
SU01	NIT09	53.85	17.16	35.57	12.74	18.28	0.08
GU12	BEN01	76.55	25.93	58.93	19.51	17.62	0.15
DUN09	GU12	48.20	17.36	35.62	19.87	12.58	0.15
NIT08	GRA03	38.59	3.82	33.90	11.73	4.70	0.39
PIL01	GRA03	46.18	14.97	45.43	11.42	0.76	0.56
BEN01	GU07	64.14	23.47	54.92	10.26	9.22	0.77
NIT08	GU07	66.33	7.28	37.83	12.73	28.50	0.02
SU01	DUN08	74.69	24.40	33.71	28.56	40.98	0.04

Table 6: Table ranking genotypes of Table 4 and 5 by leaf area consumed by *Gonipterus* sp.2, separated into two columns based on their resistance relative to *E. dunnii*.

Resistant		Susceptible	
Rank	**Genotype Code**	**Rank**	**Genotype Code**
1	GC01	1	SU01
2	GU01	2	NIT09
2	DUN10	*3*	DUN08
3	GU09	*4*	DUN09
3	GRA01	5	NIT08
3	GU10	5	GRA03
3	DUN11	5	PIL01
3	GC02	6	GU12
3	NIT07	7	BEN01
4	GU05	7	GU07
4	Nit04		
5	GRA03		
5	GU11		
5	DUN15		
5	DUN13		
5	NIT03		
5	GRA02		
5	BOT01		
6	DUN06		
6	DUN05		
7	MAC01		
8	GC03		
8	GU01		
8	CIT01		
8	OBL01		
8	OVA01		
8	SID01		
9	GON01		
10	PRO01		

Table 7: Compounds showing a statistically significant correlation between feeding and compound peak area with the r^2 value obtained by a simple linear regression of feeding to peak area (See figure 4 & 5).

Positive Correlations				Negative Correlations			
	r^2value				r^2value		
Compound	Du	GU	Ni	Compound	Du	GU	Ni
1,8-cineole	0.5	0.8	NA	*α*-pinene	0.88	0.61	NA
γ-terpinene	0.6	0.4	0.4	shikimic acid	0.38	0.51	0.1
lapachone	0.6	0.5	NA				
oxalic acid	0.8	0.6	NA				
palmitic acid	0.4	0.6	0.9				
sucrose	0.7	0.6	0.4				
terpinen-4-ol	0.6	0.9	NA				
trans-β-ocimene	0.8	0.8	NA				

Table 8: Artificial diet composition (based on the diet from Wheeler, 2001)

Compound	Amount
Agar	2.6425 g
AlphaCel	5.8 g
Ascorbic Acid	0.175 g
Casein	4.33 g
Cholesterol	0.075 g
Corn Starch	1.75 g
Glucose	1.25 g
Lecithin	0.125 g
Linseed oil	50 µl
Eucalyptus leaves	10 g
Methyl Paraben	0.08 g
Sorbic Acid	0.08 g
Sucrose	1.5 g
Vanderzant Vitamins	0.75 g
Water	100 ml
Wesson Salts	0.3875 g

Table 9: Compounds of interest separated based on their observed behavioral effects either matching or not matching the predicted effects in Table 7 with the *P-value* obtained from the *paired t-test*.

Matching behavior			Non-matching behavior		
Compound	**Conc. ($mg.mL^{-1}$)**	**P value**	**Compound**	**Conc. ($mg.mL^{-1}$)**	**P value**
1,8 - cineole	92.67 vs. 0	0.015	α-pinene	85.8 vs. 0	0.006
1,8 - cineole	9.27 vs. 0	0.102	α-pinene	8.58 vs. 0	0.319
1,8 - cineole	0.93 vs. 0	0.015	α-pinene	0.86 vs. 0	0.423
oxalic acid	196 vs. 112	0.002	γ-terpinene	850 vs. 0	0.058
oxalic acid	196 vs. 28	0.103	γ-terpinene	85 vs. 0	0.058
oxalic acid	112 vs. 28	0.571	γ-terpinene	8.5 vs. 0	0.003
shikimic acid	0.5 vs. 0.25	0.058	trans-β-ocimene	800 vs. 0	0.182
shikmic acid	0.5 vs. 0.1	0.058	trans-β-ocimene	80 vs. 0	0.444
shikmic acid	0.25 vs. 0.1	0.003	trans-β-ocimene	8 vs. 0	0.058
sucrose	700 vs. 350	0.103	palmitic Acid	7.5 vs. 5	0.007
sucrose	700 vs. 150	0.043	palmitic Acid	7.5 vs. 2.5	0.003
sucrose	350 vs. 150	0.011	palmitic Acid	5 vs. 2.5	0.638

2.8 – Figures:

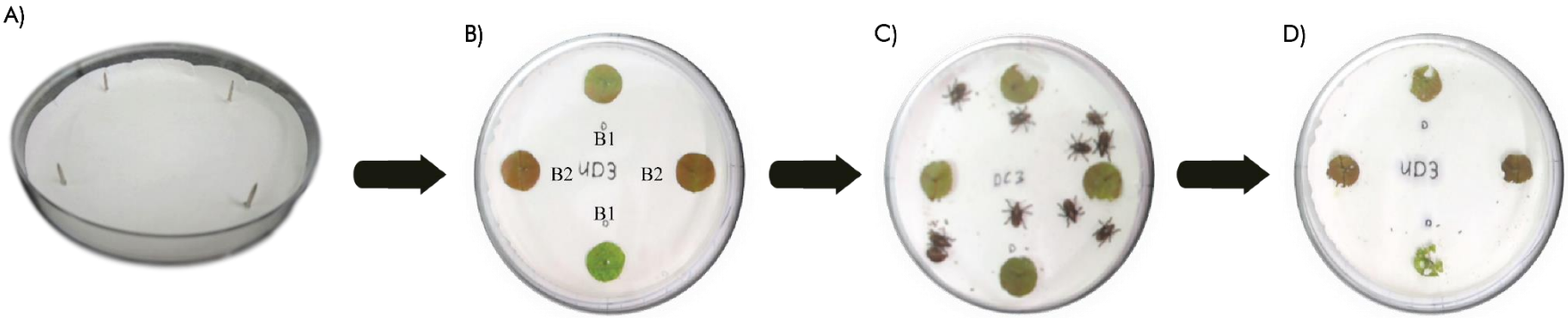


Figure 1: Leaf-disk feeding preference trial setup: A) Petri-dishes modified by inserting 4 thumb tacks around the perimeter of the plate, B) Leaf disks of a *Eucalyptus* genotype of interest (B1) and control species (Dun00) (B2) fastened to exposed tacks, C) *Eucalyptus* snout beetles were starved for 72 H before adding them to the Petri dishes, D) Amount of feeding on each disk recorded.

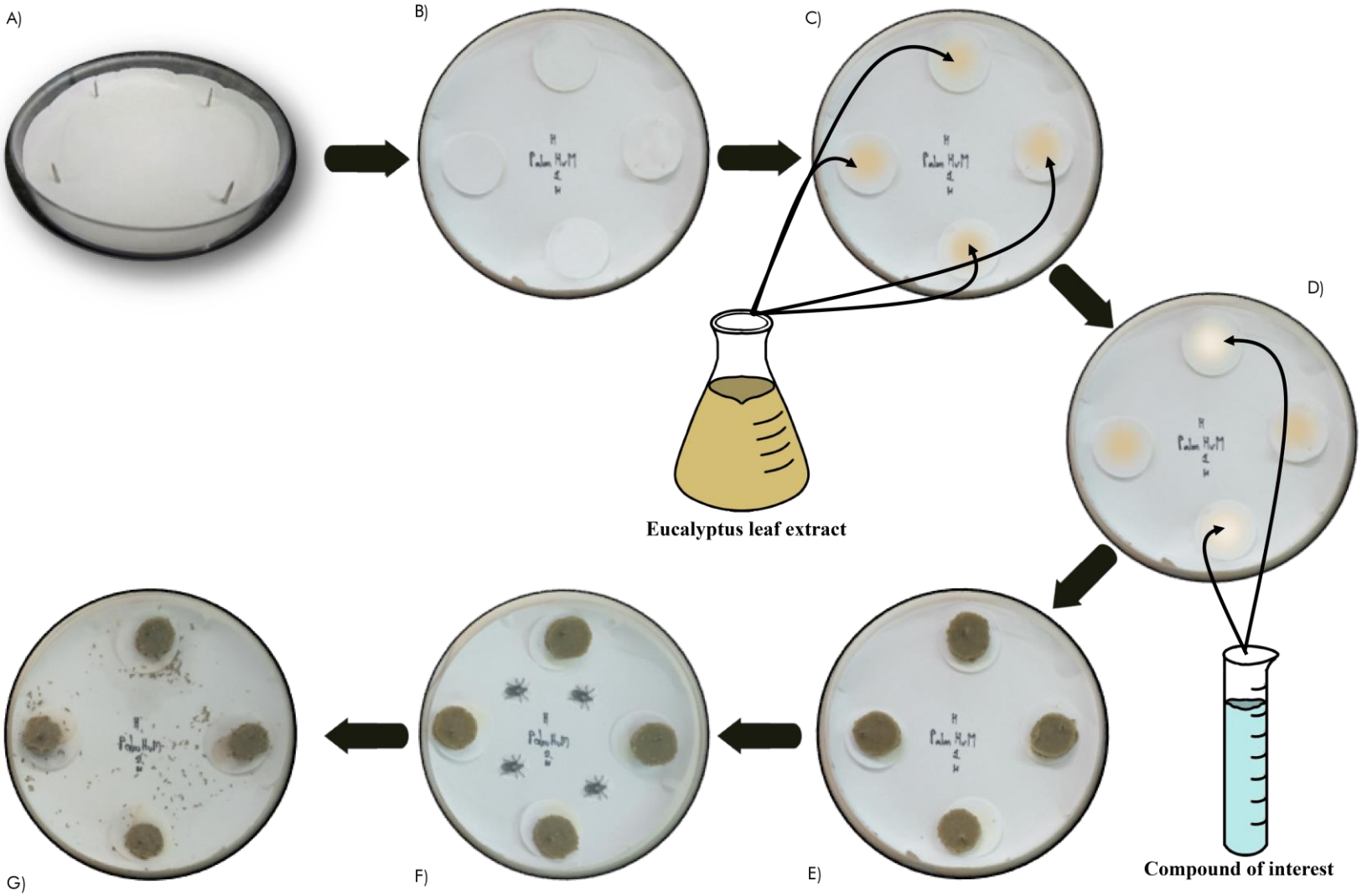


Figure 2: Artificial diet feeding preference trial setup: A) Petri-dishes modified by inserting 4 thumb tacks around the perimeter of the plate, B) four filter paper circles fastened to the exposed tacks, C) 5 µl *Eucalyptus* leaf extract added to the center the filter paper circle's D) 5 µl of the compound of interest added to the center of 2 opposite filter paper circles, E) Artificial diet disk fastened to exposed tacks covering the filter paper circle, F) *Eucalyptus* snout beetles starved for 72 H before adding them to the Petri dishes, G) Amount of feeding on each disk recorded..

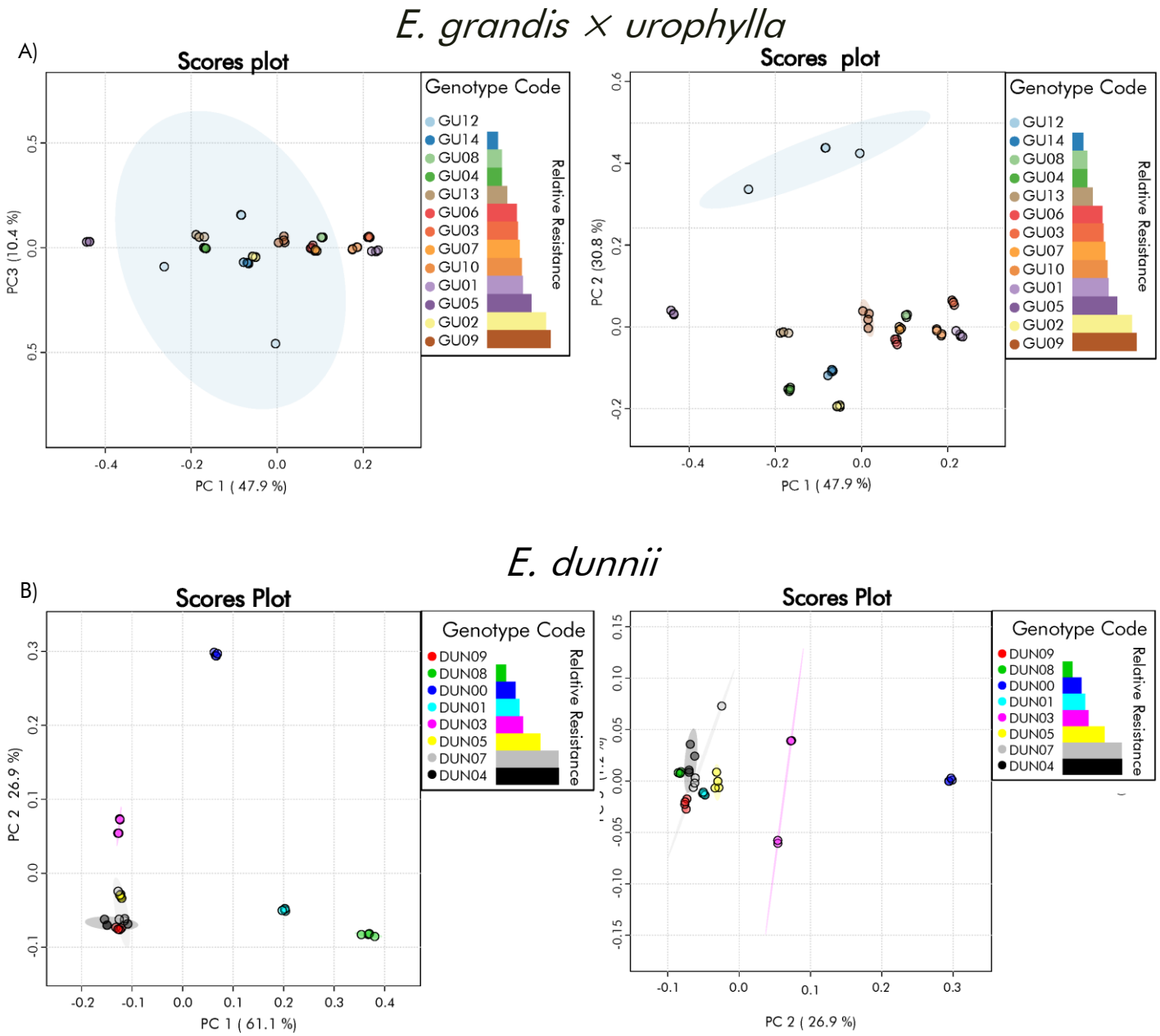


Figure 3: 2-Dimension principle component analysis of **A)** non-polar compounds found in *E. grandis x urophylla* leaves. **B)** non-polar compounds found in *E. dunnii* leaves. Horizontal bars indicate relative resistance of the clone to feeding by *Gonipterus* sp. n. 2 (relative to most susceptible tested genotype of its species (Dun09 and GU12))

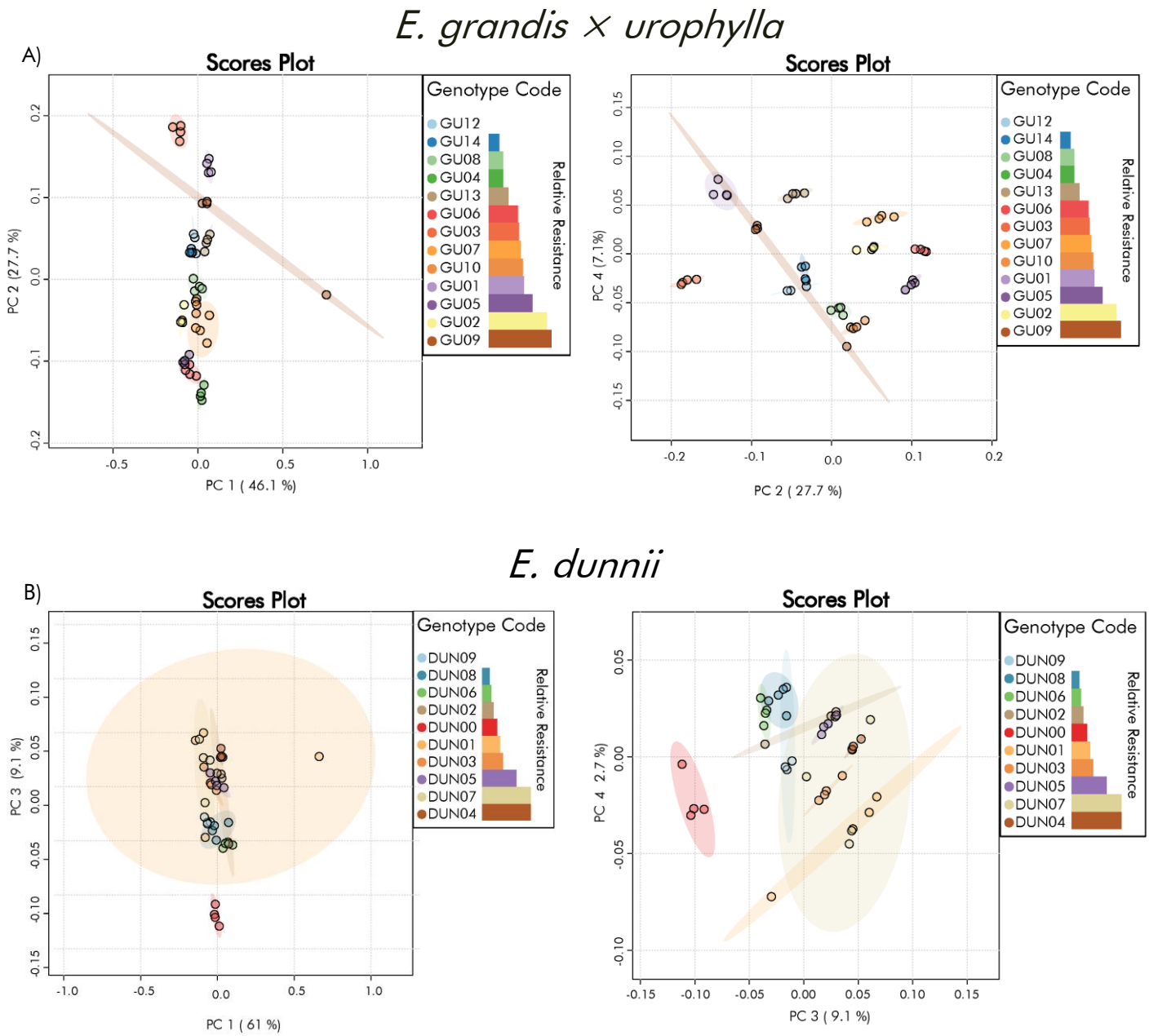


Figure 4: 2-Dimesion principle component analysis of **A)** compounds with polar functional groups found in *E. grandis x urophylla* leaves. **B)** compounds with polar functional groups found in *E. dunnii* leaves. Horizontal bars indicate relative resistance of the clone to feeding by *Gonipterus* sp. n. 2 (relative to most susceptible tested genotype of its species (Dun09 and GU12))

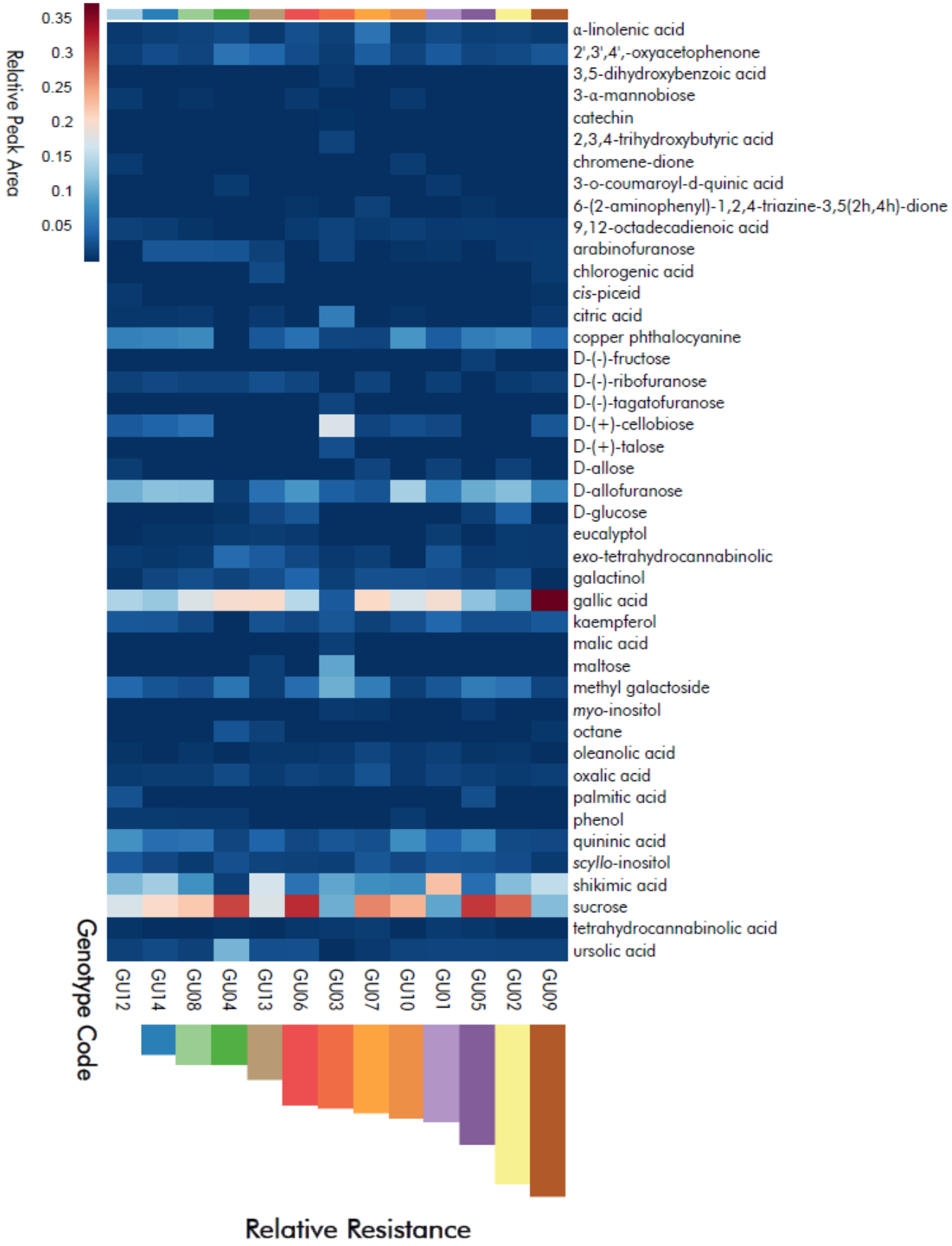


Figure 5: Heatmap of compounds with polar functional groups found in *E. grandis x urophylla* leaves. Vertical bars indicate relative resistance of the clone to feeding by *Gonipterus* sp. n. 2 (relative to most susceptible tested genotype (GU12)).

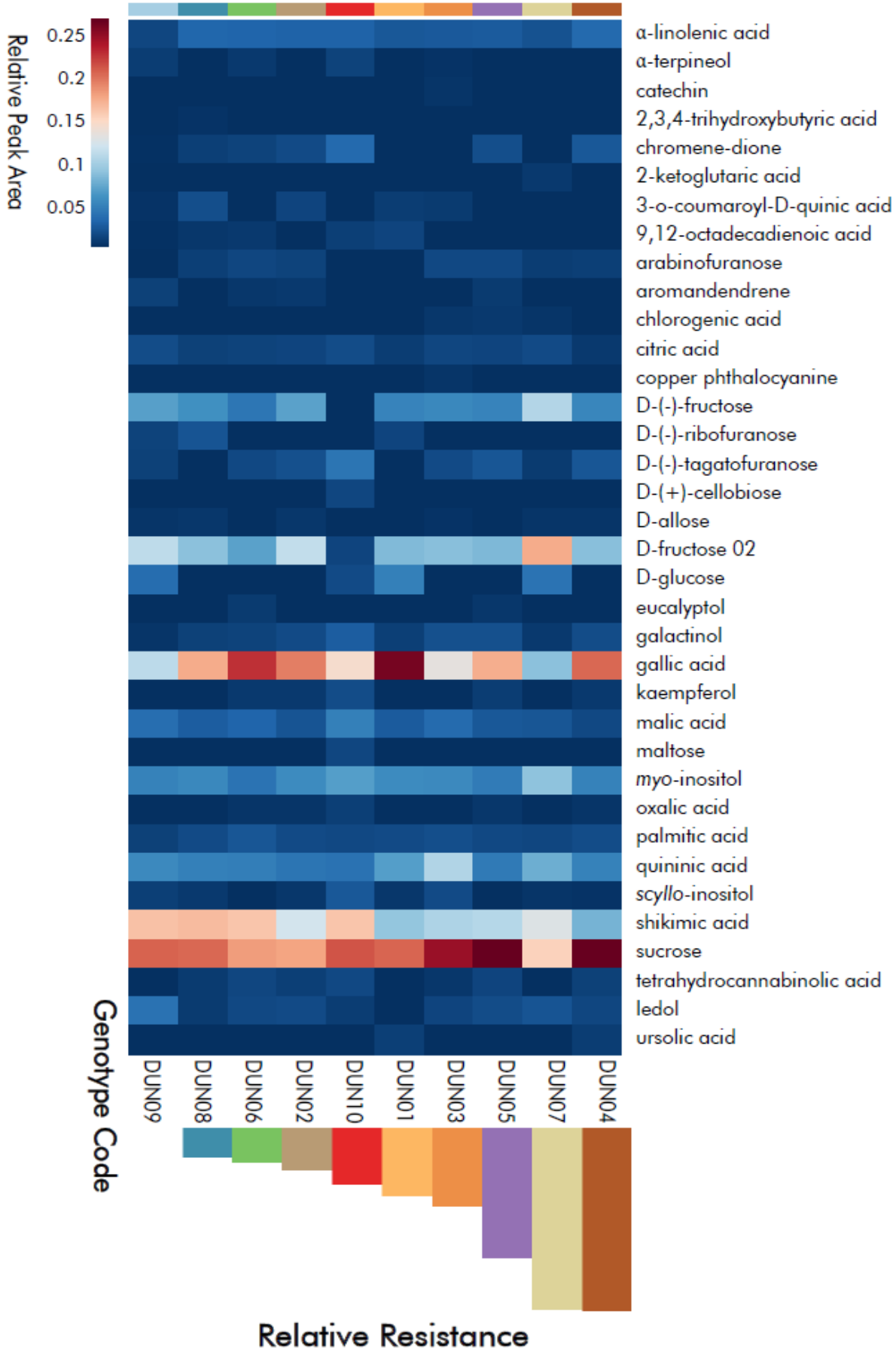


Figure 6: Heatmap of compounds with polar functional groups in *E. dunnii* leaves. Vertical bars indicate relative resistance of the clone to feeding by *Gonipterus* sp. n. 2 (relative to most susceptible tested genotype (Dun09)).

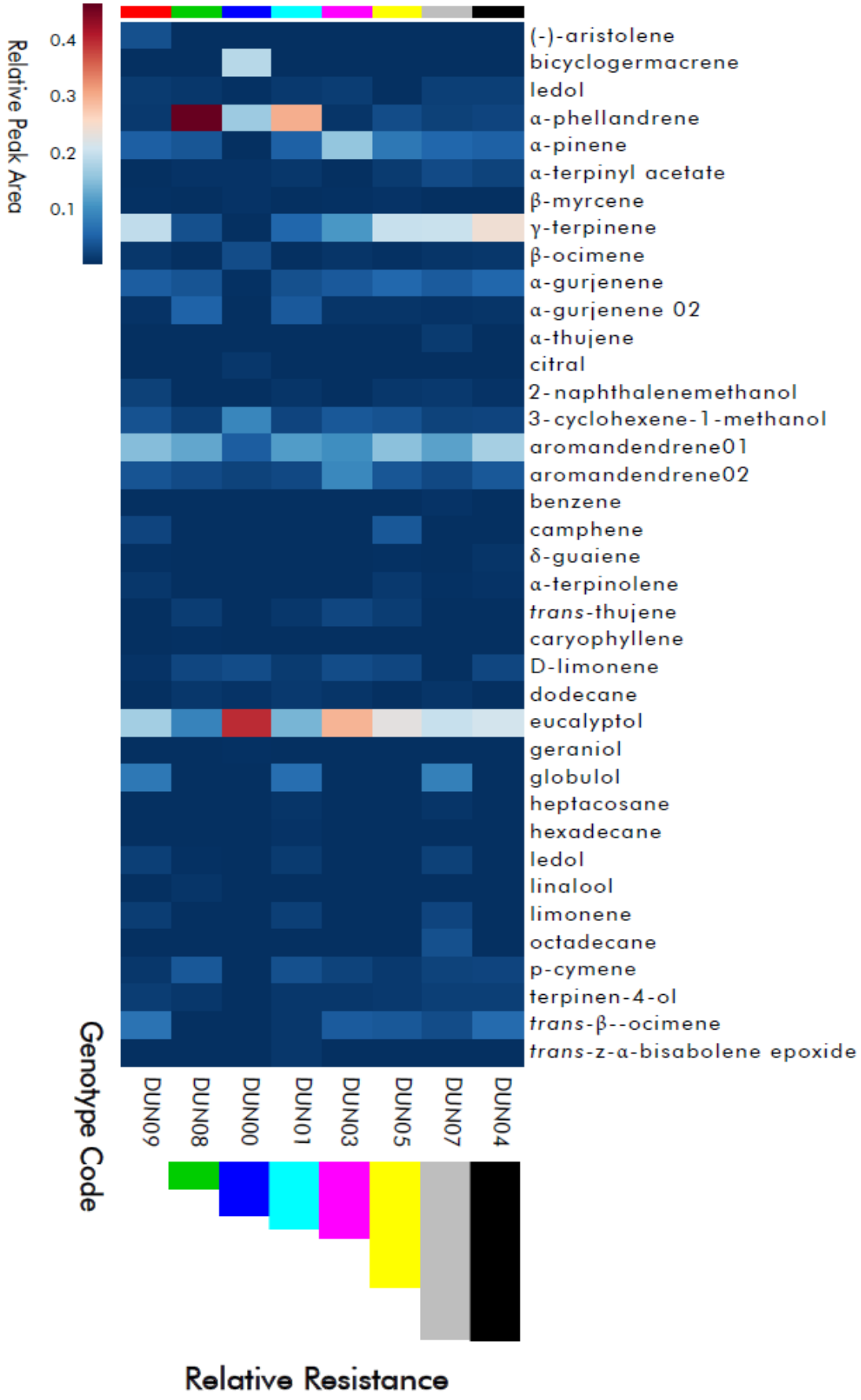


Figure 7: Heatmap of non-polar compounds found in *E. dunnii* leaves. Vertical bars indicate relative resistance of the clone to feeding by *Gonipterus* sp. n. 2 (relative to most susceptible tested genotype (Dun09)).

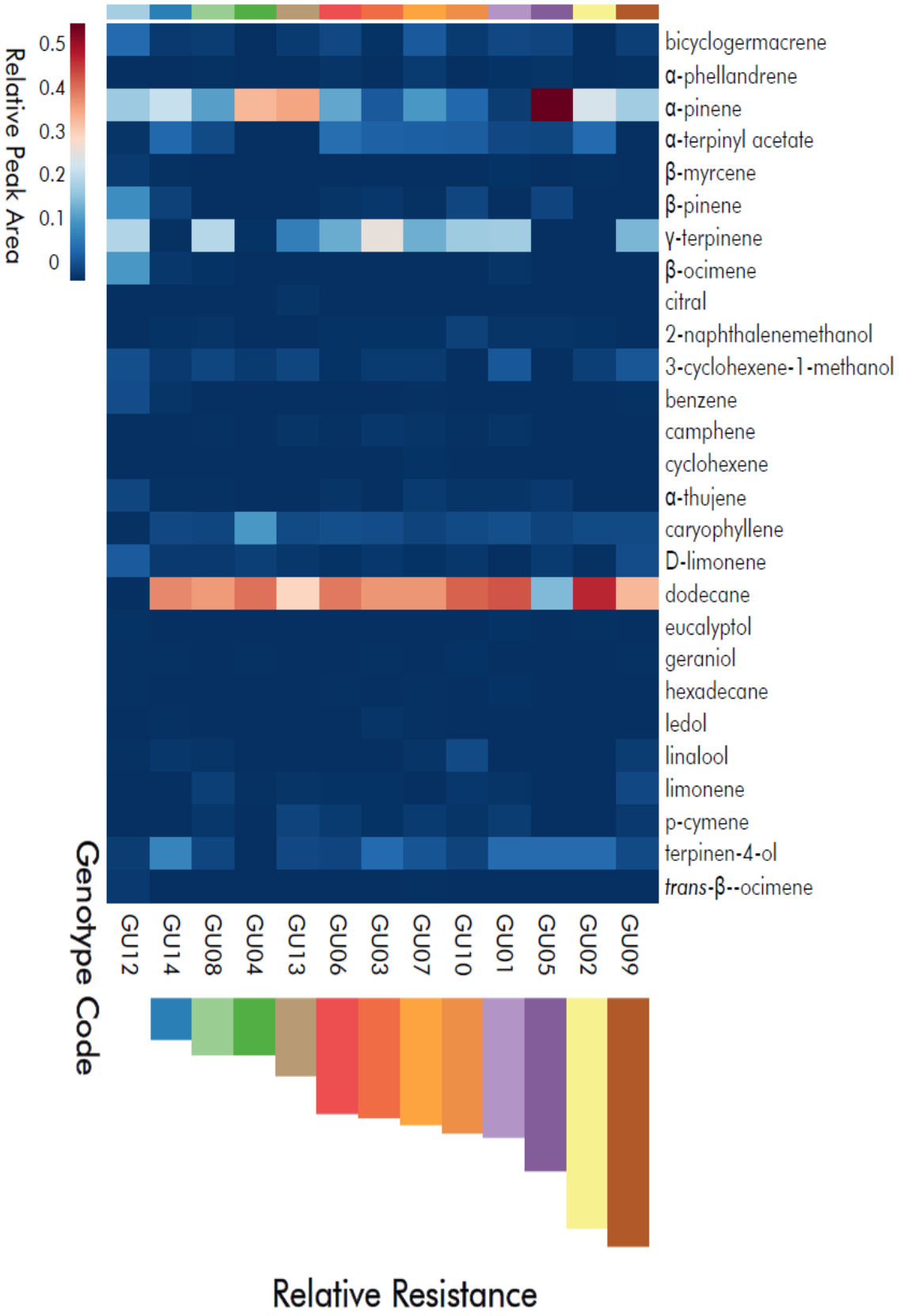


Figure 8: Heatmap of non-polar compounds found in *E. grandis x urophylla* leaves. Vertical bars indicate relative resistance of the clone to feeding by *Gonipterus* sp. n. 2 (relative to most susceptible tested genotype (GU12)).

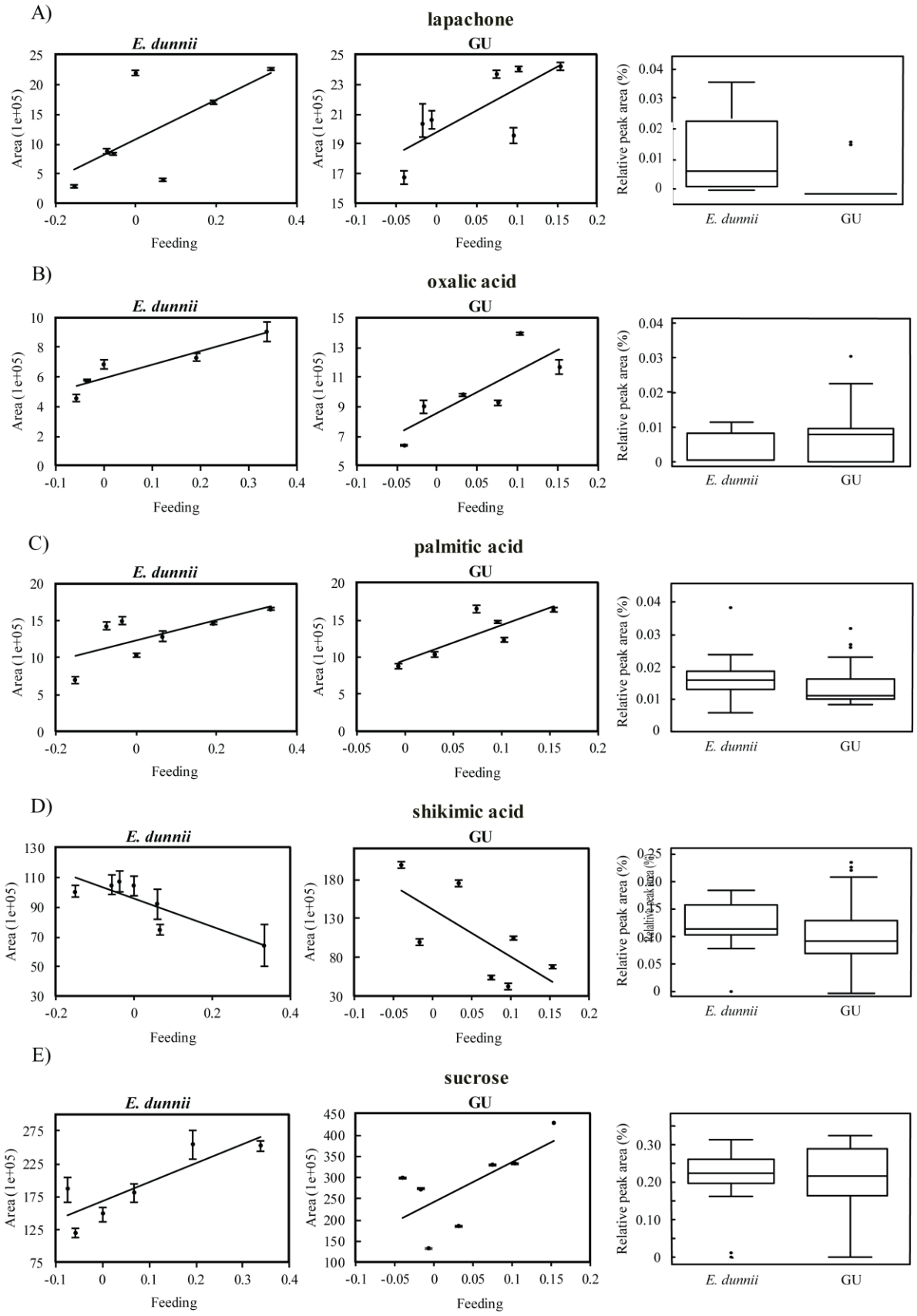


Figure 9: Linear regressions of chromatogram peak area (y axis) to feeding preference of *Gonipterus* sp. n. 2 (x axis; (See Table 7 Δ Feeding)). Separate plots for each *Eucalyptus* species (*E. dunnii* and *E. grandis x urophylla* (GU)) were tested for the compounds with polar functional groups and box plots depicting relative peak area (%) of tested species groups for **A)** lapachone **B)** oxalic acid **C)** palmitic acid **D)** shikimic acid **E)** sucrose. R^2- values for the linear correlations in Table 7.

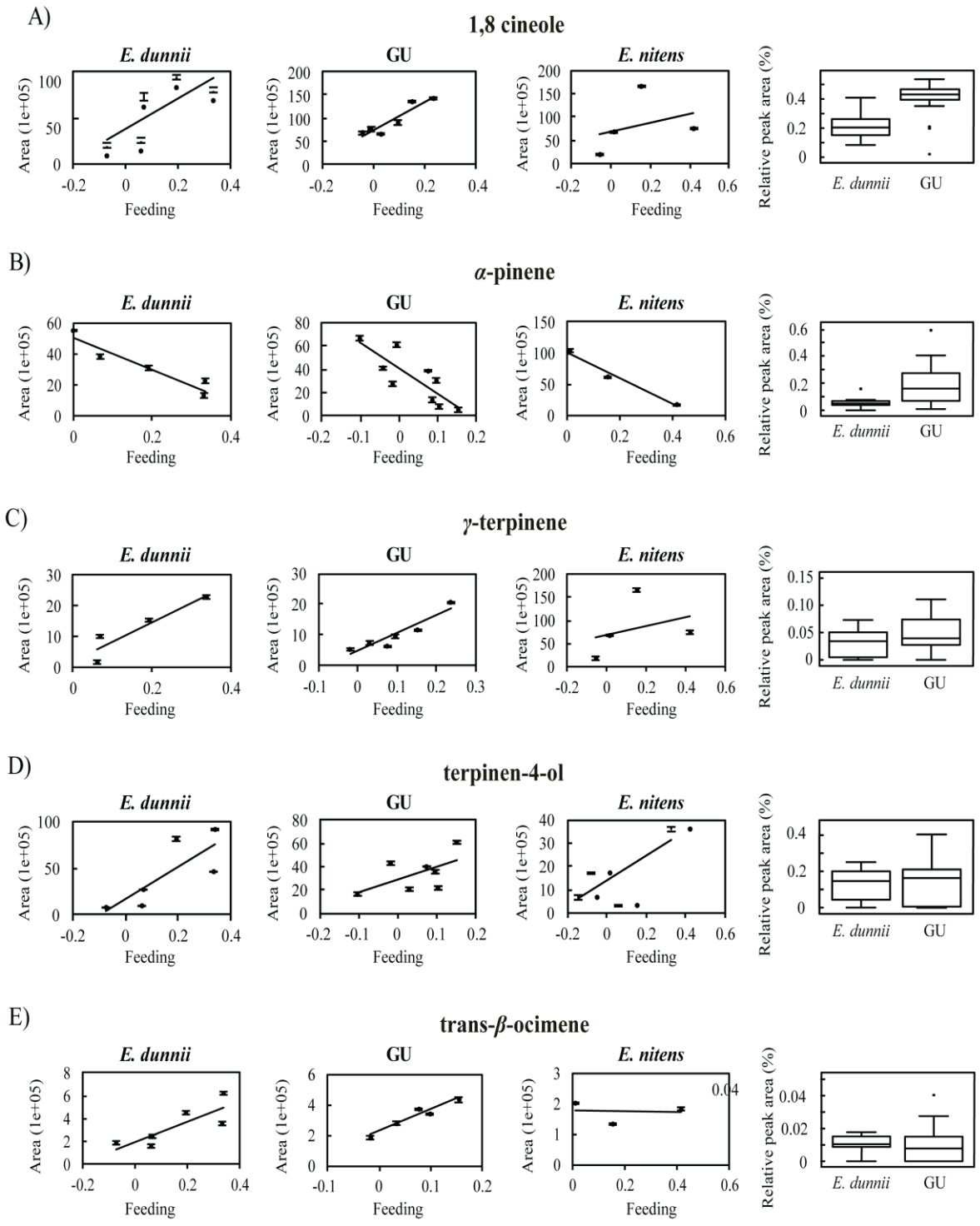
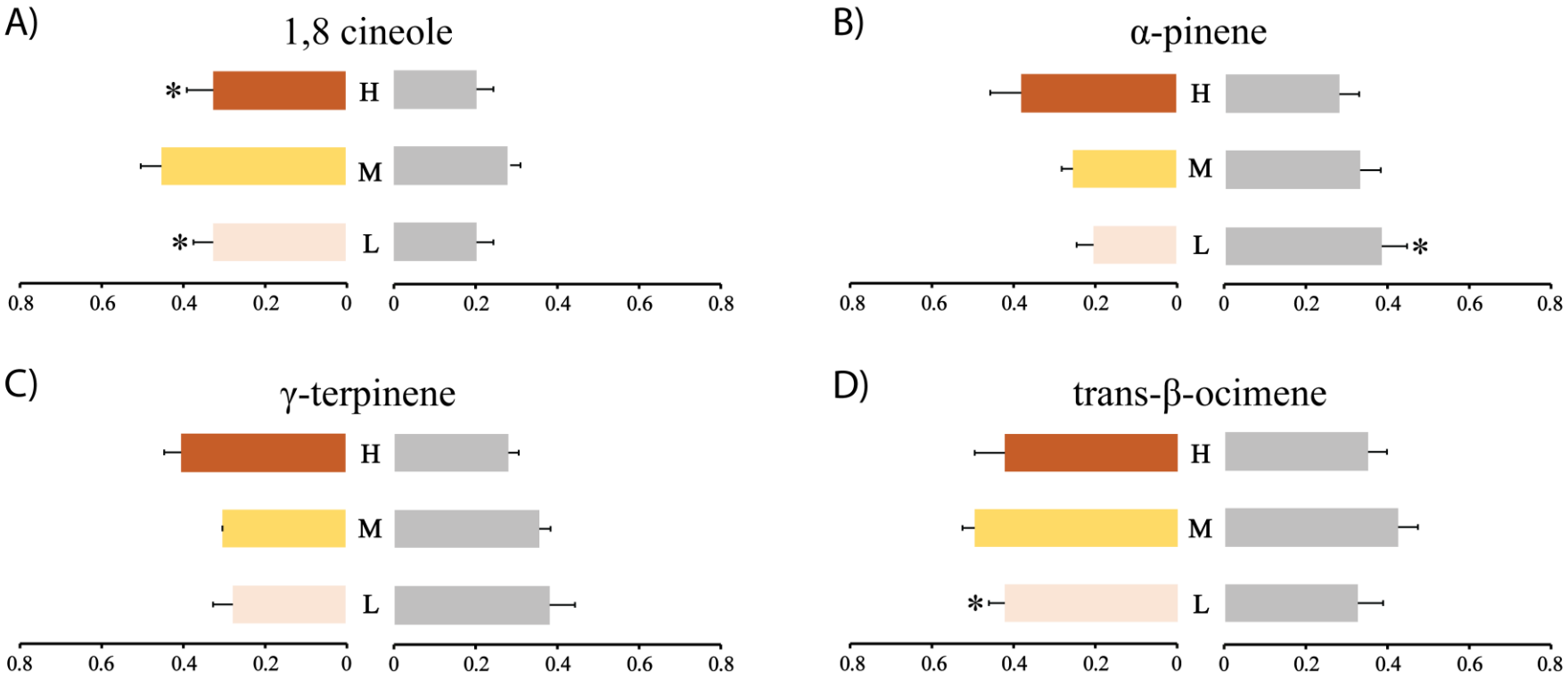


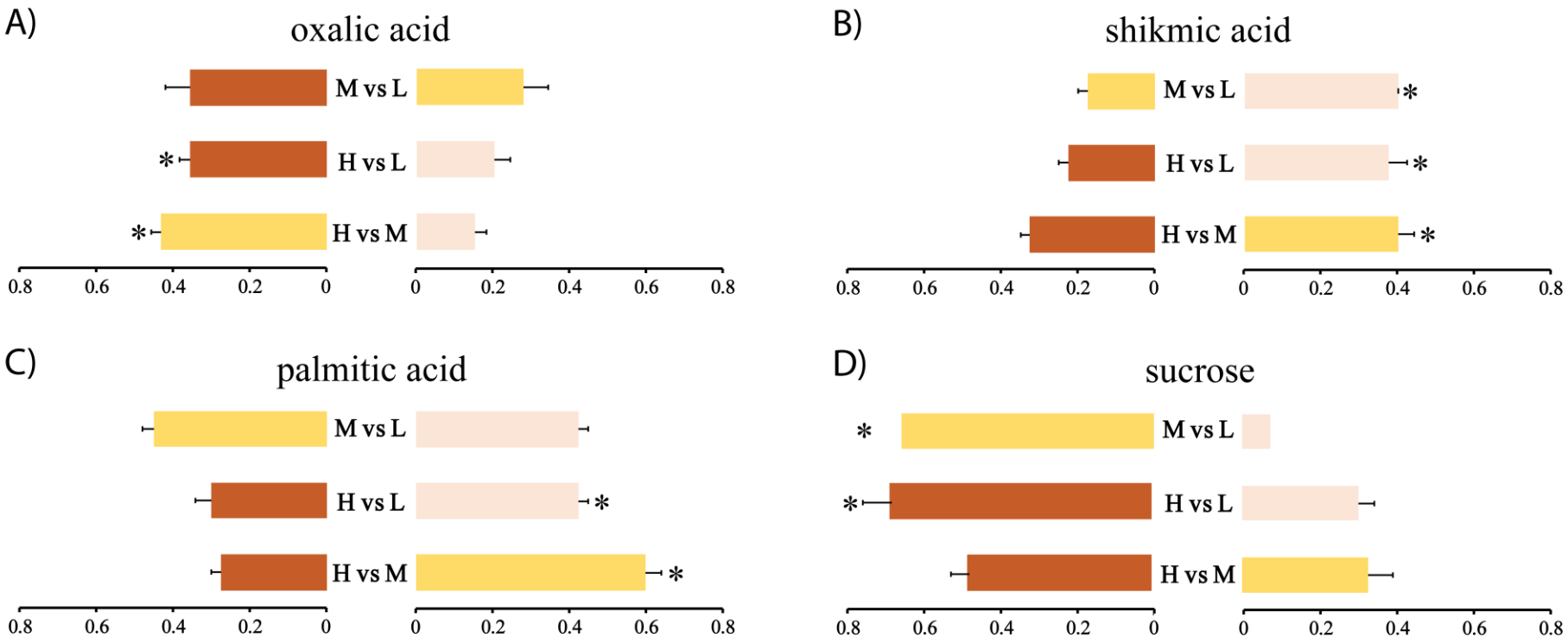
Figure 10: Linear regressions of chromatogram peak area (y axis) to feeding preference of *Gonipterus* sp. n. 2 (x axis; (See Table 7 Δ Feeding)). Separate plots for each *Eucalyptus* species (*E. dunnii*, *E. grandis x urophylla* (GU) and *E. nitens*) tested for the non-polar compound and box plots depicting relative peak area (%) of tested species groups for **A)** 1,8 cineole **B)** α-pinene C**)** γ-terpinene D**)** trans-*β*-ocimene **E)** terpinen-4-ol. R^2 values for the linear correlations in Table 7.



E)

Compound	Concentration (mg ml^{-1}) High (H)	Med (M)	Low (L)
1,8 cineole	92.7	9.27	0.93
α-pinene	85.8	8.58	0.86
γ-terpinene	850	85	8.5
trans-β-ocimene	800	80	8

Figure 11: Weight of artificial diet consumed (g) comparing an unlaced artificial diet to an artificial diet laced with different concentrations of: **A)** 1,8 cineole **B)** α-pinene **C)** γ-terpinene **D)** trans-β-ocimene **E)** Concentrations of each compound used in the assays.
* indicates significantly higher levels of feeding (*p-value* < *0.05;* pairwise t-test).



E)

Compound	Concentration ($mg\ ml^{-1}$) High (H)	Med (M)	Low (L)
oxalic acid	196	117	28
palmitic acid	7.5	5	2.5
shikimic acid	0.5	0.25	0.1
sucrose	700	350	150

Figure 12: Weight of artificial diet consumed (g) amending it with different concentrations of: **A)** oxalic acid **B)** palmitic acid **C)** shikimic acid **D)** sucrose **E)** Concentrations of each compound used in each assay.
* indicates significantly higher levels of feeding (*p-value* < *0.05;* pairwise t-test).

2.9 – References:

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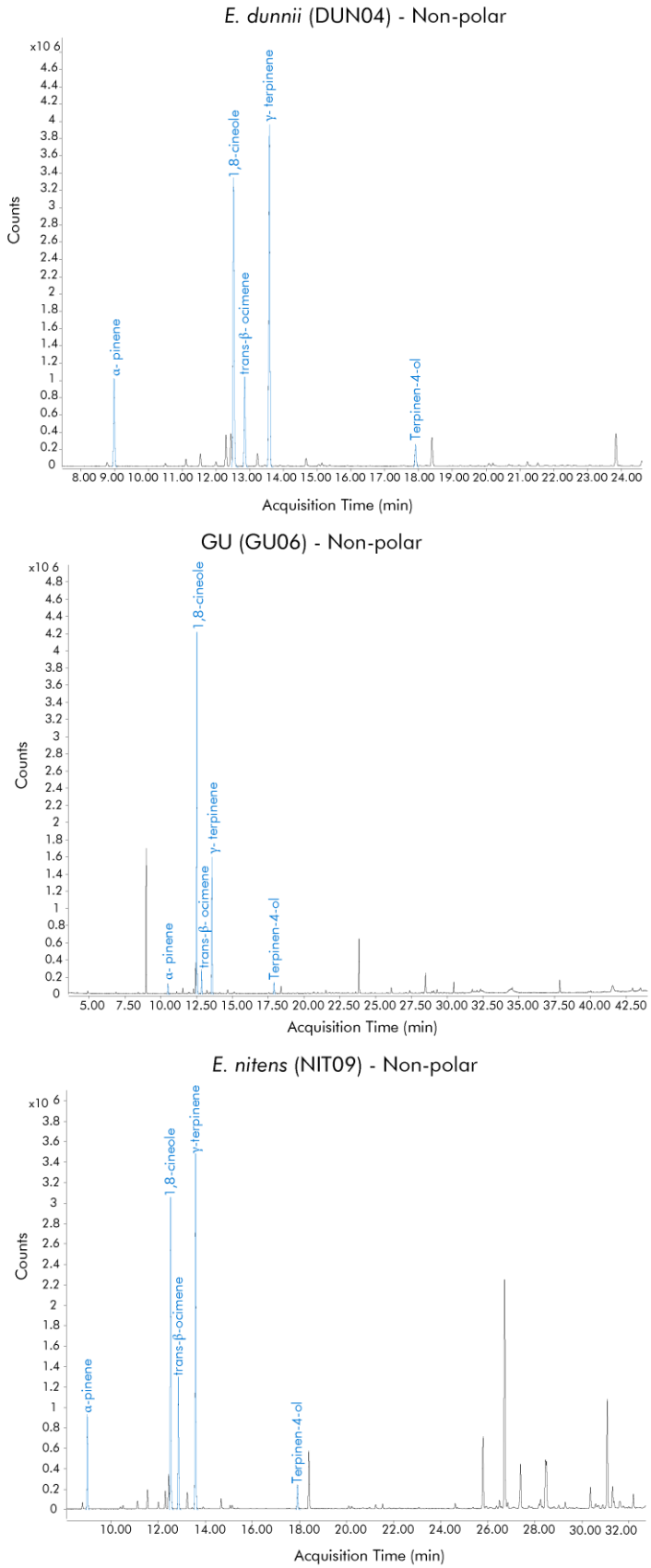
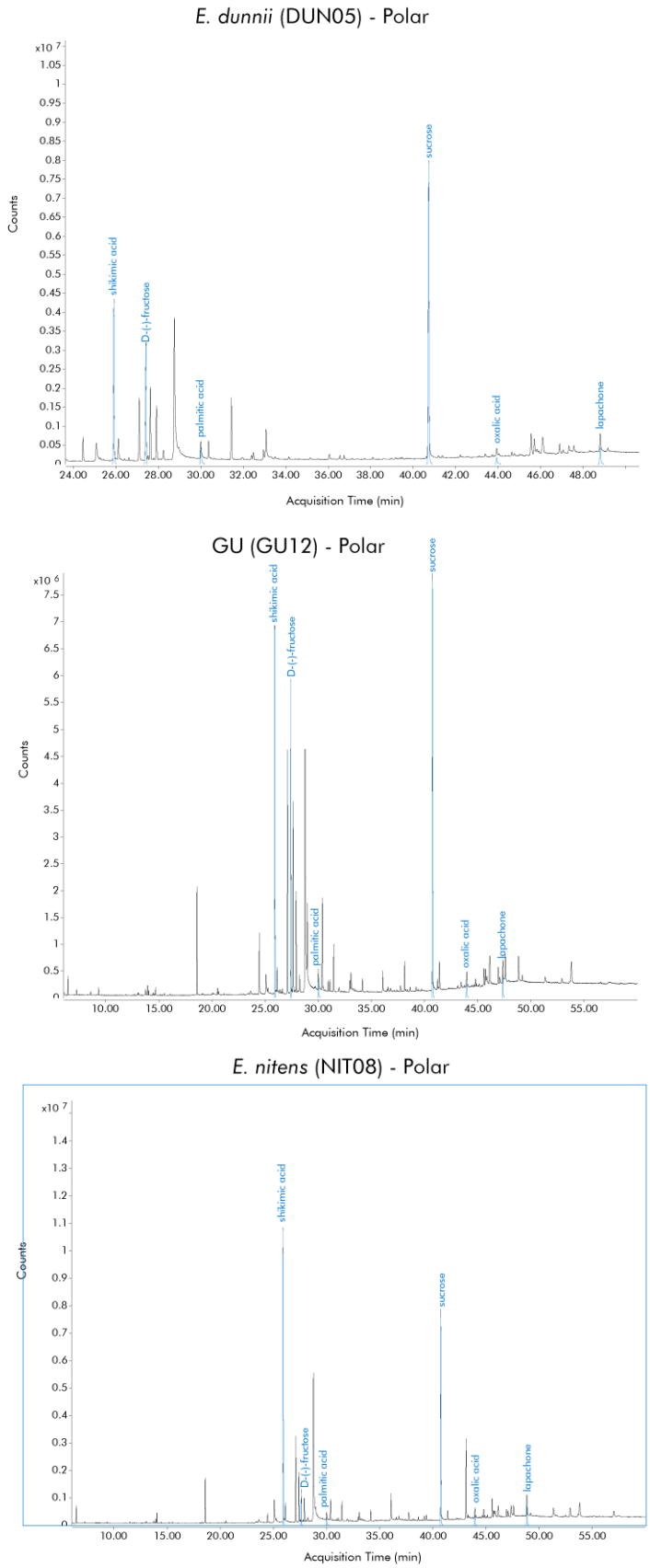
2.10 – Supplemental Material:



Supplemental Figure 1: Chromatogram of non-polar compounds of each species tested for genotype **A)** DUN04 **B)** GU06 **C)** NIT09



Supplemental Figure 2: Chromatogram of compounds with polar functional groups of each species tested for genotype **A)** DUN05 **B)** GU12 **C)** NIT08

Summary:

Gonipterus sp. n. 2 is a very important forestry pest. This insect causes massive defoliation in *Eucalyptus* plantations, specifically targeting young leaves and new shoots. *Gonipterus sp. n. 2* is controlled through a biocontrol agent *Anaphes nitens. A. nitens* was first discovered and implemented in South Africa in 1926 and by 1950 new releases of the parasitoid were ceased as the wasps were so efficient that the beetle population remained below economically significant levels for long periods. However, during the last 2 decades, massive *Gonipterus sp. 2* population outbreaks have been recorded and are causing widespread damage in commercial forestry plantations. While the reason for this is currently unknown, it has become clear that *A. nitens* may have lost its effectivity. Therefore, new management strategies are needed to help develop an effective integrated pest management programme. While many studies focused on the biocontrol agent or chemical control through pesticides/biopesticides, the plant's inherent resistance mechanism against feeding by *Gonipterus sp. 2* has not been studied in detail. *Gonipterus sp. 2* possess a very strong host preference and select certain genotypes while disregarding others. We hypothesized that these susceptible/resistant genotypes have different chemical compounds which attract or repel *Gonipterus sp. 2*. Identifying these compounds would be a key step towards optimizing the use of plant resistance as an additional control measure against the beetle. While some studies have shown that certain compounds can be detected by *Gonipterus sp. 2*, there is still very little information regarding the compounds influencing its feeding preference. Therefore, we conducted a study to identify the constitutive compounds in *Eucalyptus* which affect the feeding preference of *Gonipterus sp. 2*. Initially, we assessed the relative level of susceptibility of 59 genotypes to feeding by *Gonipterus sp. 2* through a choice bioassay. This reveled 27 genotypes from three species as good candidates for chemical analysis. We selected these genotypes as they possess a very high intraspecific variation in the beetle's feeding behavior. We then conducted an in-depth chemical analysis on the leaves of these genotypes. The abundance of each compound was correlated to the level of beetle feeding, which revealed 10 highly correlated compounds. A standardized artificial diet was developed for an *in vitro* feeding preference assay using 8 of the identified compounds, to test their effect on the feeding behavior of *Gonipterus sp. 2*. This revealed three phagostimulants (1,8-cineole, oxalic acid, and sucrose) and two repellent compounds (shikimic acid and palmitic acid) for *Gonipterus sp.* 2. Understanding the effect these behaviorally active chemicals on host preference and implementing this knowledge in tree breeding programs, may provide an opportunity to reduce damage caused by *Gonipterus sp. 2*.