INSECT – PLANT INTERACTION IN THE COWPEA BEETLE, *Callosobruchus maculatus*

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A thesis submitted for the degree of
Doctor of Philosophy

The University of Sheffield
Department of Animal and Plant Sconces

December, 2018.

FEDERAL UNIVERSITY WUKARI
Character, Excellence and Service

tetfund TERTIARY EDUCATION TRUST FUND

ABSTRACT

The cowpea weevil, *Callosobruchus maculatus* Fab. is an economically important pest of stored grain – and especially of cowpeas - in sub-Saharan Africa. It causes serious damage to stored peas, resulting in reduced food security for subsistence farmers and financial loss and economic uncertainty to larger-scale farmers. Due to the economic and nutritional importance of the crop, particularly in Nigeria, farmers take several measures to protect their produce against insect infestations. The application of pesticides has been the generic control measure due to its effectiveness, affordability and ease of application. However, the negative effects of this control strategy - including increasingly apparent health and environmental consequences – is increasingly motivating stakeholders to advocate for an alternative management approach that has less social and environmental impact and is more sustainable.

In this thesis, I investigate *C. maculatus* biology in the context of its interaction with its primary food source, *Vigna unguiculata* L. Walp. (the cowpea). I start by examining the sensory anatomy of the antenna and female external genitalia comparing individuals from a lab-adapted strain (widely used as a model system in evolutionary biology) and a wild strain. I then examine the pest's ability to detect host odour from the peas (the stored product) and the living pods (an as yet understudied aspect of the pest's infestation tactics) based on understanding olfactory cues. I also analyse volatile samples from the host plant to identify candidate attractants. The beetle is known to be plastic in its choice of host, so I finish the study by examining the nature of that plasticity in lab and field strains as well as measuring the life-history consequences of those choices.

My results document antenna sensilla types and show that gross antenna morphology does not differ between sex or strain. However, I detected sexual dimorphism in the density of specific antennal sensilla: The antenna of males has more olfactory sensilla, whereas females have more of contact-chemosensory sensilla. In terms of their behaviour, insects were attracted to mature bean pods (compared to developing pods). Interestingly, the wild strain differed from the lab-adapted strain in several key life-history parameters: although females from both strains showed a preference for particular host types as an oviposition substrate, there was no apparent adaptive correlation between their choice of host and the life-history performance of the progeny.

These results represent the first steps in developing and designing new, more integrated management strategies that focus on key life-history, behavioural and environmental bottlenecks in the pest's biology that will enable farmers to leverage more sustainable and effective control methods.

ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisor, Prof. Mike Siva-Jothy for his unconditional support and guidance since the commencement of my programme in 2016. My thanks also go to Prof. Roger Butlin and Dr. Andrew Beckerman for their input and guidance during my upgrade assessment.

To my lab group members; Dr. Sophie Evison, Dr. William Hentley, Dr. Ben Webster, Rebeca Wilson, I must confess that my association with you all contributed tremendously to the completion of my thesis. Well done!!

My gratitude also goes to the Gas Chromatography Mass-Spectrometry unit in Animal and Plant Science Department, the Arthur Willis Environment Centre (AWEC) and the Electron Microscopy lab for providing the necessary assistance I needed while conducting my experiments.

The support I received from my family throughout my PhD journey is enormous: To my lovely wife, Ugochi, my beautiful daughters; Kamsi and Kaima, my parents, my siblings and in-laws…I say thank you!

To my employer, Federal University Wukari, thank you for nominating me for TETFUND sponsorship.

May GOD be praised!!!

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CHAPTER ONE: INTRODUCTION

During the domestication of plants man unknowingly created an artificial ecosystem for pests: we selected high yield, high nutrient crops after harvesting and kept them in a local abundance (a grain store), thereby providing a resource that was 'waiting' to be exploited by nature. It is estimated that today herbivorous insects damage about one fifth of the world's crop production annually.

Most insect herbivores are specialists and feed on a specific plant (monophagy), whilst some are slightly more cosmopolitan and will feed on closely related plants (oligophagous): a few, will feed on a wide range of plants (polyphagous). Insect success in herbivory is mainly determined by (a) the evolution of their mouthparts (Bernays *et al*., 1991) and (b) the early co-invasion of land by plants and insects (that defined this new environment for many millions of years before other taxa invaded). Insects show a great diversity of feeding types on plants, and each type is restricted to (and defines) a specific Order. Leaf-chewing insects are mostly larval lepidopterans and adult coleopterans; mining and boring are mainly the province of Diptera, Hymenoptera, Lepidoptera and Coleoptera; sap suckers and gall makers are found in the Hempitera, Hymenoptera and Diptera (Labandeira & Phillips, 1996). These feeding patterns are driven by / constrained by the insects' mouth parts and probably evolved with the radiation of plants (Bernays, 1998).

Insects generally have a need for protein (due to their small size, faster development rate and inability to regulate own temperature), and this demand is higher in herbivores because most plants are low in protein (Bernays, 1998; Southwood, 1972). Consequently, insect herbivores have to feed on a host with relatively high protein content, but, when host-plant protein is low, they tend to feed more or rely on

alternate food sources. Insects use a range of visual and odour cues to identify their preferred host-plant: critical cues are shapes, contrasting colours and metabolites, with secondary metabolites being the major cue-set for identifying a suitable host and/or avoiding an unsuitable one (Bernays & Lee, 2018). For polyphagous insects, sensitivity to these chemicals varies, whereas specific feeders can be easily deterred by an unfamiliar chemical cue.

Understanding the relationship between the evolution of an insect pest and the agricultural crops it infests is important when discussing insect-host plant interactions. However, studies asking questions on how and why insects switch between hosts and attain pest status are also relevant. Insect pests of stored products are key economic pests in the tropics and sub-Saharan regions, but there is paucity of information on the evolution of this group of insects as stored products pests (Tuda *et al*., 2006).

International trade and human migration are suggested to have triggered the distribution and spread of such pests. This has been exacerbated following the reliance on large grain storage systems in order to meet export demands (Hagstrum & Phillips, 2017; King *et al*., 2014). But the original habitat of this pest prior to crop domestication still remains unknown. *Tenebrio molitor* L., the mealworm beetle had been observed using rot-holes in deciduous trees as a primary host (Palm, 1959), although, Brendell, (1975) suggested the beetle also occurs in bird nests. Similarly, another species of *Tenebrio* was found in poultry manure and on rotten timber mixed with leaf litter (Jones, 1967). These observations suggest the species was originally restricted to high concentrations of nutrients in forests. Unlike *Tenebrio sp.,* the grain weevil, *Sitophilus granarius* has only been identified on domesticated cereal crops (Buckland, 1981), and has been found on maize, oats, wheat, millet, barley (Hoffmann, 1954). Zohary, (1969)

concluded that wild cereal grasses and acorns collected and stored by man and rodents may have influenced the distribution of such weevils.

For bruchids, the evolution of oligophagy is unrelated to the close distance in storage systems, especially for dry beans (Cotton, 1956). Bean chemistry, phenology and morphology are some of the factors that drive the interaction between bruchids and their host-plant. It is important to note that sensitivity to host seeds decreases with seed dryness (due to drop in the level of seed secondary metabolites), therefore, the host-switching behaviour observed in most bruchids could have been triggered by the close proximity of alternative hosts (Tuda *et al*., 2006). The beetle's ability to adapt to these alternative hosts and survive in very dry regions also contributed to their move towards crop pest status.

The cowpea bruchid, *Callosobruchus maculatus* is an insect pest that, as a larva, feeds on its primary host, cowpea beans. Its damage on stored cowpea has always been a cause for concern for cowpea growers. As a result, farmers spray pesticides on their produce as a protection strategy against the pest. However, due to the negative health and, more recently, legislative consequences associated with this strategy (pesticide application), stakeholders are advocating the development of a sustainable non-pesticide control methods.

1.1. Economic importance of cowpea

Cowpea, (*Vigna unguiculata* [L]. Walp.), is a leguminous crop that belongs to the family, Fabaceae, and is grown in tropical and sub-tropical regions due to its ability to tolerate drought. In Sub – Saharan Africa, cowpea grains are an essential source of cheap plant protein (Ofuya & Osadahun, 2005) as most of the populace cannot afford the cost of buying animal meat. It is called "poor man's food" by the Hausas in the

western and central African regions due to its nutritional benefits. The beans also contain iron, phosphorus and calcium (Table 1.1). Other benefits of cowpea include the use of its stem and leaves as feed for livestock, maintenance of soil fertility when intercropped or cultivated in rotation with cereal crops like maize, sorghum, millet, and provision of income to local farmers and developing nations (Oparaeke & Dike, 1996). As all parts of the cowpea plant (leaves, stem, green pods, dry seeds) serve as food, this chain of cowpea 'value' makes its farming an important part of community- and economic-support to local farmers.

Table 1.1. Chemical composition of cowpea (%)

	Seeds	Hay	Leaves
Carbohydrate	56-66		8
Protein	22-24		4.7
Water	11	18	85
Crude fibre	5.9-7.3	9.6	2
Ash	3.4-3.9	23.3	
Fat	1.3-1.5	11.3	0.3
Phosphorous	0.146	2.6	0.063
Calcium	0.104-0.076		0.256
Iron	0.005		0.005

Source: Quass, (1995).

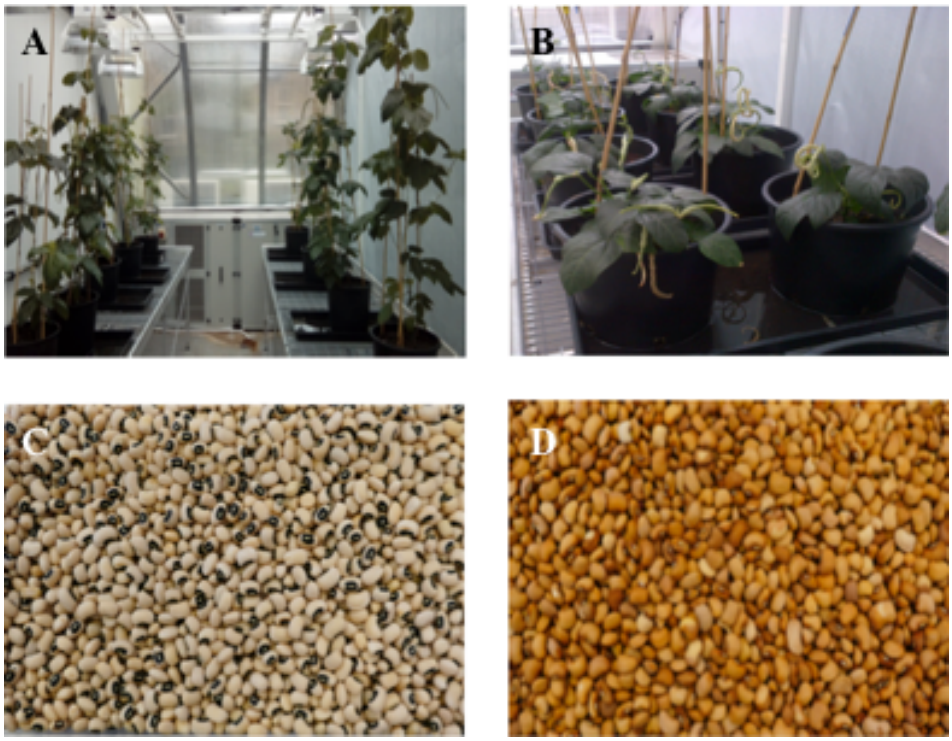

Figure 1. 1. Plants and dry seeds of cowpea cultivars. A and C are the plants and dry beans of California black-eyed cultivar; B and D represent the Borno-brown cultivar.

In Nigeria, this economically important crop is very important in all regions. It is not surprising that the country is the largest consumer, and highest producer, of cowpea in the world (FAOSTAT, 2016). Locally, cowpea production covers about 10 states, but the major producing states include Borno, Gombe, Sokoto, Zamfara, Kano and Yobe states (Figure 1.2). Demand for this cash crop has increased over the last decade, and this has created a big market. The export market for dry beans is also increasing (FAOSTAT, 2016; Figure 1.3), thus contributing to the nation's GDP. However, the decline in annual production rates is becoming problematic (Figure 1.4): The dense population (Coulibaly & Lowenberg-DeBoer, 2002) and post-harvest losses from insect infestations on stored cowpea (Ogunwolu & Odunlami, 1996) remain a big challenge.

The Indian government donated $1B to Nigerian farmers growing pulses including cowpea. Similarly, the United States Agency for International Development allocated $10M to expand cowpea production in four countries including Nigeria. These donations to boost cowpea production and meet export demands illustrate the economic and social benefits of the crop in the country.

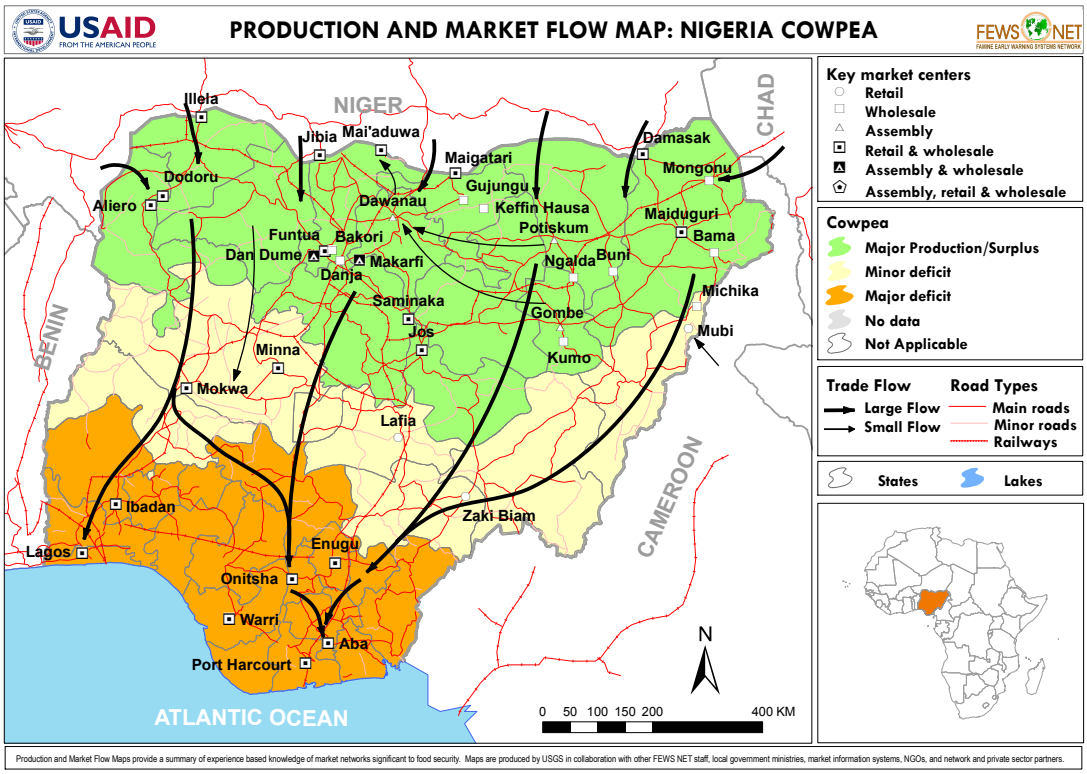

Figure 1. 2. Cowpea producing areas in Nigeria

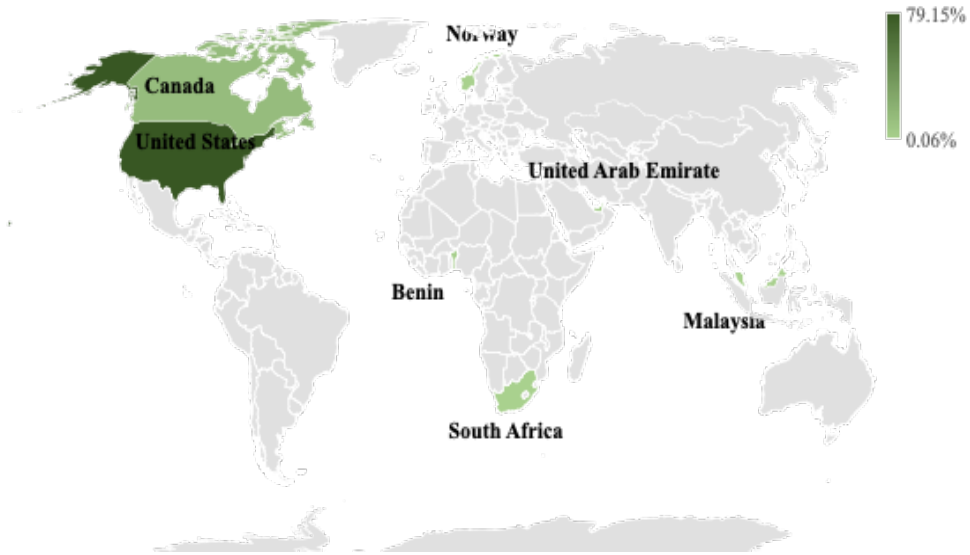

Figure 1. 3. Top cowpea export destinations from Nigeria in 2016.

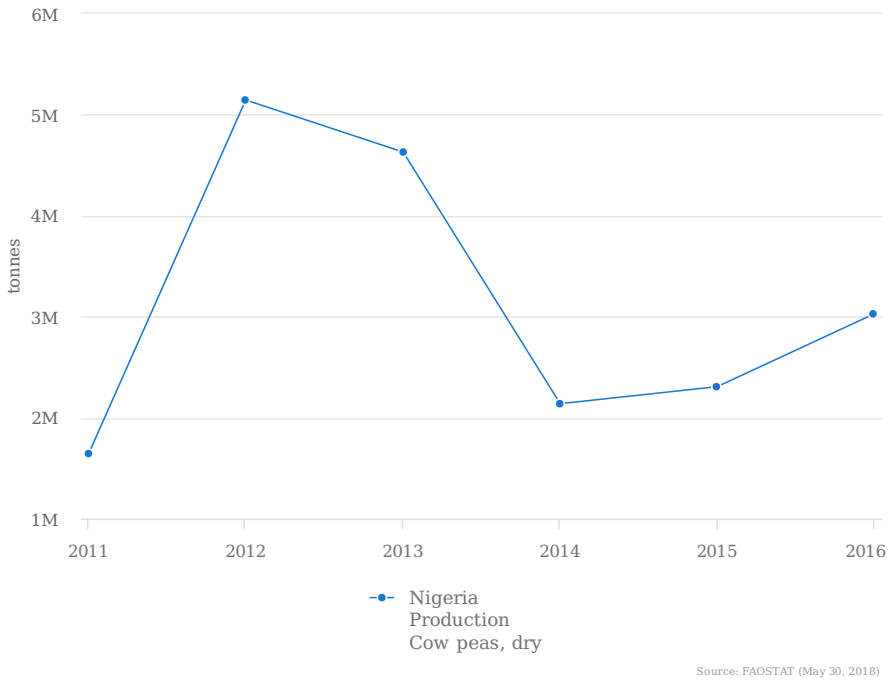

Figure 1. 4. Cowpea production in Nigeria in the past 5 years (FAOSTAT, 2016)

1.2. Cowpea distribution and ecological requirement

Cowpea has several native names in West Africa including “ewa”, “wake”, “beans” and “niebe”. In Brazil and United States, they are called “caupi” and “black-eyed” peas, respectively. In Africa, which is the primary centre of diversity of the crop, it is grown in Nigeria, Sudan, Mali, Kenya, Niger, Cameroon, Angola, Senegal, Tanzania, and Botswana. According to FAOSTAT, (2016), about 96.4 % of world cowpea production (6,991,174 T) comes from Africa, and the key cowpea producing nations in these regions include; Nigeria, Niger, Burkina Faso, Cameroon, Tanzania and Sudan (Figure 1.5). Reasonable quantities are also produced in southern and northern America, Asia and Europe.

Cowpea adapts well in most agro-ecological zones, and a temperature range of 23 ^{0}C and 32 ^{0}C are suitable for optimum growth. The crop also performs well with a rainfall amount between 500 and 1200 mm per year – it does not do well on water-logged soil. Soils rich in nitrogen affect pod formation, but the addition of phosphorus in the form of single-super phosphate, boosts flowering and podding in the plant. Growing the crop in a well-drained sandy loam to clay-loam soils that has pH between 6 and 7 is suitable for optimum yield (Dugje *et al.*, 2009).

A yellowish to brownish colouration of the pods is an indication of maturity, the pods are harvested at this stage. Harvesting of mature pods does not occur simultaneously due to the difference in anthesis (Ehlers & Hall, 1997); before storage, the pods are dried and threshed to remove chaff.

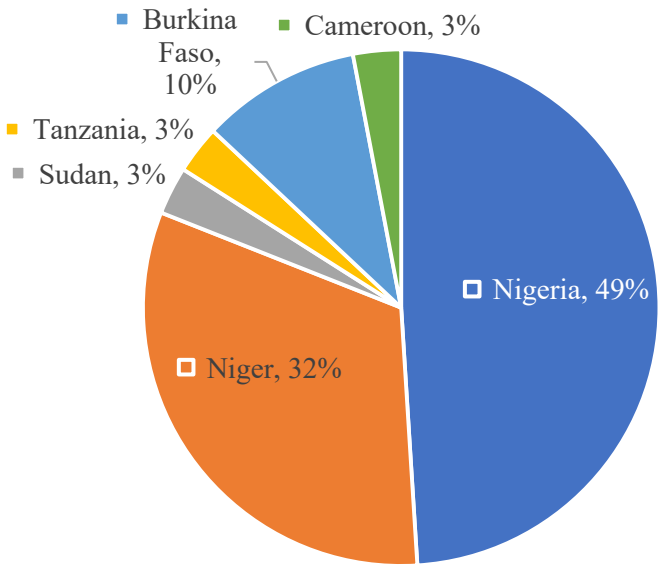

Figure 1. 5. Top six cowpea producing nations in Africa (FAOSTAT, 2016)

1.3. Economic importance of cowpea bean weevil.

Callosobruchus maculatus (Fabricius) (Coleoptera: Chrysomelidae: Bruchinae), the cowpea weevil, is the primary insect pest of stored cowpea (*V. unguiculata*) in the tropics (Abate & Ampofo, 1996; Abate *et al*. , 2000) and other locations where cowpea is grown. Due to the tremendous benefits associated with cowpea cultivation (Oparaeke *et al.*, 2004) there are serious consequences of this pest's attack on the crop. As there is high demand for cowpea all year round, farmers are always faced with the task of storing and preserving their harvest for future consumption, sales and export.

This storage-need makes infestations and damage on dry beans by *C. maculatus* the most serious problem for cowpea storage in Nigeria (Adam & Baidoo, 2008; Baidoo *et al.*, 2010; Swella & Mushobozy, 2007). Between 10-15 % of infestations in stored cowpea comes from residual eggs derived from the field (Olubayo & Port, 1997) where females deposit eggs on the host-plant pods and/or on dehisced pods . The degree of damage depends on the number of eggs laid on a bean and the infestation period. According to Rahman & Talukder, (2006), the pest can cause total destruction of stored grains within 3- 4 months. It has also been reported that about 30,000 tonnes of cowpea grain (4% of the annual production valued at over 30 million US dollars) is lost annually to damage by the pest (Caswell, 1980).

It is well established that *C. maculatus* infestations on stored cowpea result in huge financial losses to farmers and exporting nations (Caswell, 1980; Rahman & Talukder, 2006), thus the need for an effective and sustainable pest control methodology. Several measures have been taken to ameliorate the damaging effect of this stored product beetle, including the application of pesticide- and non-pesticide-dependent techniques which I discuss below.

1.4. Pesticides

Pesticide use has increased significantly and dominates pest management practices worldwide (Foster & Harris, 1997). This is principally due to changes in farming practice related to increased agricultural activity and the challenges of pest resistance (Elhag, 2000; Uygun *et al.*, 2005). The increase in use of chemicals can also be attributed to the relatively low cost, availability, ease of application and good efficacy against a wide range of pests (Calvert *et al.,* 2001; Jackai, 1993; Uygun *et al.*, 2005). Dimethoate, Cypermethrin, Carbofuran, Karate 2.5 EC., Dichlorvos (DDVP) combined with Primophos methyl or Phosphine, are frequently applied pesticides (Dugje *et al.*, 2009).

However, farmers often mishandle and misapply these chemicals: Omongo *et al.*, (1998), reported that farmers sprayed their farms about eight to ten times during each growing season: the recommended rate was two to three times. This practice exposes farmers to chemicals which pose a health challenge due to a build-up of residues in the body over time. In most cases, the accumulated residues increase the risks of developing cancer and respiratory disorders (Talukder & Howse, 2000).

In Nigeria, for example, rural agricultural areas usually have the highest level of pesticide residue exposure because more than 70 % of all pesticides are handled and applied by local farmers (Ofuya, 2003). Northeast Nigeria is a major agricultural area where pesticides are often applied on dried beans (before storage) as a control measure against bruchids. Frequent cases of poisoning have been reported from this region due to excessive use of these chemicals, probably because most farmers in this part of the country lack basic knowledge of the dangers associated with mishandling and misapplying these substances.

Awofadeji (2008), reported the poisoning, and deaths, of people in Borno State, Nigeria, after consuming beans sourced from Taraba State (Northeast, Nigeria): these were the result of the high levels of pesticide residue resulting from misapplication and multiple spraying. Laboratory analysis of the samples revealed extremely high levels of toxic organophosphates and carbamates pesticides (NAFDAC, 2004). In 2016, the European Commission placed a ban on the exportation of dried beans from Nigeria until 30th June 2019 (Juncker, 2016). The rejected beans were found to contain excessive amounts of pesticides. This decision will cause loss of revenue and considerable hardship for Nigerian farmers. Consequently, there is an urgent need for a safer, more effective, and sustainable approach to control this insect pest.

Different alternative pest management approaches including cultural and biological measures have been implemented as a consequence of the health, environmental and economic outcomes of irresponsible pesticide use. Most of these measures have shown positive outcomes although there are still challenges that needs to be addressed.

1.5. Alternatives to pesticide application

1.5.1. Plant extracts with insecticidal properties

Several plants extract show insecticidal effects against a wide range of insects pest. For example, the use of nicotine, an extract from tobacco against piercing and sucking insects is in use in some countries (eg China) where they introduce tobacco stems in rice farms as a protective strategy against maize stem borers (Thacker, 2002). Retenone is currently used to control aphids, thrips, beetles and potato beetle, (Weinzierl, 2000). Retenone is an ancient insecticide whose active component,

nicouline, is highly toxic to ectotherms but has minimal toxicity against mammals (Betarbet *et al*., 2000).

Pyrethrum is a powder extracted from the dried flowers of *Chrysanthemum spp.* .Its major active compound, pyrethrin I, is a toxic ester and is effective in controlling mites, stored product pests, and greenhouse pests. It is commercially available as pyrethroid, a synthetic form of pyrethrum which is more stable. Neem, *Azadirachta indica*, has been widely studied and used as an insecticide on variety of insects including leaf-miners, aphids, thrips, caterpillars, (Copping, 2004). Neem has been reported to act as an antifeedant, a repellent and as an insect growth inhibitor due to the presence of salannin and azadirachtins, the key active ingredients in the plant. According to Koul, (2004) volatile (di-n-propyldisulphide) from the seeds of *A. indica* showed toxicant, fumigant and antifeedant properties against the rice and floor weevil, *Sitophilus oryzae* and *Tribolium casteneum.*

Other plant products have also been used on stored grain pests and field pests. For example, seeds and root extracts of *Jatropha caucas* against *C. maculatus* (Ahuchaogu, *et al*., 2014; Ahuchaogu & Ojiako, 2015), seed extracts of *Azadirachta indica* on *C. maculatus* (Lale, & Mustapha, 2000; Lale & Abdulrahman, 1999), *Moringa oleifera* seeds and root extracts in controlling *C. maculatus* (Ojiako *et al,* 2013) and other medicinal plants (Golob *et al.*, 1982; Delobel & Malonga, 1987; Lale, 1992) have been tested. Whilst this approach has lots of potential there are issues because (1) the costs involved in the formulations, (2) the non-persistence of the plant compounds, (3) the availability of plant materials and (4) the regulatory approval required by developed nations (Regnault-Roger *et al.*, 2005) presents serious constraints affecting full adoption.

1.5.2. Plant breeding, genetic engineering and biotechnology

Many herbivore-inducing chemicals have been manipulated to be expressed in plants. For example, genetically engineered maize plant have been produced that expresses (E) - β - farnese, (E) - α - bergamotene and other herbivore-induced sesquiterpene hydrocarbons: these compounds lure the parasitic wasp, *C. marginiventris* (Schnee *et al*., 2006). The release of sterile male insects has also been utilized as an effective pest management strategy. The sterile insect release method (SIRM) has been successfully used to control *Anopheles albimanus* (Lofgren *et al*., 1974), *Culex fatigans* (Patterson *et al.,* 1970) and *Anthonomus grandis* (Carter, 1974). However, there are difficulties in replicating these processes in other regions, and cases of breakdown of approach or resurgence of the pest (as in the screwworm project in the United States) have been reported. It is likely that wild females will avoid mating with the sterile males: there is a need for detailed ecological study of the mating system dynamics and reproductive abundance and distribution of the target pest.

The use of RNAi in managing agricultural pests has also gained recent attention. Its development for and success in, crop protection has been well-reported (Gordon & Waterhouse, 2007; Huvenne & Smagghe, 2010; Zotti & Smagghe, 2015). It is a system that involves the administration of a double-stranded RNA (dsRNA) targeted at a specific gene, which is transported into a cell or body tissues, and is often used in silencing a gene of interest (Joga *et al.*, 2016). The efficacy of RNAi in controlling agricultural pests varies across insects orders - for example, higher successes of gene knockdown have been recorded in Coleopterans compared to other insect orders (Baum *et al*., 2007; Zhu *et al.*, 2011). The mode of delivery of dsRNA into an insect body has been a major bottleneck; use of nanoparticles and liposomes as delivery mechanisms have increased efficacy and lowered the degradation of dsRNA.

Other delivery systems such as spraying of RNAi-based products is gaining attention following the difficulties associated with applying previous techniques in the field (Walshe *et al.*, 2009).

The use of bacteria (Huvenne & Smagghe, 2010), viruses (Khan *et al.*, 2013; Nandety *et al.*, 2015) and transgenic plants that express dsRNA is being studied, but food and environmental safety regulations and concerns are still hindering the wider exploitation of this approach. Other challenges confronting this technique include a lack of collective adoption by farmers, inadequate qualified personnel to communicate the techniques, a lack of interest by stakeholders in developing nations to adopt the approach due to the fear of the unknown.

1.5.3. Entomopathogenic fungi

Entomopathogenic fungi are amongst the first pathogens to be integrated in the management of insect pests. Some are restricted to specific hosts, and target specific insect species. Entomopathogenic fungi can be applied on a host by (a) dipping a plant part into a spore suspension (b) foliar spraying of the fungal spores (c) introducing the spores in soils and (d) indirect transmission by a vector. A fungal spore or conidium causes infection on an insect by attaching and germinating on the insect cuticle before invading the haemolymph and the insect body (Samson *et al.,* 1988). Those mainly used in pest control are in the class of Enthomophthorales or in the Hyphocycetes. The Hyphocycetes are opportunistic pathogens, and cause host death by the secretion of toxic substances: they affect several insect orders (Roberts, 1981; Samson *et al.,* 1988). Enthomophthorales, on the other hand, causes host death *via* tissue colonisation, and do not release toxins (Humber, 1984).

Enthomophaga maimaiga and *Zoophthora radicans* are two enthomopathogenic fungi that have been successfully used in controlling a wide range

of insects (Shah & Pell, 2003). For example, E. *maimaiga* has been used to suppress the gypsy moth, *Lymantria dispar* (a pest that feeds on oaks and aspen leaves) in the United States (Elkinton *et al*.,1991; Hajek *et al*., 1996). However, in making a decision to use this approach, it is important to treat each individual pest differently and to consider its public safety and effect on non-targeted species as well as its efficacy (Goettel & Hajek, 2000; (Pell *et al*., 2001). The comparative cost implication relative to pesticide application, effects of biotic and abiotic factors on its efficacy are some the problems farmers face with its adoption.

1.5.4. Solarisation

Exposure of invertebrates to extreme temperature conditions causes sudden death, sterility (Okasha *et al.*, 1970), and behavioural disorders (Klok & Chown, 2001; Slabber & Chown, 2005). The use of solar energy (solarization) in controlling pests is a simple and traditional approach that has been in existence amongst rural farmers in tropical and sub-tropical nations for a long time. Its application in controlling insect pests of stored grains (especially, the bruchids) has been reported by many entomologists. For example, the solar heating of cowpea stores (Murdock & Shade, 1991), exposing cowpea infested seeds to solar heat (Maina & Lale, 2004), the use of plastic solar heaters has controlled *C. maculatus* infestations and sun-drying cowpea reduces infestations load and mould infection (Arogba *et al.,* 1998). Similarly, Nakayama *et al.*, (1983), used black plastic solar heaters in protecting dried millet, peaches and oatmeal from the hide beetle, moth and merchant grain beetle, respectively. The use of solar cabinets in controlling the larger grain borer in maize cobs (Mc Farlane, 1989), solar heated polyethylene in reducing the load of *Verticillium dahlia* in the soil (Ashworth & Gaona, 1982) and solar heated polyethylene sheets in reducing the population of fire-plant parasitic nematodes (Barbercheck and von

Broembsen, 1986), have all been well-documented. However, solarisation is not cost-effective for large scale production. Also, the reproductive biology of *C. maculatus* has made this approach less useful for farmers as the survival of very few eggs can still cause huge damage to stored grains.

1.5.5. Use of egg parasitoids

Another management strategy is the introduction of biological control agents. For example, *Uscana lariophaga* Steffan, and *Dinarmus basalis* Rhondani, parasitizes the developing egg and larvae of *Callosobruchus* species (Lienard *et al.*, 1993; Kapila & Agarwal, 1995; Kestenholz *et al*., 2007). The egg parasite, *U. lariophaga* prevents the egg from hatching (Kapila & Agarwal, 1995). According to Van Huis *et al*., (1998), *U. lariophaga* reduced the emergence of adult *C. maculatus.* Similarly, a study conducted in Burkina-Faso showed that the population of *C. maculatus* was reduced by 85% after inoculation of *D. basalis* when compared to the control (Sanon *et al*., 1998). The major issue with this strategy is the presence of the parasitoids' offspring on the seeds (Soundararajan *et al.,* 2012): this causes damage on the bean surface after the wasps' emergence. There are also logistic problems in sustaining the availability of parasitoids for use.

1.5.6. Intercropping and related methods

Intercropping is a cultural practice that involves growing two or more crops in a field at the same time. It includes the combination of crops, and is still being used in developing nations as a traditional farming system. Intercropping reduces pest pressure on the primary crop, and has been used to manipulate the abundance and behaviour of herbivores. It is a strategy that uses systems such as the expression of repellant chemicals, visual disruption of herbivores, physical barriers and masking of host-odour cues as protective tools against invading herbivores (Finch & Collier, 2000;

Hooks & Johnson, 2003). Consequently, habitat management in agricultural systems has great potential in pest control.

1.5.6.1. Push-Pull

The push-pull technique is a behavioural manipulation system that focuses on reducing pest abundance on a host. It is a pest control measure that uses retarding substances to push pests away from a host who are then attracted to a trap crop (Ahmed *et al*., 2008). It is a repellent and attractant system that involves intercropping for the push and trap-cropping for the pull. Push-pull has been successfully used in controlling a variety of maize pests, and most recently, the African witchweed, *Striga sp.* (Khan *et al*., 2008). A typical example is intercropping maize with a push plant, molasses grass, and trapping the pest (maize stem borer) with Napier grass (Khan *et al*., 1997). Similarly, intercropping maize with cowpea or silver-leaf, *Desmodium uncinatum* shows good results in controlling *Striga hermonthica* (Khan *et al.*, 2002). This approach of using a repellent and an attractant has also been applied in managing livestock pests and disease vectors (Gikonyo *et al*., 2003; Wanzala *et al*., 2004).

With advances in behavioural studies, the use of plant-based systems in push-pull is gaining attention. This approach involves using plants that emit these inducing substances, and provides more prospects and efficacy. For example, (*E*) ocimene and (*E*)-4,8-dimethyl-1,3,7-nonatriene have been identified to be among the active compounds that could be responsible for Molasses grass repellency against the maize stem borer (Turlings *et al*., 1990). There is a need for farmers to have an understanding of the biological and logistic basis of this approach in order for it to be successful. Its cost, availability of seeds for the push and pull crops, and concerns of cases of pests and diseases attacks on these experimental crops are current issues that mean this control measure has not gained as much traction as it should have.

1.5.6.2. Lure and kill

Lure and kill has been used for decades, and involves luring targeted insects to a location (attractant source) where they are easily killed (with an insecticide) or sterilised (using a sterilant). The efficacy of this technique depends on the target insect establishing a reasonable contact with the kill and the ability of the kill to effect mortality. Lure and kill has been used against the cotton boll weevils (Smith, 1998), housefly (Geden *et al.,* 2009) and fruit flies (El-Sayed *et al.*, 2009). Other studies have shown that the approach can be integrated into long-term management programmes that target pests of economic importance. For example, semiochemicals from the flower of *Bruchus rufimanus*'s host plant elicited a behavioural and electrophysiological attraction on the pest (Babu *et al*., 2003). Similarly, virgin and mated females of *Pteromalus cerealella* were attracted to odour stimuli from its host (Onagbola & Fadamiro 2011). Such compounds can be formulated into a lure trap which can be applied in managing these insects. A major factor affecting the acceptance of this approach is the inclusion of sterilant and/or insecticides in formulating and preparing the trap. The cost of formulating pheromones and its dependency on target-pest population are also reasons for the low take-up of this approach.

1.5.7. Pheromone manipulation

Pheromone utilization in pest management has been a major breakthrough in finding alternatives to pesticide application. A sex pheromone released by an insect attracts and excites individuals of the opposite sex, and can be used to trap sexually active pests (Jacobson, 1972). Conspecifics also use aggregation pheromones to initiate feeding and mating behaviour (Burkholder, 1990). Pheromones have been applied in pest control strategies such as mass trapping and mating disruption.

1.5.7.1. Mass trapping

Mass trapping is a technique used mainly in managing stored product pests (Buchelos & Levinson, 1993), although it has not seen large-scale adoption. The approach uses traps baited with pheromones of female adults to attract and trap males so that the females have no mates. It requires designing many traps, and a good understanding of the male's population and reproductive biology (Roelofs *et al.*, 1970). It has been successfully used in controlling *Leucinodes orbonalis* (Cork *et al*., 2003), bark beetles (Schlyter *et al.,* 2003) and banana weevil (Reddy *et al.,* 2009).

1.5.7.2. Mating disruption

Mating disruption is a pest management strategy that involves disorientation and disruption of communication between sexes and conspecifics, and has been used on many insects (Fraser & Trimble, 2001; Jones *et al.,* 2014; Stelinski & Gut, 2009). It is a complex technique that requires a comprehensive understanding of the disruption mechanism to be used. For it to be successful, a good number of males must fail to locate the females, and the females' response to the strategy has to be considered when planning the use of this technique. Mating disruption has been successfully used on *Pyralid moths* (Prevett *et al.,* 1989). There are a lot of challenges associated with pheromone-dependent approaches; The cost of making/purchasing more traps to ensure efficacy and the cost of formulating pheromones are major problems with these methods.

1.6. Plants defensive mechanisms against pests

Other than the methods designed by man to manage pest attacks on food crops, plants have evolved natural defensive tools against these attackers. A plant's defensive mechanisms can include (a) the excretion of toxic substances which cause direct mortality to the pest, (b) reducing the reproductive fitness of the pest, (c) creating

physical barriers against the pest, (d) expressing kairomones with negative effects and (e) increasing the likelihood of exposing pests to their natural enemies by delaying their development time. Most of these tools have been incorporated as a component of integrated pest management strategy, and are discussed below.

1.6.1. Constitutive and inducible defences

In order to protect itself from insect herbivours, plants have developed defensive mechanisms which include the release of compounds that attracts the pests predators and parasites (Birkett *et al.*, 2000), the expression of secondary metabolites (Baldwin, 2001; Kliebenstein *et al*., 2001), and the use of plant trichomes (Fordyce & Agrawal, 2001). In response, insects have evolved ways to avoid getting killed by these defensive tools. Such responses include the sequestration of plants poisons (Nishida, 2002), avoidance of unsuitable hosts (Zangerl, 1990) and detoxification of toxic substances (Scott & Wen, 2001).

Chemical and physical barriers are examples of plant defensive mechanisms against herbivores. A chemical defence can be constitutive or inducible, the latter (constitutive defence), uses direct toxicity to retard herbivores, whereas inducible chemicals are released when plants are attacked by a pest. The expression of chemical defences varies across plants. For example, flowers and fruits express a greater amount of chemical defences compared to other vegetative parts of a plant (Darrow & Bowers, 1999; Kozukue *et al.*, 2004; Zangerl & Rutledge, 1996). Also, young plant leaves are prone to higher herbivore infestation, thus possess a higher concentration of chemical defences (Ohnmeiss & Baldwin, 2000; Raupp & Denno, 1984). The expression of constitutive and inducive defences can be affected by several factors; according to Cipollini *et al*., (2005), invasive populations of *Alliana petiolata* expressed higher chemical defences when compared to native species. Moreira *et al*., (2014), in their

study, found that the composition and expression of defensive traits and strategies in pine trees were strongly associated with geographical and climatic conditions. Silicon, an essential plant component is one of the constitutive defensive compounds used by plants, although it has been recently identified in induced plant defences. It has a role as an inducer of aphid resistance in wheat (Gomes *et al*., 2005) and potato (Gomes *et al.*, 2008).

1.6.2. Aromatic/volatile chemical repellents

Most plants express repellency traits against a wide range of insect pests, and the identification and integration of such plants into intercropping systems has shown great prospects in providing defence against insect pests. For example, planting of *Melinitis minutifloa* (an odorous plant) in a maize field attracted the natural enemies of caterpillar pests (Khan *et al.*, 1997). Similarly, intercropping broad bean (*Vicia faba*) with *Ocimum basilicum* (an aromatic plant) in a field suppressed the infestations level of *Aphis fabae* adults (Basedow *et al.*, 2006). The ovicidal effects of *Ocimun sanctum* and *O. basilicum* on *Callosobruchus chinensis* have also been reported (Kiradoo & Srivastava, 2010). Aromatic plants can also increase the fitness of natural enemies; a study by Johanowicz and Mitchell, (2000) showed that cultivating sweet alyssum (*Lobularia maritima*) around a cabbage field increased the longevity of parasitic wasp, thus suppressing the population of cabbage field pest, and providing indirect defence to the crop.

1.6.3. Use of plant trichomes

Plant trichomes are hair-like structures on leaves, stems and reproductive structures in many plants. They are part of plants defensive tools against herbivores, and have been exploited in breeding agricultural crops for insect resistance (Levin, 1973). Trichomes

provide protection or resistance against pests by acting as a physical barrier (restraining contact with pest), expressing substances toxic to the pests, and by releasing sticky or gummy chemicals which demobilises the pest (Duffey, 1986). For example, some cotton lines rich in trichomes were more resistant to green leafhopper, *Empoasia libya* (Evans, 1965), spotted bollworm, *Earias fabia* (Mehta, 1971), cotton aphids, *Aphids gossypii* (Kamel & Elkassaby, 1965) and the boll weevil, *Anthonomus grandis* (Hunter *et al.,* 1965). However, trichome length and density, plant age, day time and weather conditions may also affect the effectiveness of these defence traits (Rembold *et al*., 1990).

1.6.4. Attraction of natural enemies

Secondary metabolites play important roles in protecting plants against herbivore attack, and can cause an antifeedant, toxicological and repellency effects on herbivores. They also help in inter- and intra-plant communication (Pare & Tumlinson, 1999) by protecting healthy plants *via* inducing defensive response. However, these metabolites are also utilized by insects to locate preferred hosts and/or in avoiding unsuitable ones (Pare and Tumlinson, 1999).

Most plant volatiles released as a result of herbivore damage are secondary metabolites, and can be expressed from any part of a plant. Each volatile compound emitted is targeted at a particular herbivore, the natural enemy, neighbouring plants, and is often associated with the plants' development stage (Hare, 2010; Rasmann *et al*., 2005). The release of these compounds triggers behavioural inducement among the invertebrates in an ecosystem (Karban *et al*., 2000; Rasmann *et al*., 2005), and they include terpenoids, amino-acids, fatty acids, benzenoids/pheny propanoids, etc. for example, (E) - β - caryophyllene emitted by maize roots due to damage by the corn rootworm, *Diabrotica virgifera* attracts *Heterorhabditis megidis,* a nematode which

feeds on the larvae of the rootworm (Rasman *et al*., 2005). Methyl silicate (Mesa), a herbivore-induced plant volatile is effective in inducing behavioural responses in most insects, and have been identified on headspace samples of soybean (Zhu & Park, 2005), lima bean (Arimura *et al*., 2002) and tomato (Ament *et al*., 2004).

"The enemy of my enemy is my friend" is an old proverb that can be used to good effect in manipulating host-pest-predator relationships in an ecology or evolution-based control system. It's a natural control strategy where a host infestation by a herbivore attracts a second herbivore whose presence aggravates the arrival of a predator that feeds on both herbivores. For example, the pine beetle is the major killing bark beetle (Safranyik *et al*., 2004), its presence on a host tree releases an induced volatile which lures another competing beetle, the pine engraver, *Ips pini* (Rankin & Borden, 1991) which also feeds on the tree. The presence of the pine engraver reduces brood production and replacement rate by the mountain pine beetle. Interestingly, the aggregation pheromone released by both beetles triggers the arrival of *Enoclerus sphegeus,* a predator which attacks both beetles. The knowledge of this interaction can be employed in manipulating the abundance of the pine engraver as a strategy in reducing pine beetle reproduction.

1.7. Seed defensive mechanisms

Plant seeds are rich in protein, carbohydrates and lipids, and have been utilised by herbivorous insects to meet food energy and nutritional needs. Bruchids have a life-history of close association with seeds of leguminous plants basically due to its rich protein content, a key dietary requirement for their development. Seeds, on the other hand, have developed defensive strategies against insect attacks most of which are constitutive, and include; tannins, cyanogenic glucosides, non-protein amino acids,

proteins (protease and amylase inhibitors). Globulin, a storage protein present in most legume seeds (especially cowpea) is suggested to be the key defensive compound in cowpea seeds. According to Macedo *et al.*, (1993), the presence of globulin fractions in cowpea variety, TVu 2027 seeds triggered resistance against *C. maculatus* infestations. Although, this compound is vulnerable to digestion by gut enzymes of other storage weevils (de Sales *et al*., 1992). Similarly, vicilin, a salt soluble globulin affected larval development of *C. maculatus* on adzuki bean, soybean and common bean (Yunes *et al*., 1998). The insecticidal effects of cys proteinase inhibitor in soybean (Botella *et al*., 1996) and amylase inhibitor in pinto bean (Pueyo *et al.,* 1993) have also been reported. Non-protein amino acids also serve as a constitutive defence mechanism in seeds (Rosenthal, 1991). Some examples of those used in legume seed defences include; $_{L}$-Canavanine (Rosenthal, 1991), GABA, γ-Aminobutyric acid (Bown *et al*., 2006; Ramputh & Bown, 1996). Little research has been done on this area, thus the need for further studies in important. Seed coat and pod pericarp also provide mechanical barriers against most bruchids, and have been reported to reduce larval survival during seed penetration. Seed morphology (seed size, seed shape and seed colour) may also serve as defensive tools against herbivore attacks (Yang *et al.*, 2006). It is, however, important to establish if seed morphology correlates with the amount of constitutive defensive compounds present in host seeds.

In summary, insects have developed successful behavioural and biological mechanisms to suppress most plants' defensive tactics, thus explaining why crops are still attacked by pests. As a result, man has to intervene to ensure adequate food is available for consumption. But successful intervention measures that are easy to adopt by farmers (excluding the application of pesticides - a primary crop protection method) still remains a problem. In some developed nations, the commercialisation of plant-

based and biocontrol insecticidal products has already commenced. However, adopting these measures by farmers in local areas in developing nations remains a big challenge. The lack of interest is linked to the fear of the unknow relative to chemical method. Consequently, pesticide application still stands as a major pest control measure used by farmers in these regions due its availability, efficacy and affordability.

Figure 1. 6. Dry cowpea seeds infested by *C. maculatus.*

1.8. *C. maculatus* as an experimental tool in evolutionary studies

C. maculatus has been an important tool in studies that address evolutionary behaviours in insects. This is due to the ease of culturing them in a controlled environment (laboratory) and the fact they do not feed as adults. Over the years, studies on lab-adapted stock of this insect has provided important advances in practical understanding and tests of life-history issues and fitness consequences, but the relevance of such behaviours in the management of the pest has not gained much attention.

It is worth noting that if populations from the wild provide similar life-history and other biological data to the lab-adapted stock much of the extant evolutionary and ecological inference and theory can be directly applied in pest management. Such practical understanding of the pest's behaviour will be relevant in improving food production systems.

1.9. Life cycle of C. maculatus

Eggs laid by female *C. maculatus,* which can range from a single to multiple eggs, stick firmly on the bean's surface. The eggs are oval in shape, smooth and clear when laid (Beck & Blumer, 2014). Larval development has four stages; within 6-7 days after eggs are laid, the first instar larva hatches and tunnels to the seed endosperm. The latter larval stages (2^{nd}, 3^{rd} & 4^{th}) then feed on the endosperm before pupation occurs. The pupa emerges by creating a hole on the bean surface (Figure 1.5). After emergence, mated adults can live up to two weeks, whereas the virgins may stay alive for 30 days. During this period, they do not require food nor water; they only live to reproduce (Devi & Devi, 2014).

1.10. Development time in *C. maculatus*

Different development times from laying of eggs to the emergence of adult *C. maculatus* have been reported in many studies. According to Howe & Currie, (1964), relative humidity (RH) and temperature (T) are key factors that determine the beetle's development time when cultured on a susceptible host. For example, with RH and T set at 40-60% and 25 ^{0}C, respectively, *C. maculatus* adults emerged within 5 weeks. But, when adjusted to 30 % and 30 ^{0}C, the beetles started emerging after 3-4 weeks (Beck & Blumer, 2014).

The type of rearing host also determines how fast the beetles can develop. Paukku & Kotiaho, (2008) reported that *C. maculatus* reared on mung beans had a faster development rate compared to those reared on black-eyed beans. Similarly, the emergence of *C. maculatus* was delayed for an extra 3 weeks compared to 3-4 weeks recorded on mung beans (Beck & Blumer, 2014).

1.11. Sex identification

Morphological features are used in identifying gender: The colour on the plate covering the abdomen (elytra pattern) is a key tool in distinguishing the sexes (Utida, 1954): On females, there is a dark strip on both side of the dorsal abdomen which is not present on the males in most populations. Furthermore, in most strains, females are black coloured, and males appear brownish.

Figure 1. 7. Life cycle of *C. maculatus* (Devi, and Devi, 2014)

1.12. Life - history theory and its relevance in pest management

1.12.1. Life-history theory

Life-history theory explains how differences in key traits lead to variation in individual performance in a population. It focuses on natural selection, fitness, adaptation and constraints (Stearns, 1992) and broadly explains the features of an organism's life cycle in the context of variation in traits such as; age and size at maturity, growth, offspring number and size and reproductive investment, which interact to explain individual fitness (growth rate, senescence and mortality, frequency of reproduction, etc).

Life history theory is captured as three separate but related theories.

- Theory regarding demography and population dynamics

This theory explains that increasing rate of population growth is directly determined by age-specific reproduction, and survival of those categories, which makes study of Life-history an integral part of population ecology (Begon, 1996). This theory is important because it is used in pest forecasting and monitoring.

- Fitness and Optimality

The theory states that the relative fitness of a particular trait will be influenced by the life-history of individuals with such trait. It centres on understanding the evolution of fitness variances amongst conspecifics.

- Theory of Life Cycle and Life-History Adaptations

This theory centres on what determines the life-history and shape of life cycles.

1.12.2. Life-history traits

Life history traits focus mainly on reproduction and survival and explain features like longevity, offspring number, age and mass of offspring especially at first generation (Stearns, 1992). In all, these traits focus on the life cycle of an organism which defers from their physiological, morphological and behavioural traits (Nylin, 2001). Life history traits explain the developmental stages associated with an organism's lifecycle, and determine the changes in population and fitness.

1.12.3. Application of life-history theory in pest management

The application of the theory of demography and population dynamics in pest forecasting (an integral part of pest management) demonstrates the relevance of life-history studies in designing pest control strategies. Integrated pest management (IPM) involves the combination of different control measures, and monitoring of the pest is one principle of IPM. It is a strategy that is key in predicting future pests outbreaks. By understanding the environmental factors that drive the changes in insect number, the chances of making an erratic forecast is limited. For example, the fluctuations in temperature and abundance of natural enemies are key factors in explaining the fluctuations in the population of the giant phasmatids *Didymuria violescens* (Readshaw, 1965) and psyllid *Cardiaspina albitextura* (Clark & Dallwitz, 1975).

Another important determinant of fluctuations in population size of an insect is the life-cycle of the insect (Tammaru & Haukioja, 1996). Achieving an effective and accurate forecasting system requires having a practical understanding of the pest's lifecycle; the number of generations per year, and how it is affected by environmental conditions. Effect of food quality on life-cycle and female preference for an alternative host are also factors to be considered while making forecasting decisions (Nylin,

2001). The presence of natural enemies and how they affect the pests' life-cycle and population levels are also important. Life-history theory has also enriched our understanding of the behaviour of natural enemies and how such knowledge can be applied in biological control method (Luck, 1990). The theory has provided practical knowledge in studies on insect-host interactions (Bernays & Chapman, 1994).

Therefore, investigating the behavioural mechanism that drives insect attraction to semiochemicals and integrating it with their life-history performances will be an effective way to approach this challenge. As an evolving scientist with basic applied research skills, harvesting my residual knowledge in applied studies and incorporating it with both behavioural and life-history skills will not only lead to the development of a new approach to research designs, but also diversify my research experience and expertise.

With this in mind, I first examined the antennae properties of a lab-adapted strain of this beetle (which has been serving as an experimental tool in insects behavioural studies) including hairs associated with the sense of smell and touch behaviour and compared it with a wild-type (farmers' strain). Next, I looked at the ability of females of the beetle (wild strain) to use its sense of smell in locating a preferred bean type both in storage and field situations and tentatively identified candidate compounds that could be driving such attraction. Then, I studied how females of both strains select a bean to lay eggs on and examined how such a choice affect the well-being of their progenies.

I believe the findings will contribute to developing a sustainable and effective management measure for the pest.

1.13. Thesis outline

The work in this thesis examines how *C. maculatus* interacts with its host, *V. unguiculata*. It seeks to understand the cues that drive this relationship and the evolution of host selection by females.

Chapter 2. Explores the sensory anatomy of host detection in *C. maculatus* by examining the antennae and female ovipositor sensilla which are the key communication tool that guides the insect in host-seeking, egg laying and copulation tasks. The sensilla types, shapes, length, middle and basal diameter, basal socket abundance, stain penetration and ultrastructural features are discussed.

Chapter 3. Measures the responses of the beetle (wild strain) to odour stimuli from five bean types, and the headspace volatile samples of three most preferred bean using an olfactometer. The headspace samples of preferred bean types are subjected to Coupled Gas Chromatography-Mass spectrometry for volatile compound identification.

Chapter 4. Investigates the attraction of the beetle to headspace volatile samples from pods of cowpea plant harvested at different growth stages using the olfactometer designed in Chapter 3, and subjects the samples for analysis using Coupled Gas Chromatography-Mass Spectrometry.

Chapter 5. Examines how the beetles select a preferred host, and the consequences of such choices on the progenies performances.

Chapter 6. Discusses the key findings from the thesis, and also indicates areas of interest for future research.

CHAPTER TWO: THE SENSORY ANATOMY OF HOST DETECTION IN *C. maculatus*

2.1. Introduction

Callosobruchus maculatus is an economic insect pest that causes huge financial loss to farmers growing cowpea in the tropical and sub-tropical world. Due to its infestation routes as a field-to-store pest, managing this pest in these regions has been a major challenge. Consequently, understanding the sensory modalities that mould host identification by the pest is an important step towards unlocking its host-finding tactics.

Insects possess sensory receptors on their body surface (mainly, the antennae, tarsi, ovipositor, palpi) which aid them in host selection, and they potentially do this *via* several different sensory modalities (visual, thermal, chemical and mechanical) (Chapman, 1982; Chapman & Thomas, 1978). A sensory stimulus received by a sensory organ is processed by the central nervous system which provides information that informs an insect's behaviour. This is a very complex process that involves balancing an array of stimuli. These array of cues can lead to host-finding and oviposition behaviour (Renwick & Chew, 1994). For example, in gravid moths and butterflies, searching, orientation, encounter and landing, surface evaluation and acceptance have been reported as a sequence of host finding behavioural patterns that lead to oviposition (Renwick & Chew, 1994; Morris & Kareiva, 1991).

Both chemical and mechanical cues are some of the signals an insect first perceives while examining host suitability (Renwick & Chew, 1994). For example, egg-laden seeds were avoided as oviposition substrates by gravid females of *Callosobruchus spp* (Messina, *et al.,* 1987; Messina & Renwick, 1985; Parr *et al*., 1998) suggesting the

ability to detect oviposition deterring pheromones and/or the physical presence of eggs on the seeds. This avoidance behaviour has been reported to be triggered by the presence of oleic acid on bruchid eggs (Sakai *et al.*, 1986), indicating the role of a chemical cue. Similarly, the artificial attachment of "egg model" on seeds deterred oviposition in *Callosobruchus maculatus*, signifying the utilisation of mechanoreceptors in making egg laying decisions (Messina *et al*., 1987). Insects also make use of residual experience while choosing a preferred host, and this has been established as an important tactic used by insects to avoid unsuitable choices in the field (Bernays & Wrubel, 1985; Blaney & Simmonds, 1985). Bernays & Chapman, (1973), also suggested that the time since last meal affects host acceptability. Ovipositing females may therefore employ any or all of these cues before selecting a preferred host.

The majority of host-finding signals are detected by insects *via* the sensilla embedded in the exoskeleton. These hair-like or peg-like structures arise from the cuticle (in which they are embedded). Each functional type of sensillum has a specific form which can be used to identify, or predict the functional characteristics of the sensillum. For example, a sensillum with a sharp tip is often associated with the sense of touch, whereas those with blunt tips often detect volatile chemicals (Cribb & Merritt, 2012). Blunt tipped sensilla have openings (pores) in their surfaces through which the volatiles diffuse and interact with the sensory neurones. Sharp-tipped sensilla lack these pores (figure 2.1 A&B).

There are two major groups of sensilla in terms of numerical representation on an insect's body: Those that respond to mechanical stimuli (mechanoreceptors) and those that detect chemical signals (chemoreceptors).

Mechanoreceptors respond to mechanical distortion in the insect's cuticle (eg touch, change in body position, cuticular stress) and in the surrounding environment (eg, vibration, gravity, air pressure). They have two main forms: The trichoid and the campaniform sensilla (Chapman, 2012). Both lacks pores on the cuticular surface. Trichoid sensilla are sharp tipped; campaniform sensilla have a dome-like appearance (figure 2.1 A & C). Taste and smell/olfaction are afforded by chemosensilla: these usually have blunt tips with one or more pores in the cuticular surface. Sensilla with one single pore tend to respond to a sense of taste, whereas those with many pores confer a sense of smell/olfaction (Chapman & Matson, 2013). Other chemo-sensory sensilla types include sensilla basiconica, sensilla chaetica, coeloconic sensilla, and all have been reported to influence the behaviour of insect pests (Ritcey & Mciver, 1990; Baker, 2001; Onagbola & Fadamiro, 2008; Renthal *et al.*, 2003; Schafer & Sanchez, 1976; Obonyo *et al.*, 2011; Eilers *et al.*, 2012; Ali *et al.*, 2016).

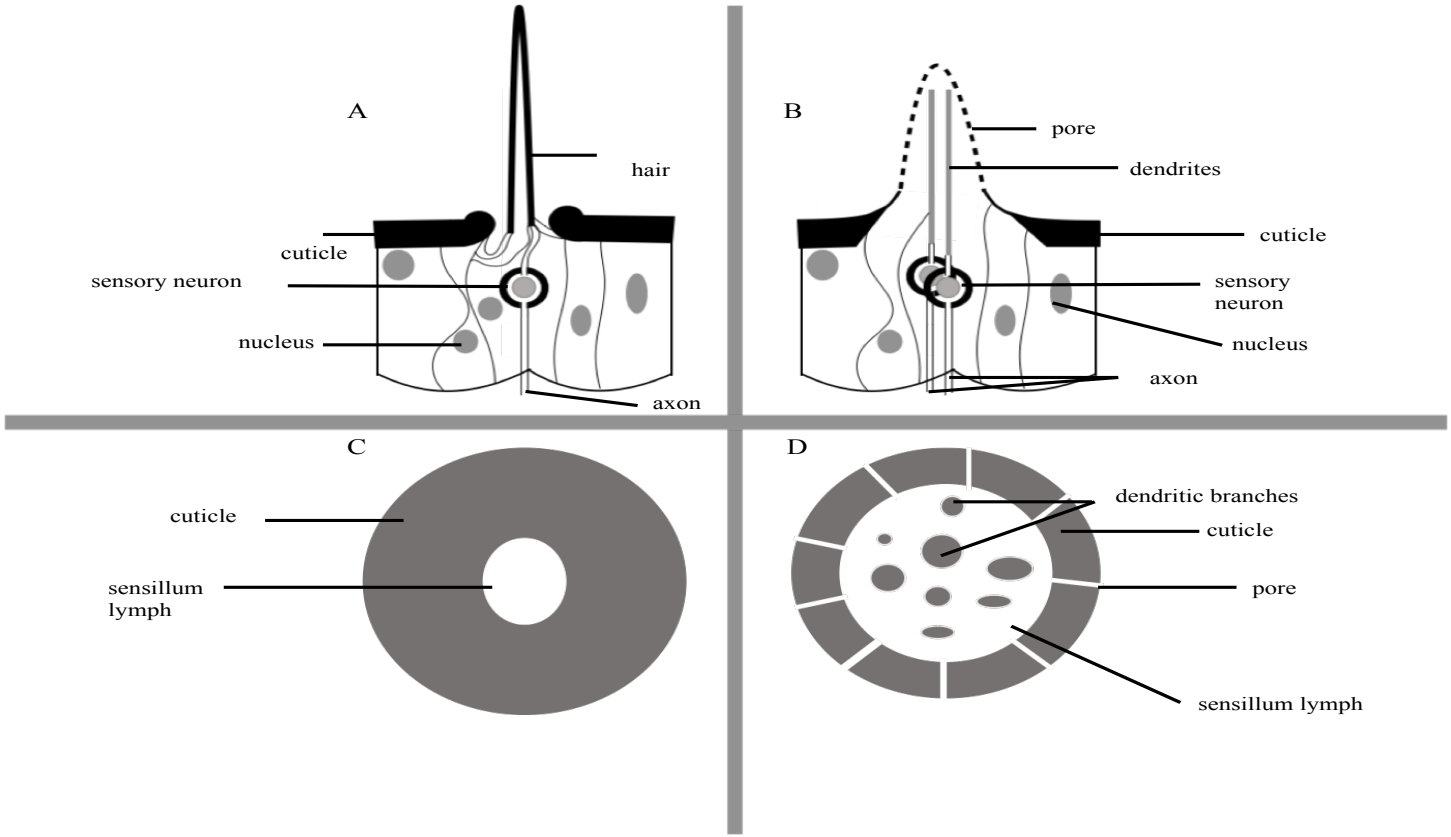

Figure 2. 1. Schematic drawings showing longitudinal and transverse sections of a sensillum. A: Longitudinal section of a mechanoreceptor (trichoid) sensillum; B: Longitudinal section of a chemoreceptor (olfactory) sensillum, C: Transverse section of a mechanoreceptor (trichoid) sensillum; D: Transverse section of a chemoreceptor (olfactory) sensillum

Understanding how the signals from these sensors initiate a host acceptance response is very important, and to perceive and accept a potential host as food or oviposition site may be the responsibilities of some specialised sensilla. The abundance and distribution of sensilla may affect host-making decisions in insects; according to Chapman, (1988), not all sensilla neurons are used in initiating a host acceptance behavioural response at one time. Blaney & Chapman, (1970) showed that about 80 out of nearly 400 sensilla were enough to establish a host recognition response in first-instar larvae of *Locusta migratoria*. This does not imply that a portion of the sensilla are irrelevant, but could mean that they are involved in other functions like contact sensitivity and sensory adaptation. The abundance of sensilla on specialist and generalist insects is also known to differ; the findings of Chapman & Thomas, (1978) revealed that monophagous species of grasshopper had fewer sensilla on their mouthpart compared to the polyphagous species. This further indicates that recognition of host-specific cues may require less sensory receptors, and that each sensillum neuron in a specialist insect might have evolved to respond to specific chemical cues.

Most sensilla types, especially those responsible for the sense of olfaction, are housed on the antenna (Klowden, 2013; Krieger & Breer, 1999; Ceballos *et al.*, 2015) a sensory organ that plays key roles in many behavioural responses including courtship, copulation, host location, oviposition and host examination (Isidoro *et al*., 1996). For example, the antennae have been reported to be used in identifying plant volatile organic compounds that drive insect-plant interactions in the bean aphid (Webster *et al.*, 2008), the pea weevil (Ceballos *et al.,* 2015), the common bean beetle (Khelfane-Goucem *et al*., 2014) and the palm borer moth (Ruschioni *et al.*, 2015). Because of the importance of the antennae in reproduction and mate detection, it is

common to find sexual dimorphism in antennal structure and the number and distribution of antennal sensilla (Onagbola & Fadamiro, 2008). Female *C. maculatus* produce pheromones that attract males (Phillips *et al.*, 1996; Shu *et al.*, 1996), and also have to identify suitable hosts for her developing offspring. The sexually dimorphic life-history 'tasks' that adult *C. maculatus* undertake could select for sexual dimorphism in sensilla morphology and density. Another variable that might affect the sensory anatomy of *C. maculatus* is differences in a selective regime driven by laboratory rearing and field exposure to farming practices. Wild populations are faced with a complex ecosystem that houses a spectrum of cues which they have to screen before isolating a preferred host or switching to an alternative. Consequently, host searching tactics/behaviours are expected to differ between populations from varying ecological conditions. For example, different behavioural responses were detected among field- and lab-strains of some lepidopteran populations (Kareiva, 1985; Morris & Kareiva, 1991), and was linked to specific physiological cues including, shape, colour, visual. Schafer & Sanchez, (1976) also identified quantitative differences in the distribution of olfactory sensilla on the antennae of different field-collection sites in a cockroach.

Studies on insect sensilla have centred on the antenna and mouthparts with little or no work on female external genitalia. Over the years, the diversity in the morphology of insect genitalic sensilla have been studied by evolutionary biologists, and their application in insect classification have been reported (eg Hubbell, 1932). The male genitalia are involved in transferring, receiving, storing and ejecting sperm. The female genitalia are proposed to have evolved to avoid heterospecific mating with males (Sirot, 2003), thus implying sexual selection. The sensilla on female genitalia can be exploited by males during sperm competition. For example, during copulation,

adult males use their genitalia to exploit the sensory systems in the female genitalia which leads to the ejection of any rival sperm stored in the spermatheca (Siva-Jothy, 1987), and this behaviour has been reported in damselfly (Córdoba-Aguilar, 1999; Waage, 1979).

Female genitalic sensilla are also suggested to play a role in egg laying behaviours in insects (Simmons, 2013). Earlier studies on *Callosobruchus spp.* have revealed that choice of oviposition substrate is influenced by difference in seed size (Cope & Fox, 2003; Kawecki & Mery, 2003), seed surface area (Bhattachary & Banerjee, 2001; Mainali *et al*., 2015) and chemical cues on seed surfaces (Credland & Wright, 1989). The trichoid and basiconic sensilla have been identified on the genitalia of female *Stomorhina disolor* and *Ceylonomyia nigripes*, and other studies showed that the female genitalic sensilla are used in copulation (Acebes *et al.*, 2003; Rossignol & McIver, 1977) and oviposition (Rossignol & McIver, 1977; (Simmons, 2013). Examining the genitalic sensilla of female *C. maculatus* will help us understand the sensory physical environment, their forms, and aid in suggesting their probable roles in making sexual selection and oviposition choices.

This chapter examines the morphology and abundance of the sensilla on the antennae of a wild and a lab-adapted strain of *C. maculatus,* and predicts that sensilla abundance between both strains will vary due to their ecological differences.

2.1.1. Chapter objectives

This study aims to;

- Define and characterise the antennal sensilla of *C. maculatus*
- Examine the quantitative differences in the abundance of the antennal sensilla between lab and wild strains.

- Examine the quantitative differences in the abundance of the antennal sensilla between the sexes.
- Define and characterise the sensilla types on the female genitalia of *C. maculatus.*

2.2. Methods

2.2.1. Insects

Two *C. maculatus* stocks were used: A wild strain (from a farmer's field in Taraba State, Nigeria) and a lab-adapted strain (maintained in Sheffield for more than 3 decades). Both stocks were cultured by placing individuals from each strain separately into breeding containers (17 x 11.5 cm) containing 200 g of uninfested whole Borno – brown beans. The lids of the containers were perforated for ventilation. The cultures were kept in controlled climate conditions of 28 ± 2 ^{0}C and relative humidity of 30 ± 5 %.

2.2.2. Quantitative morphology of antennal sensilla

Newly emerged adult (male and female) progeny were prepared for morphological examination by anaesthetising them in a freezer at -20 ^{0}C for 15 min and then removing their heads. The heads were fixed in 70 % ethanol for 24 h after which they were sonicated in an Ultrasonic bath (Ultrawave Ltd, Cardiff) for 2 mins before being dehydrated in a graded ethanol series of 75, 80, 85, 90, 100%, over a period of 1 h. The dehydrated heads were allowed to air-dry for 24 hrs in an incubator at 25 ^{0}C, after which they were mounted individually on aluminium stubs with double-sided adhesive carbon tape and/or silver - Electro - DAG, and sputter - coated with gold (Edwards

S150B sputter coater). Prepared specimens were examined with a scanning electron microscope (SEM) (Tescan Vega3 LMU) at 10 – 15 kV.

To measure antennal sensilla abundance, the number of sensilla basal sockets per 1000 um^2 were counted from the scanning electron micrographs. The abundance of type IV and type VI antennal sensilla were also counted from the SEM micrographs.

2.2.3. Silver nitrate staining of sensillum pores

To identify porous and non-porous sensilla types, adult beetles were anaesthetised in a freezer at -20 ^{0}C for 15 min, and the antennae removed from the head with forceps. The samples were transferred into a 2ml glass vial containing 1% aqueous silver nitrate solution for 48hrs, followed by dehydration in graded ethanol concentrations (70%, 90%, 100%). They were then placed in xylene overnight and mounted in "fluoromount" aqueous medium for observation using a compound microscope (Olympus BX51). The staining allowed me to identify sensilla with and without pores, and map their distribution on the sensilla types identified in the SEM.

2.2.4. Ultrastructure of antennal sensilla

The antennae of *C. maculatus* were obtained by anaesthetising the adults in a freezer at -20 ^{0}C for 15 min, and the excised antennae immersed in 5% sucrose and 2.5% glutaraldehyde in 0.1 M phosphate buffer (PBS, pH 7.2 – 7.4), and left overnight at 5 ^{0}C. The samples were then fixed in 2 % w/v osmium tetroxide and washed in a solution containing 0.1M phosphate buffer (PBS, pH 7.2 – 7.4). They were sequentially dehydrated in an ascending series of ethanol dilutions, cleared in epoxypropane (EPP) and infiltrated overnight in a 50/50 mixture of epoxypropane (EPP) and Araldite resin (CY212) on a laboratory rotor.

They were then processed through two changes of fresh Araldite resin mixture for 8 hours and finally embedded in fresh resin mixture, mounted in gel capsules, and oven dried for 48-72 hours at 60 ^{0}C. Ultra-thin sections were cut with a diamond knife (Diatome) at ~85nm using an ultramicrotome (Reichert – Jung Ultracut E) and mounted on Formvar-coated 200 mesh grids. The sections were examined using a TEM (FEI Tecnai T12 Spirit) at an operating voltage of 80 Kv and images were recorded using a Gatan Orius 1000B digital camera (Gatan Digital Micrograph software). The ultrastructural characteristics (number of pores, the thickness of the cuticular wall, sectional diameter, number of dendrite branches, and sensilla surface texture) of the sensilla types were identified from the transmission electron micrographs.

2.2.5. Sensilla identification

Key morphological features associated with each sensilla type identified from the TEM, SEM and light microscope studies described above were used to cross-match features to generate a "matrix" identification tool (Table 2.3 in results section). Information from the table was used to define the unique characteristics I used to define each sensilla type.

2.2.6. Sensilla associated with the female external genitalia

To examine the morphological features of the female external genital sensilla, female adults were anaesthetised by putting them in a -20^{0}C freezer for 15 min. After de-frosting, the abdomen was pressed using forceps to extend and evert the external genital and expose the sensilla. Females with protruding ovipositors were mounted on an aluminium stub fitted with double-sided carbon tape and examined using a

Benchtop scanning electron microscope (Hitachi TM 3030 Plus) set at EDX and MIX (BSE & SE) observational conditions. Data on the shape of the sensilla, their length and basal diameter were taken from the electron micrographs.

2.2.7. Statistical analysis

Variations among the variables were analysed using permanova. Principal component analysis was used to show the how the abundance of sensilla basal sockets, sensilla type IV & V are related. R (R Core Team, 2013) statistical software was used for all analyses.

2.3. Results

2.3.1. Antennal morphology

The antennae of lab and wild strains were morphologically similar (Figure 2.2). The antennae consist of 11 antennomeres; the scape, pedicel and 9 flagellomeres. The pedicel (second antennae segment) was the shortest (75 ± 2.0 μm) antennomere, while the distal antennal antennomere (eleventh segment) were the longest (303 ± 7.0 μm) in length (Table 2.1). The antennal lengths measurements showed that males of the lab strain had the longest antennae (2264 ± 121.0 μm), followed by the wild-type males (2019 ± 103.0 μm). Females of the lab strain and the wild-type, each measured (1825 ± 46.0 μm) and (1653 ± 49.0 μm), respectively (Table 2.1).

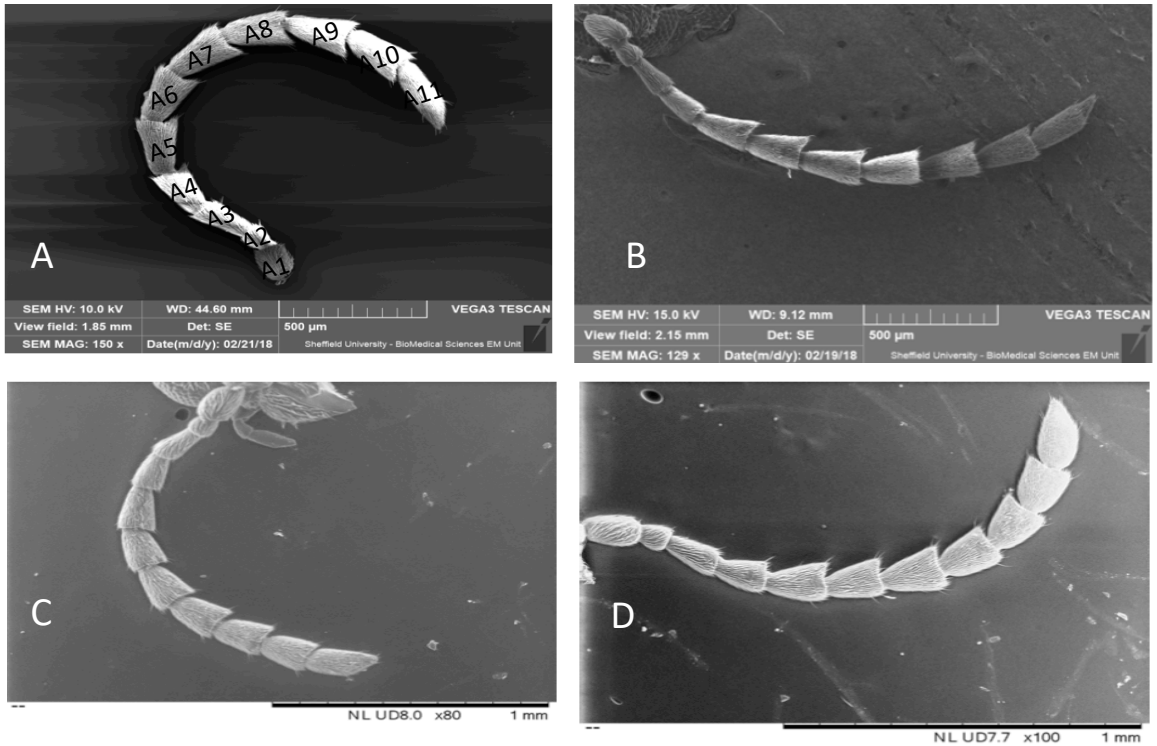

Figure 2. 2. Antennae of two *C. maculatus* strains. Image shows scanning electron micrographs of excised antennae of male and female of both beetle strains. A: Lab strain male; B: Wild strain male; C: Lab strain female; D: Wild strain female. A1: Scape; A2: Pedicel; A3-A11: Flagellomeres.

2.3.2. Sensilla morphology

Eight types of morphologically distinct antennae sensilla were identified on the examined antennae (Figure 2.3; Figure 2.4).

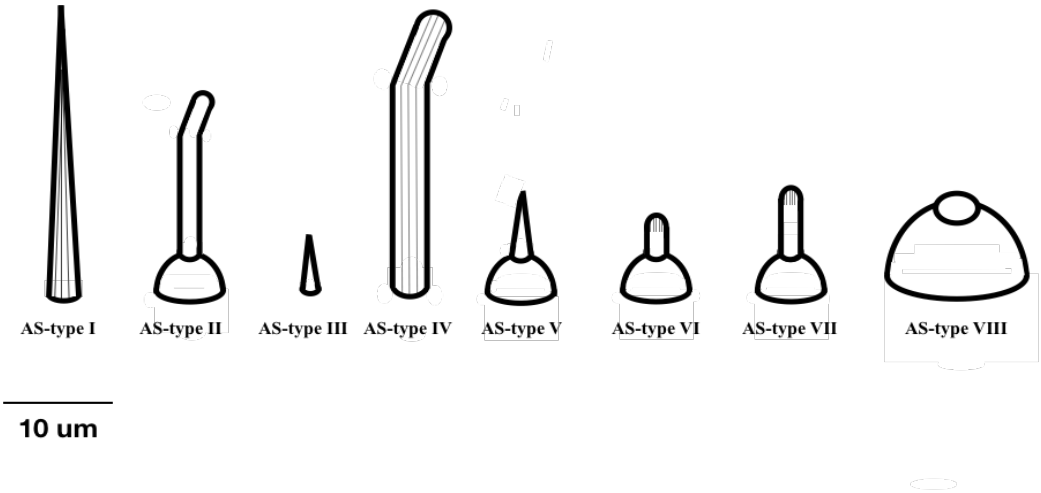

Figure 2. 3. A schematic drawing of antennal sensilla types identified in *C. maculatus*.

2.3.2.1. Antennal sensilla (AS) -Type I

This type of sensillum occurs on all the segments of the antennae. They have longitudinal grooves/ridges on their cuticular surface, and sharp tips (Figure 2.4 – B1). They are straight or slightly curved towards the antennae shaft. These sensilla range from 27.40 ± 0.52μm in length, 0.95 ± 0.02 μm in mid diameter and 1.76 ± 0.12 μm (n=114) in basal diameter.

2.3.2.2. Antennal sensilla (AS) -Type II

This type is characterized by a smooth surface and a blunt curved tip (Figure 2.4 – A1), and are found mainly on segments 8 – 11. They measure 14.88 ± 0.86 μm, 1.11

± 0.05 μm and 2.21 ± 0.07 μm (n=35) in length, mid-point and basal diameters, respectively.

2.3.2.3. Antennal sensilla (AS) -Type III

This sensillum occurs mainly on the base of the scape, the pedicel and occasionally on the first flagellomere (Figure 2.4 – C1). It has a smooth cuticle with a triangular tapering peg-like structure. They are 4.70 ± 0.44 μm long, with a mid and basal diameter of 0.95 ± 0.03 μm and 0.10 ± 0.08 μm, (n=30) respectively.

2.3.2.4. Antennal sensilla (AS) -Type IV

This type is characterized by a longitudinally grooved cuticular surface with a curved blunt tip. They project outwards and are symmetrically arranged around the distal margin of all but the terminal antennomere (Figure 2.4 – B2). They are the longest type of sensilla examined and are 38.27 ± 4.81 μm long. The mid and basal diameter are 1.59 ± 0.15 μm and 2.64 ± 0.28 μm, (n=87) respectively.

2.3.2.5. Antennal sensilla (AS) -Type V

This short sensilla type occurs only on the flagellomeres, and is characterized by a smooth cuticular surface and relatively blunt tip (Figure 2.4 – A2). They range from 11.58 ± 1.87 μm in length; 1.72 ± 0.03 μm in mid diameter and 3.21 ± 0.05 μm (n=93) in basal diameter.

2.3.2.6. Antennal sensilla (AS) -Type VI

This very short peg is distinguished by a bulbous base and a clavate tip (Figure 2.4 – D2) with short grooves on the surface. They occur together on the distal-ventral margins of flagellomeres 4 - 10 and are located in the depression on the distal antennal segment. They are usually surrounded by type V sensilla. The peg-like hairs range

from 1.69 ± 0.23 μm in length, 0.38 ± 0.04 μm in mid diameter and 2.56 ± 0.26 μm (n=15) in basal diameter.

2.3.2.7. Antennal sensilla (AS) -Type VII sensilla

This sensilla type is short but longer than the type VI sensilla which share similar morphological features and location (Figure 2.4 – D1)**.** They range from 3.78 ± 0.12 in length, 0.77 ± 0.07 in mid diameter and 2.46 ± 0.06 (n=26) in basal diameter.

2.3.2.8. Antennal sensilla (AS) -Type VIII

This very short campaniform - like sensilla type occurs with types VII & VIII sensilla and has a wide-flat base (Figure 2.4 – D3).

2.3.3. Analysis of antennal sensilla abundance

The results of the principal component analysis on the abundance of Type IV, V and antenna basal sockets, showed that the first principal component explains 76.51 % of variability, whereas the second and third components explain 15.70 and 7.78 % variance, respectively (Table 2.2). The first component is positively correlated (0.60, 0.54 & 0.59) with all the three variables. An increase in this component increases the values of these variables. The second principal component however shows a strong positive relationship with Type V sensillum. A high correlation of 0.83 indicates that this component is primarily a measure of Type V sensillum. The third component is also strongly correlated (0.74) with Type IV sensillum. This component increases with an increase in this variable, but a decrease in number of basal sockets and Type V sensillum.

The result of permanova shows that the abundance of Type IV sensillum, Type V sensillum and sensilla basal sockets do not defer between beetle strain ($F = 0.157$, $df = 1, 172$, $P=0.842$; Table 2.4, Figure 2.5), but on beetle sex ($F = 9.342$, $df = 1, 172$, $P < 0.01$; Table 2.4, Figure 2.5). A strain vs sex interaction was not detected ($F = 0.655$, $df = 1, 172$, $P = 0.473$; Table 2.4, Figure 2.5) . A post-hoc test showed that Type V sensillum is more abundant on male antenna, whereas, female antennae has more antennal sensilla basal sockets. Type IV sensillum does not differ between sexes.

Table 2.1. Mean length of antennal segments of each beetle strain

				Antennomeres		
Beetle Strain	Sex	A1	A2	A3	A4	A5
Lab Strain	F	155 ± 2.0	75 ± 2.0	164 ± 4.0	153 ± 2.0	178 ± 6.0
	M	154 ± 12.0	88 ± 30.0	76 ± 3.0	196 ± 12.0	226 ± 11.0
Wild Strain	F	140±8.0	82±4.0	140±3.0	141±2.0	155±3.0
	M	146±5.0	78±7.0	144±7.0	158±8.0	191±8.0
		A6	A7	A8	A9	A10
Lab Strains	F	171 ± 5.0	178 ± 7.0	173 ± 6.0	170 ± 5.0	168 ± 2.0
	M	220 ± 12.0	228 ± 7.0	221 ± 8.0	223 ± 10.0	229 ± 9.0
Wild Strain	F	148 ± 3.0	160 ± 4.0	157 ± 5.0	148 ± 9.0	145 ± 6.0
	M	184 ± 11.0	207 ± 14.0	209 ± 9.0	203 ± 15.0	209 ± 12.0
		A11	Total			
Lab Strains	F	240 ± 5.0	1825 ± 46.0			
	M	303 ± 7.0	2264 ± 121			
Wild Strain	F	225 ± 0.8	1653 ± 49.0			
	M	290 ± 7.0	2019 ± 103			

Values show mean length (μm ± S.E).

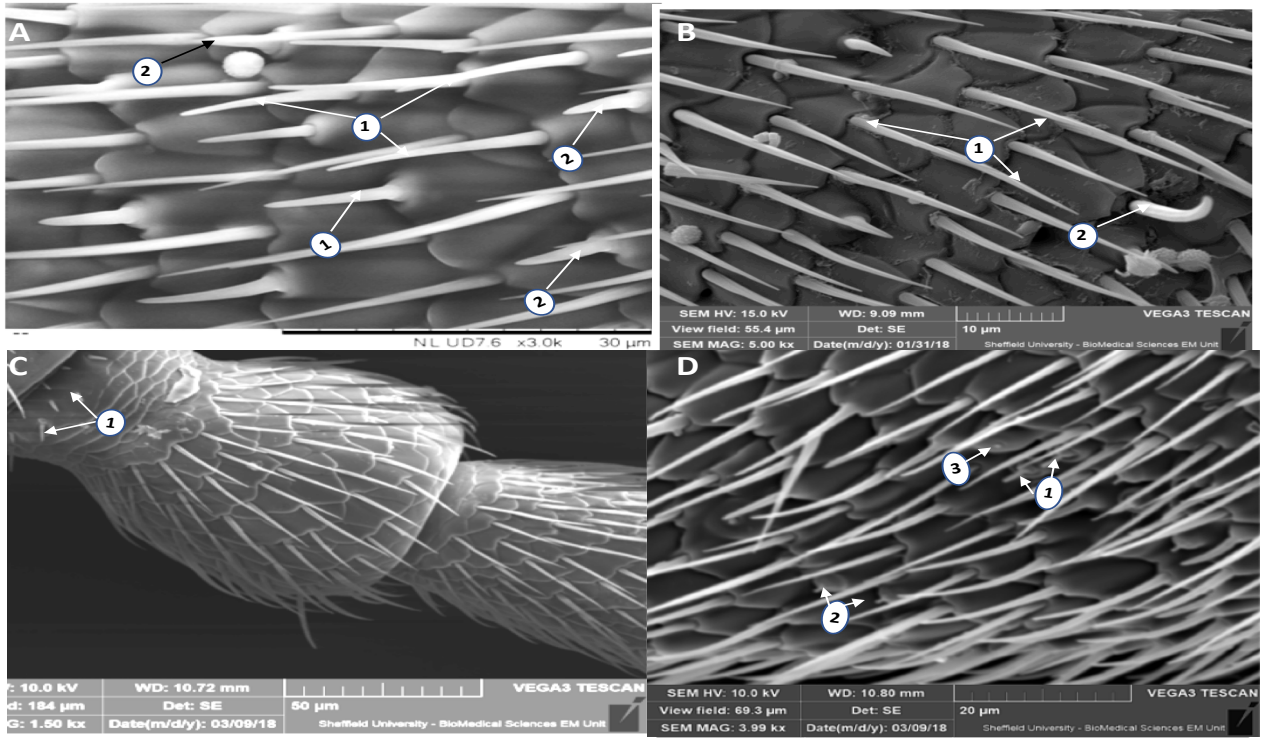

Figure 2. 4. The sensilla types recorded on antennae of *C. maculatus* strains. Plate shows scanning electron micrographs of antennal sensilla (AS) Type II (A-1); sensilla Type V (A-2); sensilla Type I (B-1); sensilla Type IV (B-2), sensilla Type III (C1); Type VI (D-2); Type VII (D-1), and Type VIII sensilla (D-3).

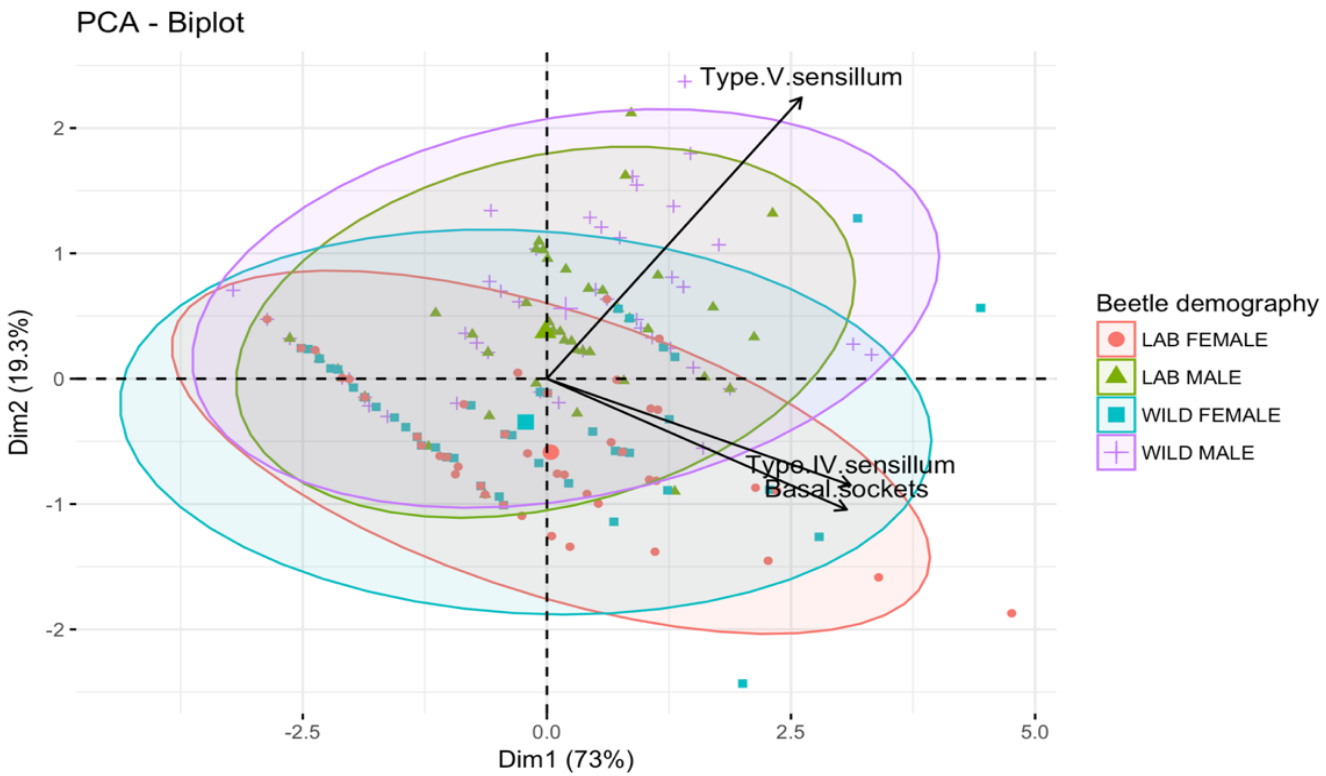

Figure 2. 5. A scatter plot of the first two principal components from a principal component analysis of sensilla measurement differences. Each coloured dot represents an individual sensillum and the ellipses accounts for 80 % confidence interval for the group (beetle demography).

Table 2.2. Principal component analysis loadings of sensilla abundances on *C. maculatus* antennae

Rotation:	**PC1**	**PC2**	**PC3**
Type IV sensillum	0.6019752	-0.2852378	0.7458319
Type V sensillum	0.5400924	0.8334069	-0.1171887
Basal sockets	0.5881548	-0.4733628	-0.6557450
Standard deviation	1.5150	0.6864	0.48322
Proportion of Variance	0.7651	0.1570	0.07783
Cumulative Proportion	0.7651	0.9222	1.00000

2.3.4. Sensilla pores

The presence of silver deposits (dark stains) was used to identify silver nitrate penetration in sensilla pores. Type I and type III sensilla showed no cuticular pores, while type IV and V showed staining (Figure 2.6). The stain was distributed on the shaft of type V sensilla but, appeared on the tip of type IV sensilla

Sensilla types II, VI, VII and VIII showed no stain penetration.

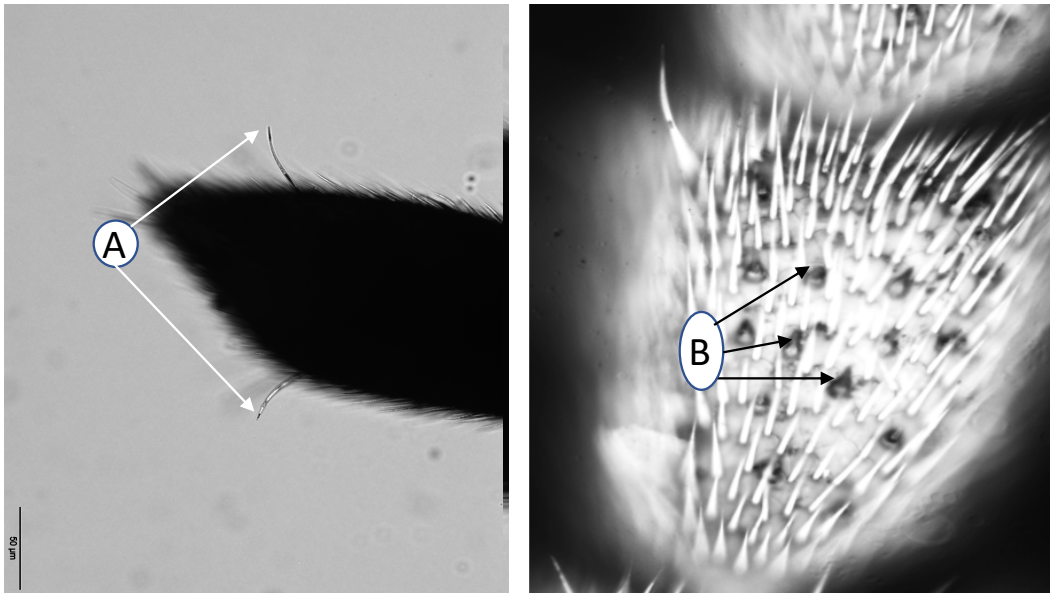

Figure 2. 6. Identification of porous sensilla by silver staining technique. A & B represent Type IV and V antennal sensilla, respectively.

2.3.5. Ultrastructural classification of antennal sensilla

TEM examination showed 3 distinct categories based on the presence of grooves on the surfaces and the diameter of each sensillum sections.

2.3.5.1. Group I

This sensilla type had longitudinal grooved surfaces, and lacked wall pores. Some had thin sensillum walls and dendritic branches (Figure 2.7 - B.) while, others were characterised by thick sensillum walls, and lacked dendritic branches (Figure 2.7 - A, C & D). They had a sensilla wall thickness of 0.33 ± 0.02 μm (n=12).

2.3.5.2. Group II

This type of sensilla had a grooved surface, a single dendritic branch, thin (0.25 ± 0.03 μm, n=9) sensilla walls and pores. They had a small sensilla mid – diameter (Figure 2. 8 A-D).

2.3.5.3. Group III

The majority of the porous sensilla identified were in this category: They are characterised by the presence of two or more dendritic branches in the sensillum interior, the presence of pores, a smooth external surface (without grooves) and thin cuticle walls (0.23 ± 0.01 μm, n=17; Figure 2.9 A-L).

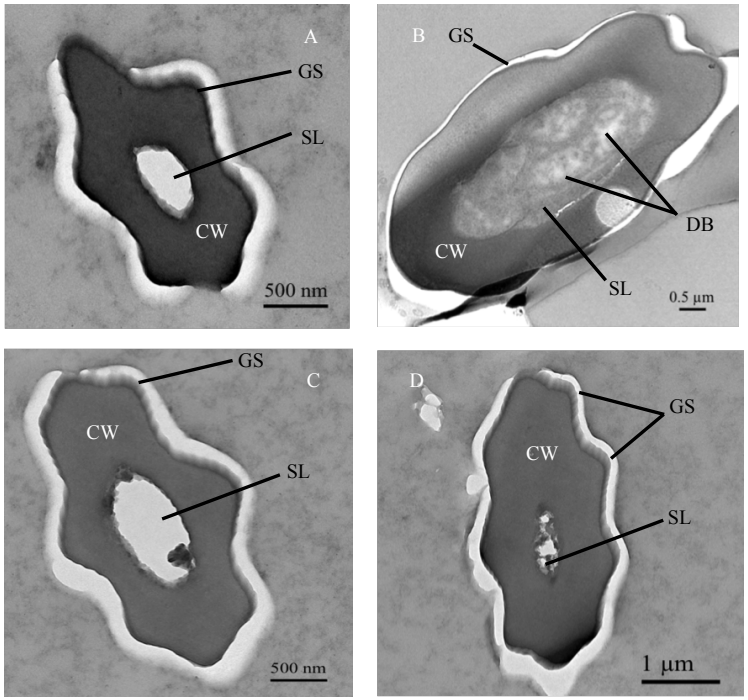

Figure 2. 7. Transmission electron microscopy micrographs of *C. maculatus* antennal sensilla. A,C and D: Showing non – porous cuticle walls and grooved surfaces. B: Showing non – porous cuticle walls, dendritic branches and grooved surfaces. (SL): Sensilla lymph, (CW):cuticle wall, (DB): dendritic branches, and (GS): grooved surfaces.

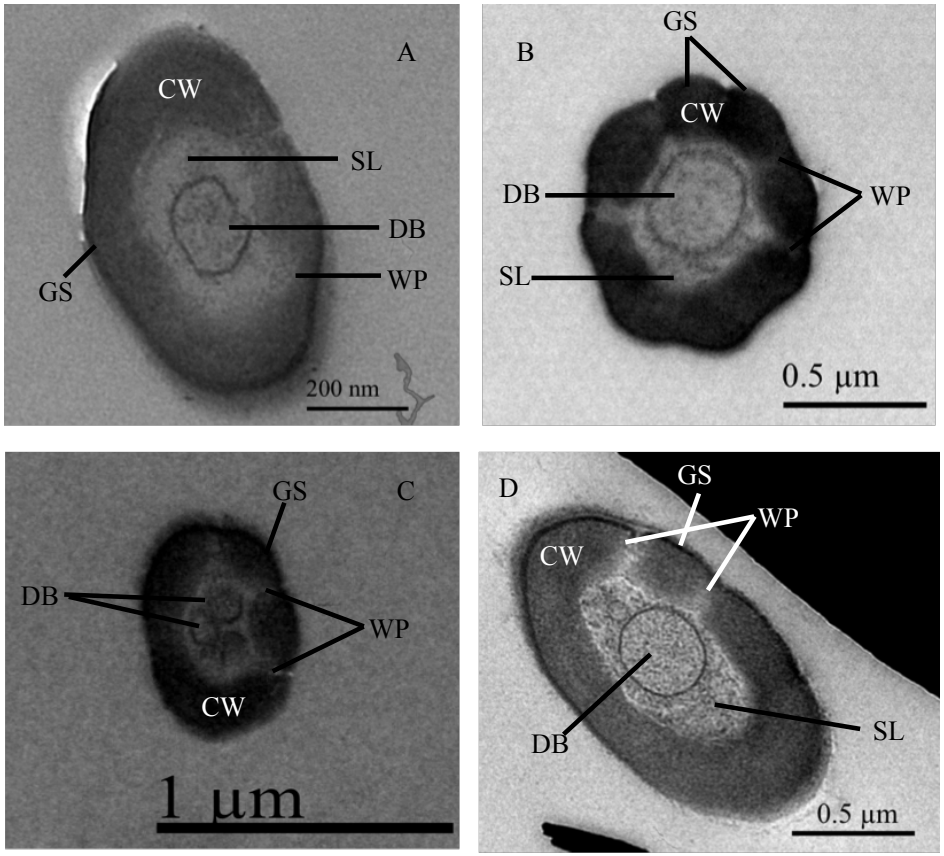

Figure 2. 8. Transmission electron microscopy micrographs of *C. maculatus* antennal sensilla. A-D: Showing porous cuticle walls, and grooved surfaces (SL): Sensilla lymph, (CW): cuticle wall, (DB): dendritic branches, (GS): grooved surfaces and (WP): wall pores.

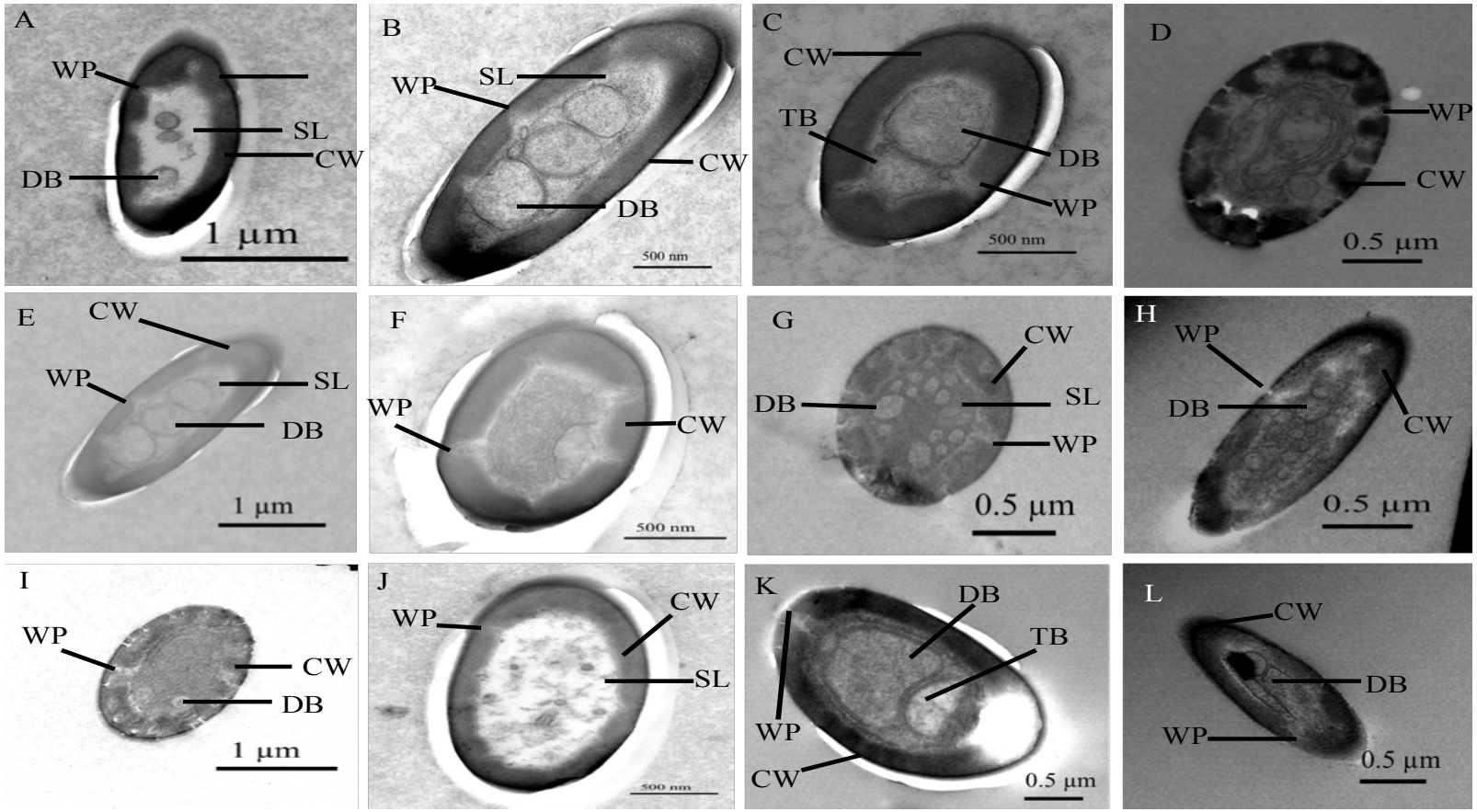

Figure 2. 9. Transmission electron microscopy micrographs of *C. maculatus* antennal sensilla. A-L: Showing diversity of the porous sensilla with smooth cuticular surfaces. (SL): Sensilla lymph, (CW): cuticle wall, (DB): dendritic branches, (TB): tubular body and (WP): wall pores.

TEM observations					SEM observations				LM observations
Sensilla types	Grooved surface	Wall pores	Dendritic Branch	Relative wall thickness	Grooved surface	Sensilla tip	Sensilla length	Mid diameter	Stain penetration
I		×	×		√√				×
II	×	?	?	?	×				?
III	×	×	×		×				×
IV		√√	√√		√√√				√
V	×	√√√	√√√		×				√√√
VI		?	?	?	√√				?
VII		?	?	?	√√				?
VIII	?	?	?	?	?	?	?	?	?

Table 2.3. Ultrastructural and morphological tool for defining the various sensilla types in *C. maculatus*.

Key: √√√ - High; √√ - Moderate; √ - Low; × - Absent; ? – No data.

2.3.6. Ultrastructural characteristics of antennal sensilla types identified

2.3.6.1. Antennal sensilla (AS) - Type I

Using information from the key developed above, this sensillum type has grooved surfaces, thick cell walls, and lack wall pores and dendritic branches (Figure 2.7 - A, C & D).

2.3.6.2. Antennal sensilla (AS) - Type II

Morphological appearance of this sensillum type (smooth cuticular surface) suggests they have wall pores, dendritic branches and thin sensillum walls.

2.3.6.3. Antennal sensilla (AS) - Type III

This aporous sensillum type has thick cuticle walls (though not verified in this study) and an absence of pores. There is no evidence of their ultrastructural morphology in this study which could be due to the location of the sensillum on the antennomeres (joint between scape and pedicel) which might have made it difficult for ultrathin sectioning to cut.

2.3.6.4. Antennal sensilla (AS) - Type IV

This type of sensillum is characterised by longitudinally grooved cuticular surfaces, thin cuticle walls and dendritic branches (Figure 2.7 - B). Although the fine structural features of this sensillum type showed a sensillum wall which lacks pores, however, this suggests the sensilla was cut through a non-porous section thus explains the spotted stains observed in the silver nitrate study.

2.3.6.5. Antennal sensilla (AS) - Type V

This sensilla type is characterized by a smooth cuticular surface, and the silver nitrate stain on the entire sensillum shaft suggest they are associated with high pores density, multiple dendritic branches and thin sensillum walls. Figure (2.9 - D & I) matches the description of this type of sensillum, however, the variation in the diameter of the sensillum shaft suggests other multiparous sections in the Plate may be associated with the sensillum type. It is worth noting the presence of tubular bodies on Figure (2.9 - C & K) suggesting a sensilla type with a chemo and mechanoreceptive function.

2.3.6.6. Antennal sensilla (AS) - Type VI & VII

These sensilla types were not examined for silver nitrate stain test. The small mid-diameter together with a short-grooved cuticle suggests these sensilla types have wall pores, thin cuticle walls and dendritic branches (Figure 2.8 A-D).

2.3.7. Sensilla associated with the female external genitalia

C. maculatus females have a substitutional ovipositor (the abdominal segments extends posteriorly). The external genitalia is not visible (Figure 2.11 - A) unless pressure is applied on the abdomen to extend the structure (Figure 2.11 - B). This suggests that they are pushed-out during oviposition by the female, probably to assess host suitability as an oviposition substrate. The genitalia has a pair of styles on the terminal segment. Each is 32.64 ± 2.01 μm in length, 13.13 ± 0.23 μm in md – diameter and 18.75 ± 1.44 μm in basal diameter, and also bears two types of genital sensilla (Type IV and VI) at the tips. The base of each style is surrounded by five types of genital sensilla (GS: I, II, III, IV and V). Sensilla types II and IV are the most abundant while very few of type V sensilla occur on the plates (Figure 2.11. – C).

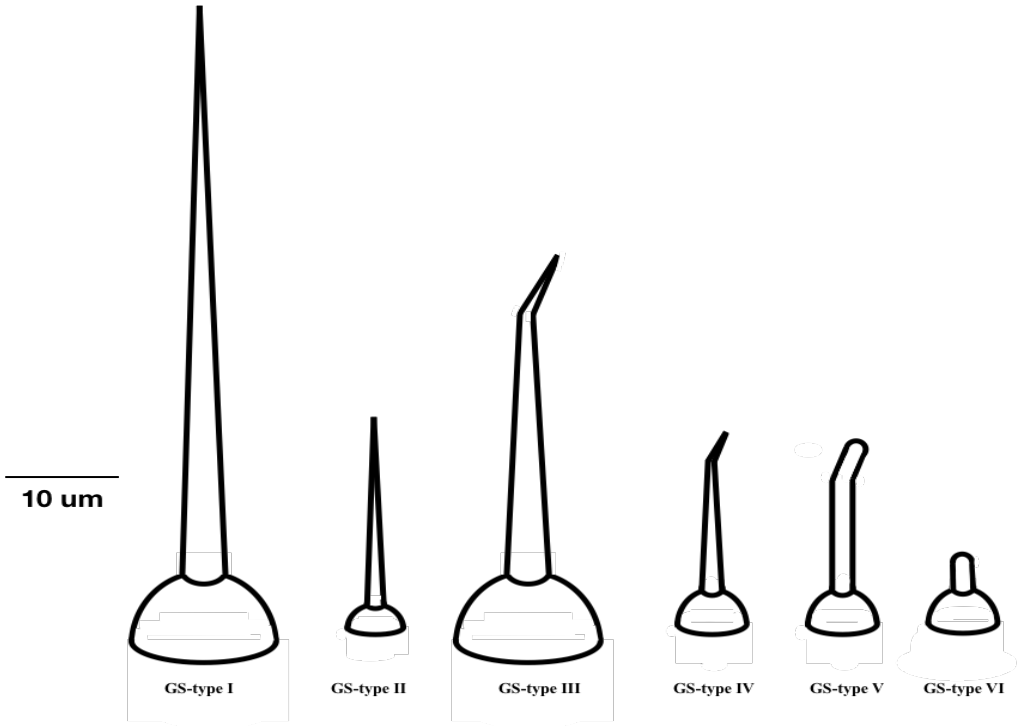

Figure 2. 10. A schematic drawing of genital sensilla types identified in *C. maculatus.*

2.3.8.1. Genital sensilla (GS) -Type I .

This type of sensillum is the longest with sharp tips, and are ranged from 88.64 ± 5.73 µm in length, 1.93 ± 0.11 µm in mid diameter and 3.25 ± 0.13µm (n=41)in basal diameter.

2.3.8.2. Genital sensilla (GS) - Type II.

This sensillum type has the smallest mid diameter, and are sharp tipped. Their length, mid and basal diameter are ranged from 26.35 ± 1.95µm, 0.90 ± 0.05µm and 1.98 ± 0.09 µm (n=75).

2.3.8.3. Genital sensilla (GS) - Type III.

This sensillum is the second longest and has a thick diameter at the base tapering at the tip. They are ranged from 58.16 ± 3.46 μm in length, 2.49 ± 0.08 μm in mid-diameter and 3.67 ± 0.15 μm (n=47) in basal diameter.

2.3.8.4. Genital sensilla (GS) - Type IV.

The type IV sensillum is short with a thick sensillum diameter at the base tapering at the tip. The length, mid and basal diameters is 13.64 ± 1.25μm, 1.19 ± 0.04μm and 2.04 ± 0.06 μm (n=35), respectively. A single sensillum of this type occurs occasionally at the tip of the style.

2.3.8.5. Genital sensilla (GS) - Type V.

This type of sensillum is slender, blunt-tipped, and occur close to the style. It is ranged from 25.75 ± 1.62μm, 1.39 ± 0.05μm and 2.22 ± 0.07 μm (n=40) in length, mid and basal diameters, respectively.

2.3.8.6. Genital sensilla (GS) - Type VI.

This sensillum type is short-stout, blunt-tipped, and occur on the tip of the style. They are 3.53 ± 0.51 μm in length, 1.59 ± 0.10 μm in mid diameter and 1.97 ± 0.13 μm (n=28) in basal diameter.

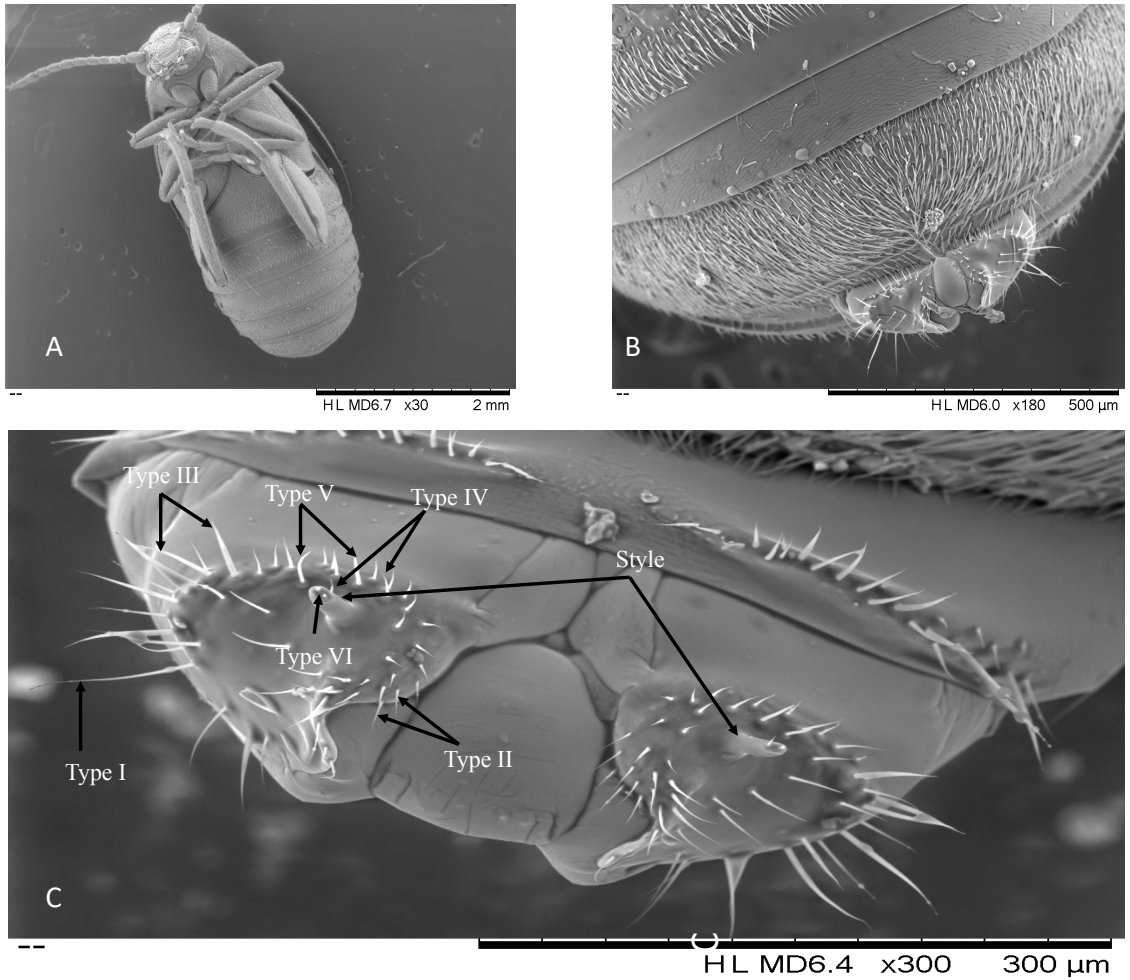

Figure 2. 11. A and B show female *C. maculatus* before and after genitalia extension. C; magnified image of genitalia sensilla types.

Table 2.4. Permanova analysis on sensilla abundance

	Df	Sum of Squares	F-value	Pr(>F)
Beetle strain	1	0.0042	0.1575	0.842
Beetle sex	1	0.2518	9.3427	0.004 **
Beetle strain x Beetle sex	1	0.0177	0.6559	0.473
Residual	172	4.6349		

Table 2.5. Two – way ANOVA results on antennal length of *C. maculatus*.

	df	Sum Squares	Mean Square	F - value	P - value
Beetle strain	1	0.2271	0.2271	24.263	0.00035 ***
Beetle sex	1	0.6964	0.6964	74.416	1.72e-06 ***
Beetle strain x Beetle sex	1	0.0014	0.0014	0.146	0.70879
Residuals	12	0.1123	0.0094		

2.4. Discussion

This study identified eight types of antennal sensilla in both sexes and strains, and six types of female genitalic sensilla in *C. maculatus.* The antennal length was different between beetle strain and beetle sex, but the abundance of sensilla basal sockets and Type V sensilla only differed between sexes.

The characteristics of the antennal sensilla defined in this study agrees with previous studies on antennae of *Callosobruchus sp*. (Fukuda *et al*., 2016; Hu *et al*., 2009) The antennae consist of the scape, pedicel and flagellum (with nine flagellomeres), and is serrate in shape. Examination of the antennal sensilla revealed nine morphologically distinct types on males and females in both strains. The antennae are longer in males than females, and their abundance differ between sexes (which does not differ between the strains). These differences suggest a difference between the roles each sex plays during mating, food searching and egg-laying behaviours. Differences in antennal length and antennal sensilla abundance have been reported in sex (Ali *et al.*, 2016; Dyer & Seabrook, 2018; Onagbola & Fadamiro, 2008; Fukuda *et al*., 2016), strain (Schafer & Sanchez, 1976) and species (Ritcey & Mciver, 1990; Hu *et al*., 2009) of other insects. Onagbola & Fadamiro, (2008), recorded major differences in the distribution of antennal sensilla types between the sexes of *Pteromalus ceraelallae.* Significant differences between the antennal segments of *C. chinensis and C. maculatus* have also been reported (Hu *et al*., 2009). Although this study did not identify any difference in sensilla abundance between strains, the result could indicate the importance of associative learning or experience in host identification by female *C. maculatus.*

Eight different types of antennal sensilla was identified in this study. The antennal sensilla - Type I with thick sensillum wall, longitudinally grooved surface,

sharp tips and absence of dendritic branches and wall pores strongly suggests they respond to stimuli associated with tactile mechanoreception and/or body position. Sensillum with similar morphology have been reported on the antennae of other coleopterans (Ali *et al*., 2016; Palma *et al*., 2013; Ritcey & Mciver, 1990). This sensillum type had been described as; aporous type II trachod sensillum (Onagbola & Fadamiro, 2008); sensillum trachodea type I (Ali *et al.*, 2016; Ritcey & Mciver, 1990; Hu *et al*., 2009; Fukuda *et al*., 2016) and tactile mechanoreceptors (Gullan & Cranston, 1994).

The type II sensillum reported in this study is similar to those described on the antennae of flea beetles species (Ritcey & Mciver, 1990), *Concotrachelus nenuphar* (Alm & Hall, 1986), as sensilla trichoid 2 on *C. maculatus and C. chinensis* (Hu *et al*., 2009). The smooth cuticular surface of this sensillum suggests it plays a probable role in the beetle's olfaction.

Another type of sensillum defined in this work is antennal sensilla type III which had been described as 'hair plates' on antennae of flea beetles (Ritcey & Mciver, 1990); as type IV sensilla trichodea on *Pteromalus cerealellae* antennae (Onagbola & Fadamiro, 2008); as proprioceptors (Chapman, 1982), as basiconic sensillum on antennae of *Trichogramma australicum* (Amornsak, *et al.*, 1998) and as Bohm bristles on antennae of other *C. sp.* (Hu *et al*., 2009; Fukuda et al., 2016). These studies reported that this sensillum occurs only on the scape and pedicel, but in my work, it also occurs on the joint between the second and third antennal segments. The silver nitrate stain test showed a lack of pores in the sensillum cuticle, and confirms its probable role in maintaining the orientation of the insect's body parts (Chapman, 1988).

The antennal sensilla - type IV in this study have been described in previous studies (Ali *et al.*, 2016; Palma *et al*., 2013; Schafer & Sanchez, 1976; Hu *et al*., 2009; Fukuda *et al*., 2016). The outward projection of this sensillum from the antennae shaft indicates it may be detecting stimuli prior to other antennal sensilla types (Schneider, 1964) and thus plays a protective role over them. The silver stain and TEM results showed the presence of dendritic branches, a thin cell wall and few pores which suggests a role in contact or smell chemoreception (Seada, 2015; Sen & Mitchell, 2001).

Antennal sensilla - Type V is commonly found on antennae of most insects. Its function as an olfactory sensillum on the antennae of *Paysandisia archon* (Ruschioni *et al*., 2015), *Solenopsis invicta* (Renthal *et al*., 2003), *Tribolium castaneum* (Ali *et al*., 2016), two *Callosobruchus sp*. (Hu *et al*., 2009), four *Periplaneta* sp. (Schafer & Sanchez, 1976), four species of flea beetles (Ritcey & McIver, 1990) and *Hylastinus obscurus* (Palma *et al*., 2013) have been reported. Findings from these studies agree with the multiple silver stains, multiple wall pores, thin cuticle wall and presence of dendritic branch identified as features of the sensillum in this study. As this sensillum is surrounded by type I (mechanoreceptor) and type II (chemoreceptor) sensilla which are longer in length, they may be protecting it against mechanical and chemical damage (Sen & Mitchell, 2001).

The type VI & VII antennal sensilla of *C. maculatus* as described in this study have been found on the antennae sensilla of flea beetles (Ritcey & McIver, 1990), cockroaches (Altner, *et al*., 1978), locusts (Boeckh, 1967), moths (Faucheux, *et al*., 2006), *C. rhodsianus* (Fukuda *et al*., 2016), *C. maculatus and C. chinensis* (Hu *et al*., 2009; Fukuda *et al*., 2016) and Colorado potato beetle (Sen & Mitchell, 2001) . Their location, shape and size designate them as airborne chemoreceptors. As they

occur in the company of other longer porous sensilla (type II & V), this reveals they are not contact chemoreceptors, and suggests each type of multiporous sensillum may have evolved to detect a specialised type of airborne chemical. A probable role as a thermo or hygroreceptor has been reported on this sensillum (Altner & Loftus, 1985; Yokohari, 1981).

The flat broad basal base sensillum reported in this study as antennal sensilla - type VIII is similar in morphology to the sensillum reported as a short basiconic mouthpart on antennae of *Phoracantha recurus* (Faucheux, 2013). But, was not reported by other *Callosobruchus sp*. (Hu *et al*., 2009; Fukuda *et al*., 2016). Its similarities (location and sparsity) with type VI & VII sensilla suggest it may be involved in thermoregulatory function although it has been described as campaniform sensillum and /or stress receptors (McIver, 1985).

The structure of the extended female genitalia shows that genital sensilla - type IV and VI that occur on the tip of the style may be the first to have a direct contact with any host surface. These sensilla types may play a role in the examination of host surface texture and host size determination. The type I and III of the genital sensilla which occur at the marginal regions of the cuticular plate probably probes an extended area of a host surface. Similarly, the marginal position of the genital sensilla - type II suggests it may be used to assess host surfaces, distally. This agrees with the reports of Simmons, (2013) on the directional sensitivity of trichoid mechanoreceptors on female genitalia. Other studies have shown that host physical cues such as seed surface area (Bhattacharya & Banerjee, 2001; Mainali *et al*., 2015), seed sizes (Cope & Fox, 2003; Kawecki & Mery, 2003) affect female oviposition behaviour in *C. maculatus.* The trichoid genitallic sensilla could also be playing a role in sexual selection by detecting physical aspects of the male genitalia during copulation. This is important

because sexual selection in females has been linked with the evolution of male mating structures (Sirot, 2003).

The type V sensillum of the female genitalia which occurs ventrally, and close to the styles is assumed to be chemoreceptor. Its ventral position and closeness to the styles reveal its inability to establish immediate contact with any surface consequently, a mechanoreceptive function is ruled out. It is therefore, suggested to regulate egg crowding during oviposition by detecting airborne stimuli from eggs and/or larvae deposited on the host surface. Presence of chemoreceptors in ovipositors of insects have been reported in the work of Simmons, (2013). Basiconic sensilla, a chemoreceptor have also been identified on the genitalia of female *Stomorhina disolor* and *Ceylonomyia nigripes*. According to (Credland & Wright, 1989), the chemical cues of seed surfaces induced a host-acceptance or avoidance behaviour in gravid females. Olfactory sensilla could also be used by females to detect semiochemicals secreted by heterospecific males during copulation, thus triggering an avoidance response.

In summary, I have defined and characterised the antennal sensilla types of *C. maculatu*s as well as the female genitalic sensilla, and shown that sexual dimorphism on antennal sensilla abundance exists, but that there are no strain differences. The antennal sensilla type I, III and VIII are mechanoreceptors, while type II, V, VI and VII are chemoreceptors. The type IV antennal sensillum is a contact chemoreceptor but, a mechanoreceptive role is feasible; whereas, the genital sensilla identified in this study are mechanoreceptors except for the type V sensillum with a probable olfactory role.

CHAPTER THREE: ATTRACTION of FEMALE *C. maculatus* TO HOST ODOURS.

3.1. Introduction

The application of pesticides as a pest control strategy has been gaining negative attention due to the health and environmental consequences associated with its use. The dry seeds of cowpea, an important food and cash crop to farmers (mainly, cowpea growers in tropical regions) are heavily infested by *C. maculatus* during storage, causing huge economic loss. As a result, these farmers have to spray pesticides on their harvest to control the pest attack without understanding the consequences of their actions. Consequently, stakeholders are seeking alternative safer routes to handling this infestation problem.

Studies on how insects relate with their host-plants have revealed the prospects of managing pests' attacks using semiochemical-based approach (Cai *et al*., 2015). Odour cues detected over a distance drive many insect-plant interactions and many of the chemicals involved are volatile organic compounds (VOCs) (Dudareva, *et al*., 2004). These substances can be released from the flowers, developing pods or seeds of the host plant and are used by pest insects to identify, home-in on and utilise a preferred host type (Ignacimuthu *et al*., 2000; Uechi *et al*., 2007; Webster *et al.*, 2008). The use of plant VOCs in pest control has produced some remarkable outcomes (Agelopoulos *et al*., 1999). For example, the cosmopolitan granary pest *Acanthoscelides obtectus* (Say), the pea weevil, *Bruchus pisorum* L. and the legume pod borer (*Maruca vitrata* Fab.) are attracted to volatile compounds from dry bean cultivars (Khelfane-Goucem *et al*., 2014), *Pisum sativum* L. (Ceballos *et al.*, 2015) and *Vigna unguiculata* (Bendera *et al.*, 2015; Zhou *et al*., 2015), respectively. This

attraction has been used to control its effects on these agriculturally important products.

A range of volatile blends as well as a single compound and variations in chemical profiles have been suggested to influence host discrimination in many insects (De Bruyne & Baker, 2008; Smith, 1998). A study by Bruce & Pickett, (2011), showed that insects use a combination of 3-10 compounds as chemical cues during host location. In another study, Bruce *et al.*, (2005), identified 3-octane and 1-octanol as volatile inducing compounds against insect pests of stored grains. According to Ajayi *et al*. (2015), *C. maculatus,* showed 90-95 % attraction to 3 legume cultivars, and identified 2-ethyl hexanol as a key volatile compound driving the responses. Arnold *et al.* (2012) also reported that higher concentrations of methyl silicate, a botanically derived compound, repelled a subgroup of in-active *C. chinensis* adults compared to active adults. Another study on *C. chinensis* revealed that tridecane, a volatile with the highest amount from cowpea seeds infested with fourth instar larvae repelled conspecific females (Babu *et al*., 2003).

In many insect species, female egg-laying behaviour determines host acceptance or preference, and differs with populations (Carrière & Roitberg, 1996) and other factors. Gravid female *C. maculatus* use a combination of chemical and physical cues associated with host seed-surface to discriminate among seeds of legume cultivars (Messina *et al.* 1987; Credland & Wright, 1989), and has exhibited behavioural attraction to different legume cultivars. For example, females avoid beans that already have eggs and/or larvae (Messina & Renwick, 1985; Messina *et a*l., 1987), and such egg-laying behaviour is influenced, in part, by the presence of the 'deterring' pheromones of conspecifics (Messina *et al*., 1987; Shu *et al.*, 1996). They also consider host surface texture (Cope & Fox, 2003), host bean size (Beck & Blumer,

2014) and egg-load on a bean (Messina *et al*., 1987) when choosing an oviposition substrate. Furthermore, *C. chinensis*, was suggested to be attracted to volatiles from un-infested and egg-carrying seeds of cowpea, and repelled by seeds carrying developing larvae (Ignacimuthu *et al*., 2000). Geographical location, beetle sex and morph also affect host preference in *Callosobruchus spp.* According to Messina & Slade, (1997), egg-laying female *C. maculatus* from Africa preferred cowpea to mung bean as an oviposition substrate, whereas, strains from Asia could not distinguish between bean types. Also, Arnold *et al.*, (2012) found that female adults and normal forms of *C. maculatus* show stronger attraction to cowpea odours compared to males and active forms.

To examine how female *C. maculatus* uses olfactory cues in host selection during oviposition, their behavioural responses when exposed to odour from different bean types (both suitable and unsuitable host) was examined using a wild strain of the beetle. The choice of a wild type over a lab-adapted strain is based on the assumption that populations from the wild have evolved to develop special mechanisms that aid them in identifying and isolating a preferred host in a mixture of familiar and unfamiliar hosts in the field. Thus, the unlocking of a host searching cue is very likely. Understanding the connection between the preference behaviour of this stored-product beetle towards host plants, and identifying the VOCs responsible for such response would be an important step forward in designing novel control measures that will focus on monitoring, predicting and controlling infestation outbreaks. The work in this chapter is driven by the notion that behavioural attraction and preference for a bean type by female *C. maculatus* is mediated by host odour cues.

3.1.1. Chapter objectives

This chapter's objectives are as follows;

- To examine the preference of the mated female *C. maculatus* to odour from different agriculturally important bean types.
- To identify and quantify candidate headspace volatile compounds from preferred bean types.
- To analyse the volatile compounds to identify compounds that are more abundant on the various bean types.

3.2. Methods

3.2.1. Insects

A wild strain of *C. maculatus* was collected from infested Borno-brown beans in a farmer's field in Taraba State, Nigeria, and cultured in breeding containers (17 x 11.5 cm) containing 200 g of uninfested whole Borno – brown bean. Lids of the containers were perforated to allow for ventilation. The cultures were kept in a laboratory at a temperature of 28 $\pm$ 2 ^{0}C and relative humidity of 30 $\pm$ 5 %.

3.2.2. Beans

Seeds of five bean types were used in this study; "Borno brown", black-eyed bean (cultivars of *Vigna unguiculata* L. Walper), adzuki bean (*Vigna angularis* Wild), mung bean (*Vigna radiata* L. Wilzek) and pinto bean (*Phaseolus vulgaris* L.). With the exception of "Borno brown" from Nigeria, all were sourced from a local Whole Food Store (in Sheffield). Three kg of each bean type was frozen at -20 ^{0}C for 10 days to ensure the seeds were free of infestation before experimental use. They were equilibrated for 2 weeks at 28 $\pm$ 2 ^{0}C and 60 + 5 % relative humidity.

3.2.3. Does female *C. maculatus* discriminate amongst hosts?

To examine the preference of the beetle to odour from a mixture of beans, three bean types were used: A bean familiar to the wild strain (Borno brown), an unfamiliar bean of the same genus (adzuki bean) and another unfamiliar host of a different genus (pinto bean). This bean choice is not unrealistic in a field situation where the beetle is often faced with a wide range of host types.

A four arm olfactometer with three layers (Floor, observation and cover) was used. The floor was fitted with a Whatman filter paper base (110 mm) to provide friction while the observation layer had four edges drilled into the four arms of the olfactometer. A hole (4 mm diameter) was also drilled at the centre of the third layer (cover) for air suction. Four (60 ml) BD plastipak's were used as odour chambers. Each of the bean types was placed in one of the odour chambers, while the fourth chamber served as a control (clean air). A PTFE (polytetrafluoroethylene) connecting tubing (1.5mm ID x 3.2mm OD) was used to link each of the chambers to the four arms of the olfactometer, and the connections sealed with PTFE tape. The four odour chambers were surrounded with brown paper to prevent the beetle from viewing the samples. A 60 W light bulb was positioned above the olfactometer to provide illumination.

A mated female (2 days old) was introduced into the observation arena, and airflow was generated using a vacuum air pressure pulling air through the four arms of the olfactometer at a rate of 200 ml/min. After the beetle's introduction, it was given 3 min to acclimatize before being allowed to make a choice (15 min). Beetles that made no decision within 5 min after introduction were discarded. After testing five beetles, the odour source was replaced. The olfactometer arm together with the filter paper was rotated after each test to reduce any positional effects. Before the commencement of each experiment, the olfactometer, Teflon tubing and the BD plastipaks (odour chambers) were washed with a detergent, rinsed with distilled water, and then cleaned with 70 % ethanol. Each weevil was tested only once. Data on the beetle's attraction to odour was measured as the mean time spent in each odour chamber (arm).

3.2.4. Is female *C. maculatus* attracted to host-bean odour?

To test the attraction of the beetle to odour from a particular bean, five different bean types were used: Borno brown, black-eyed bean, adzuki bean, mung bean and pinto bean. This approach was designed to measure the beetle's preference for a particular host which is a familiar situation in most storage conditions.

A two-arm olfactometer was used for the study. It consists of three layers representing the base (floor), the observation layer and the cover clipped together to form an eight-sided shape with a two-arm exposure chamber. Each layer was made of a transparent Perpex base 6 mm thick. The first layer (floor) was lined with a Whatman filter paper base (110 mm) to provide traction for the beetle. Another layer, the observation arena had a hole (3 mm diameter) drilled from both edges into the two arms to accommodate the odour chambers. Then, a third layer (cover), all of the same size and shape, had a hole (4 mm diameter) drilled at the centre. Two 60 ml BD plastiak's (syringes) served as the odour chambers. A Teflon tube (1.5mm ID x 3.2mm OD) was used to connect each of the chambers to both arms of the olfactometer, and the connections were tightened with a PTFE tape. A bean type was placed into one of the odour chambers, while the second chamber was used as a control (clean air). Both chambers were covered with a brown paper to prevent the beetle from having a visual cue of the host. A 60 W light bulb was positioned 1m above the olfactometer to provide uniform illumination.

A mated female was introduced into the centre of the olfactometer (observation arena). Air was drawn through both arms using a vacuum air pressure, and regulated with a flow meter at a rate of 100 ml/min. After the introduction, each weevil was given 3 min to settle in the observation arena, and the movement towards both arms was observed for 15 min. Beetles that do not make any choice after 5 min of

introduction were regarded as “non-responders”, and discarded. After testing five adults, the odour source was replaced. The olfactometer arm together with the filter paper was rotated after each test to reduce any positional effects. Before the commencement of each experiment, the olfactometer, Teflon tubing and the BD plastipaks (odour chambers) were washed with a detergent, rinsed with distilled water, and then cleaned with 70 % ethanol to eliminate any organic residue. Each weevil was tested only ones. Each odour source was tested with 8-10 individuals, and data on bruchid response was determined by calculating the mean time spent in each arm by the beetle.

3.2.5. Collection of headspace VOC’s

Headspace collection of organic compounds released from the three beans in the two-arm trial above was carried out for a 24-hr period. 100 g of each bean type was placed in a glass vessel (190 mm high x 100 mm wide), open at the top for an inlet and outlet ports. A volatile collection trap (8 cm long, 5 mm diameter) containing Porapak Q absorbent (50 mg, 80/100 mesh) was connected to the glass vessel to trap the VOCs. Charcoal filtered air was passed through the Porapak Q absorbent at a constant rate of 1 L/ min (Figure 3.1). All the connections were made with PTFE tubing and tape. VOCs absorbed on Porapak Q were eluted with 1 ml of acetone. Extracted samples were stored in glass vials in a freezer at -80 ^{0}C until used for analyses.

3.2.6. Beetle attraction to headspace volatiles.

To investigate the attractiveness of the beetle to volatile samples collected, a two-arm olfactometer was used as described in 3.2.5.

Twenty microliters (20 µl) of volatile samples from the 3 bean types (Borno brown, adzuki bean and black-eyed bean) were applied on a piece of filter paper, and 1 min was allowed for solvent evaporation. The treated filter paper was then put into one of the odour chambers, while the second chamber was used as a control which contained a piece of filter paper treated with 20 µl of hexane. A mated female was then introduced into the centre of the olfactometer (observation arena). Air was pulled through both arms using a vacuum air pressure and regulated with a flow meter at a rate of 100 ml/min. After introduction, each weevil was given 3 min to settle in the observation arena, and the movement towards both arms was observed for 15 min. Weevils that made no choice after 5 min of introduction were regarded as "non-responders" and discarded. Before the commencement of each experiment, the olfactometer, Teflon tubing and the BD plastipaks (odour chambers) were washed with a detergent, rinsed with distilled water, and then cleaned with 70 % ethanol. Each beetle was tested only once.

3.2.7. Identification of volatile compounds

Tentative identification of candidate compounds associated with the volatile samples from the bean types was achieved using GC – MS. A 2 µL of the air headspace sample was injected onto a capillary GC column (30 m x 0.25 mm ID, 0.25 µm film thickness), which was directly coupled to a mass spectrometer (PerkinElmer, Clarus® SQ 8T). The carrier gas was Helium with a flow rate of 1.02 mL min^{-1}. Ionization was achieved by electron impact at 70 eV, 230 ^{0}C. The injection port was maintained on a splitless mode. The GC initial oven temperature was maintained at 30 ^{0}C min^{-1}, then ramped at 5 ^{0}C min^{-1} to 240 ^{0}C, and held for 20 min. Mass spectrum acquisition was scanned using a mass/charge (*m/z*) range of 35 to 450.

Candidate compounds were identified by comparing the chromatograph retention index and mass spectra with a library database spectra using the National Institute of Standards and Technology (NIST) mass spectra search programme (version 2.2, NIST 14, Gaithersburg, Maryland, USA). The retention index of each compound identified was calculated using a series of straight alkanes (C_8 – C_{20}). The abundance of each identified compound was calculated by integrating the peak areas of the total ion chromatograph and averaged.

3.2.8. Statistical analyses

The four-choice data on the beetles' responses to VOCs from each bean type was subjected to ANOVA, whereas, the two-arm result was analysed using Chi-square (χ^2) test. Stacked bars were used to present the proportion of time spent by the beetles in the two-choice olfactometer. The chemical analysis data on the abundance of volatile compounds from each bean type examined was subjected to permanova analysis to identify variances amongst compounds. The similarities of the compounds based on their abundance were interpreted using cluster analysis (by Ward's method); whereas, principal component analysis (PCA) was used to indicate the ordination of the compounds and their relationships. Finally, a Venn diagram was plotted to interpret the number of unique and shared compounds among the bean types examined. R statistical software (R Core Team, 2013) was used for all analyses.

Figure 3. 1. Collection of headspace VOC's from Borno-brown beans

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3.3. Results

3.3.1. Female *C. maculatus* responses to odour from bean types.

The four-arm choice test showed that mated females spent more time in the arm containing Borno brown beans when compared with arms housing adzuki bean, pinto bean, and the clean air (control), respectively ($F = 7.68$, $df = 3, 36$, $P < 0.001$; Table 3.1, Figure 3.2). The females did not distinguish the difference in odour stimulus from adzuki bean and pinto bean seeds when compared with the clean air (control).

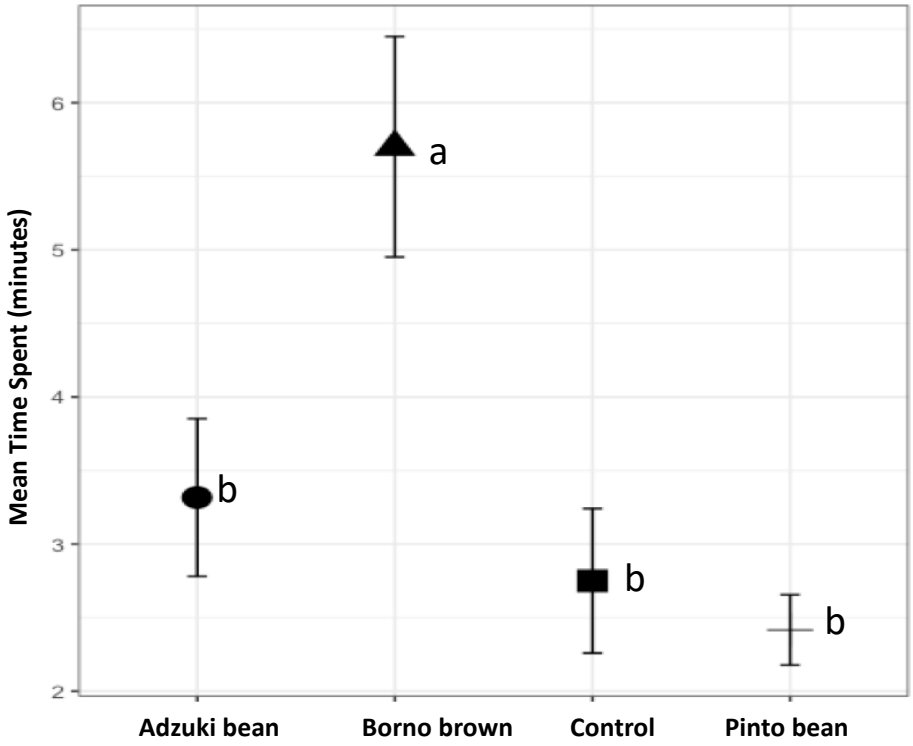

Figure 3. 2. Mean time spent by mated female *C. maculatus* in response to odours from a mixture of three bean types.

3.3.2. Female *C. maculatus* attraction to a host-bean odour.

Mated females spent significantly more time in the arm containing odour from adzuki bean seeds ($\chi^2 = 11.77$, df = 1, $P < 0.001$; Figure 3.3). A similar clear-cut behavioural response was detected on seeds of black-eyed bean ($\chi^2 = 10.98$, df = 1, $P < 0.001$; Figure 3.3) and Borno-brown ($\chi^2 = 5.28$, df = 1, $P = 0.022$; Figure 3.3) cultivars, respectively. However, when pinto beans and clean air were used as odour sources, there was no statistical difference ($\chi^2 = 0.65$, df = 1, $P <= 0.422$; Figure 3.3) in the time spent by the bruchids in both arms. Likewise, in the case of mung beans the weevils also spent equal time in both arms ($\chi^2 = 2.51$, df = 1, $P = 0.113$; Figure 3.3).

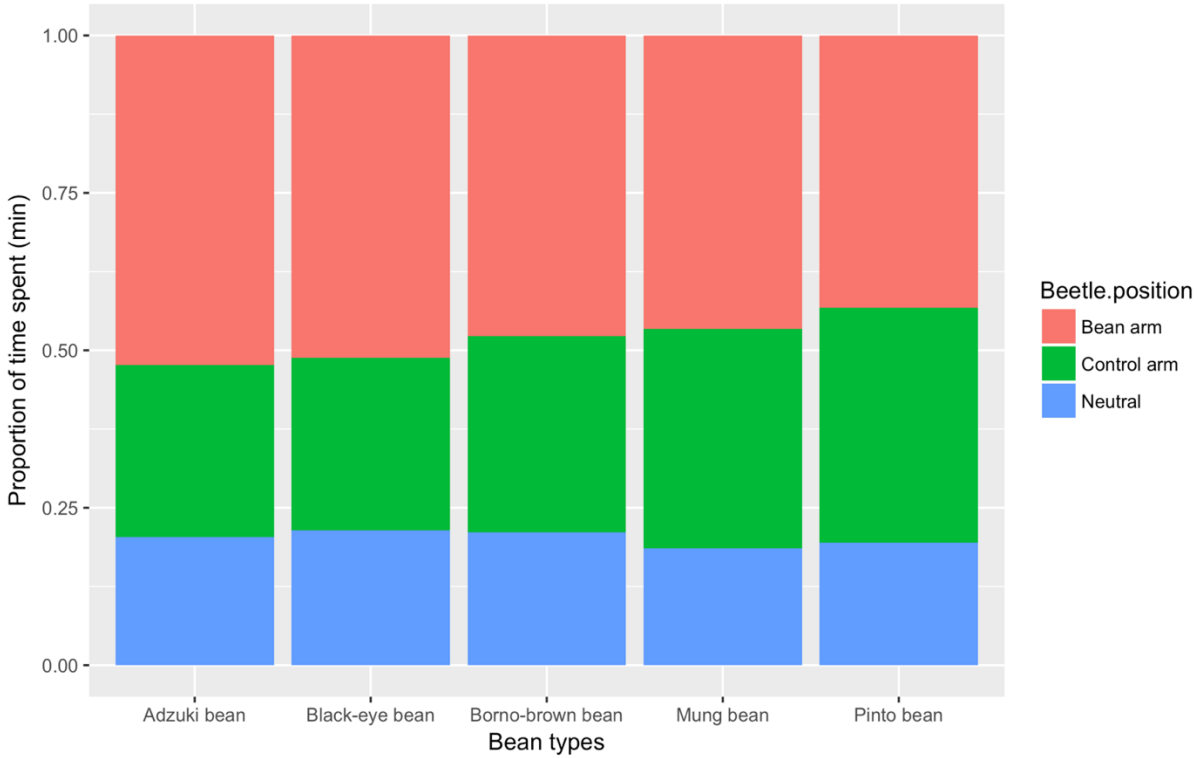

Figure 3. 3. Proportion of time spent by mated female *C. maculatus* in response to volatile odours from seeds of legume cultivars compared to control in a two-arm olfactometer.

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3.3.3 Beetle attraction to headspace volatiles.

Mated females spent significantly more time in the arm containing volatile samples from the 3 bean types (Borno-brown, black-eyed and Adzuki bean), respectively. However, a weak attraction was observed when volatiles from Adzuki bean (χ^2 = 4.219, df = 1, p = 0.039; Figure 3.4) and Borno-brown (χ^2 = 3.956, df = 1, p = 0.046; Figure 3.4) were used as odour sources compared to black-eyed bean (χ^2 = 5.581, df = 1, p = 0.018; Figure 3.4).

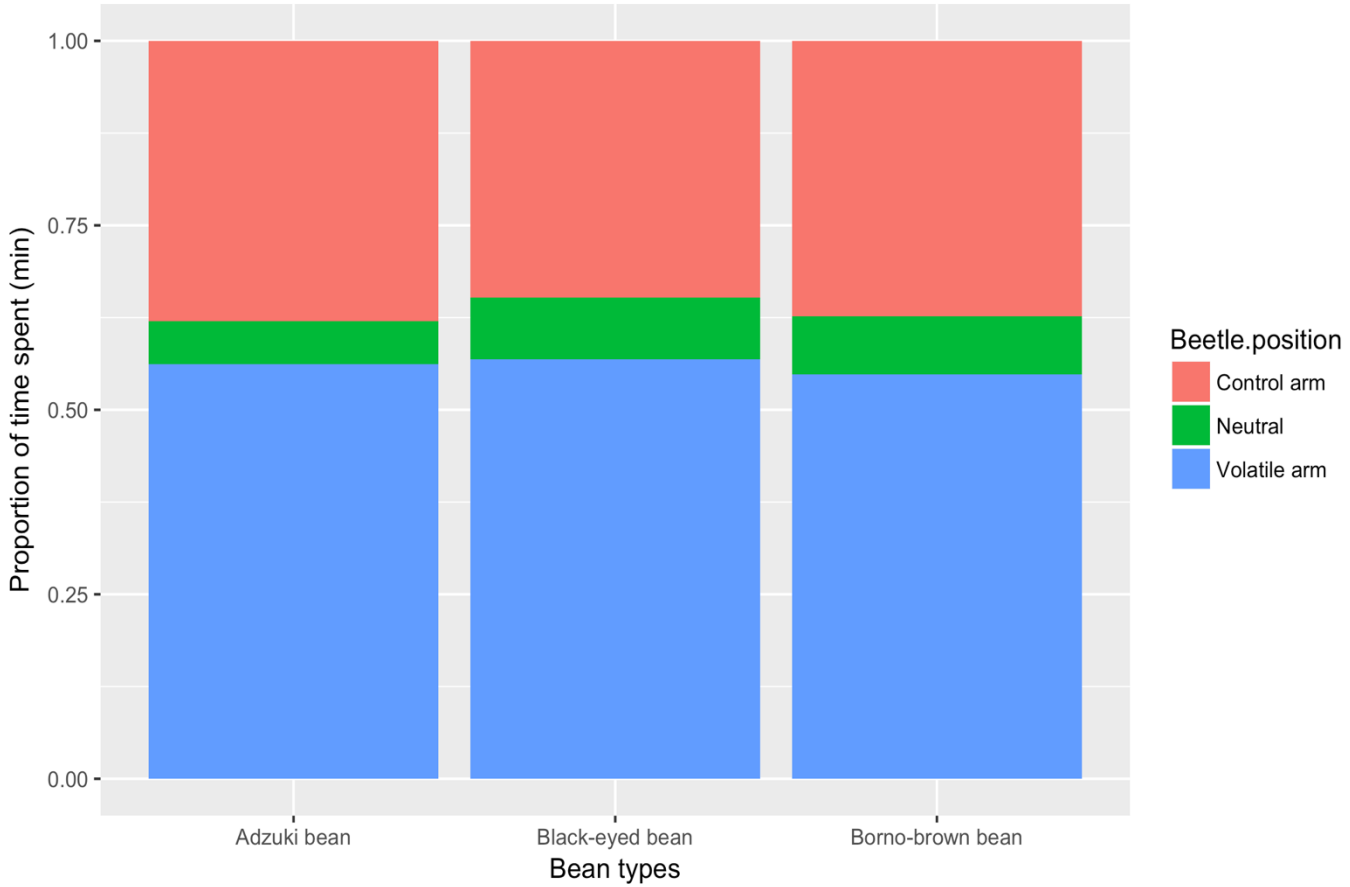


Figure 3. 4. Proportion of time spent by mated female *C. maculatus* in response to volatile samples from three bean types compared to a control.

3.3.4. Identification and chemical analyses of volatile compounds

A total of 18 compounds were identified from the three most attractive volatile bean samples. All eighteen compounds were detected in black-eye beans, while eighteen and seventeen compounds were identified in Borno-brown and adzuki beans, respectively (Table 3.2; Figure 3.7). O-xylene was not detected in Borno-brown while, naphthalene, 1,5-dimethyl and 2,4-dimethyl -1- heptane were not detected in adzuki beans. The PCA showed that components, 1 and 2 explained more than 97 % of the variance in the abundance of VOCs examined (Figure 3.6), and the cluster analysis grouped the compounds in three clusters. Limonene, representing cluster 1 has no similarity with any other compound (Figure 3.5). Compounds in the same cluster share a similar abundance profile.

The chemical analyses of the compounds indicated that they varied significantly ($F = 402.96$, $df = 17, 53$, $P < 0.01$; Table 3.4) within and among the bean types tested. A post-hoc test further revealed how they varied (Table 3.4). Limonene was the dominant compound, followed by benzyl alcohol and nonanal in adzuki bean; whereas, in Borno-brown bean, Limonene was also dominant, followed by benzyl alcohol and 2,4-dimethyl-1-heptane. Nonanal was the dominant compound in the black-eye bean, followed by limonene and benzyl alcohol (Table 3.2). However, p-xylene, hexanal and benze,1,2,3,4-trimethyl were the least abundant compounds in adzuki bean. Both p-xylene and hexanal were the least abundant in Borno-brown bean; whereas, only benze,1,2,3,4-trimethyl was the least abundant in the black-eye bean.

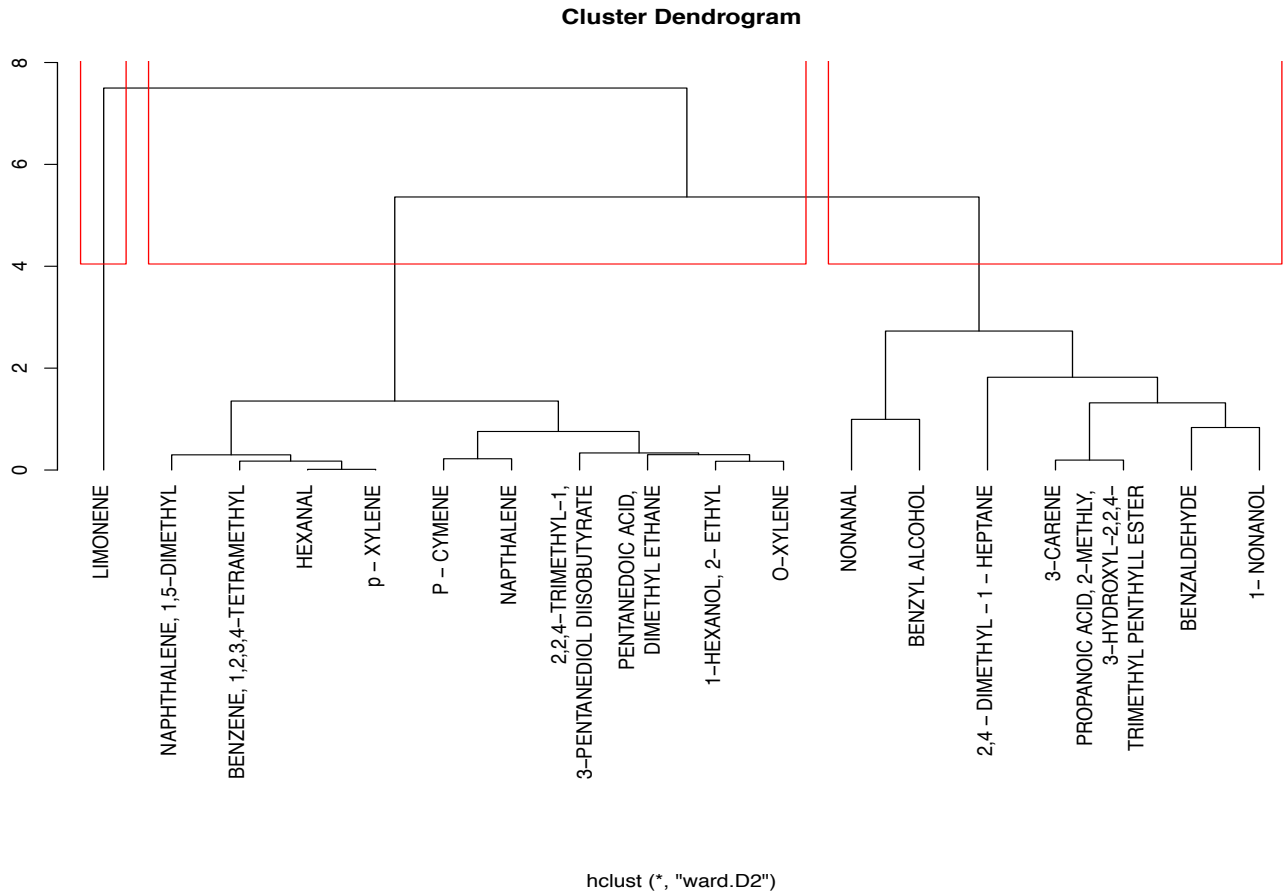


Figure 3. 5. Dendrogram showing relationship among 18 volatile compounds from three bean types based on their relative abundance. The red rectangular boxes represent each cluster.

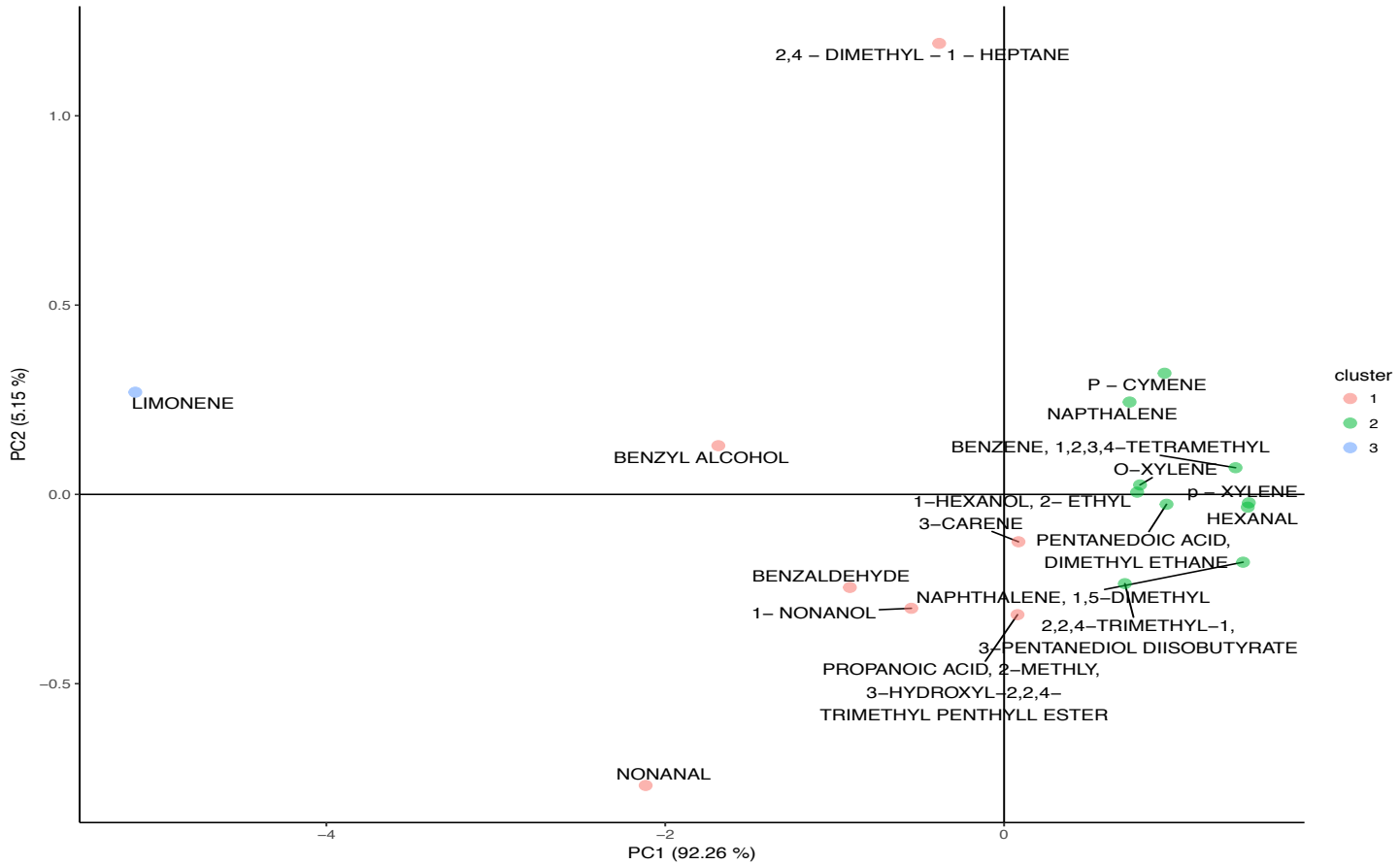


Figure 3. 6. Biplot showing the ordination of the clustered volatile compounds

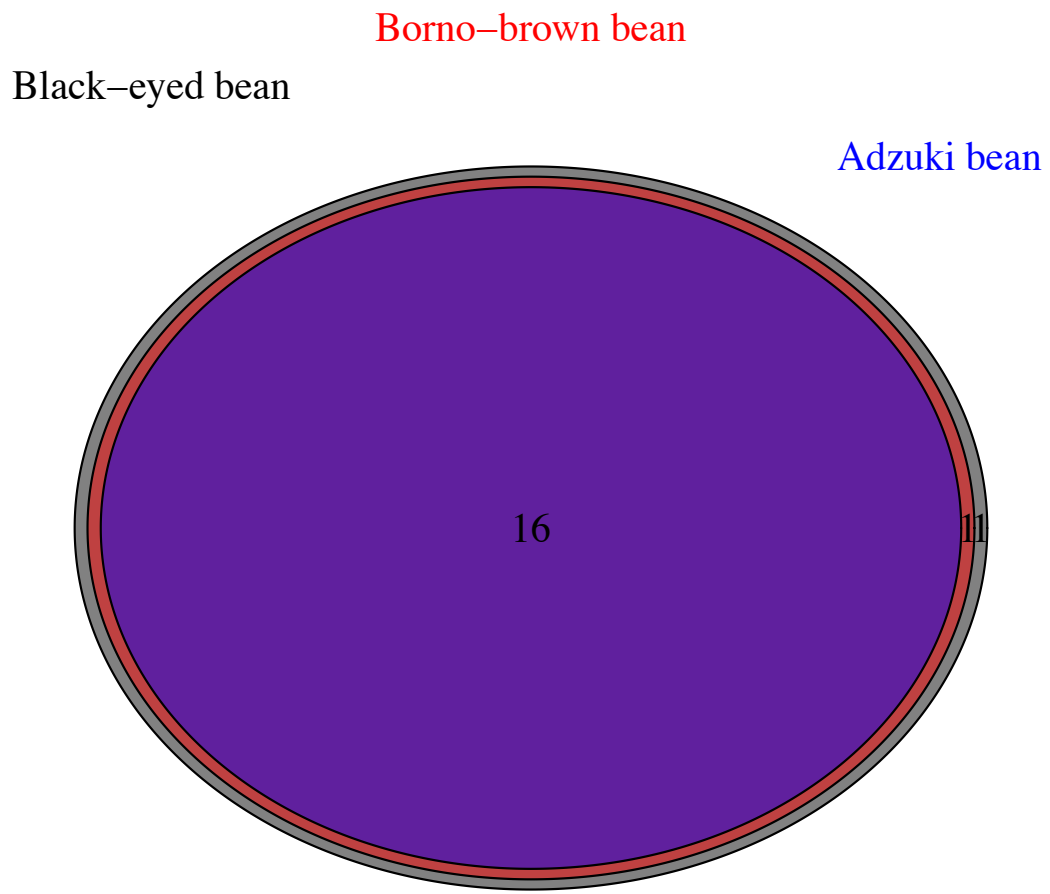


Figure 3. 7. A Venn diagram showing the number of unique and shared compounds from the bean types

Table 3.1. One – way ANOVA result on olfactory attraction of female *C. maculatus* to odour stimuli from different bean types.

	df	Sum Sq.	Mean Sq.	F value	Pr(>F)
Treatments	3	66.01	22.004	7.684	0.00043 ***
Residuals	36	103.10	2.864		

Table 3.2. GC-MS analysis of volatile organic compounds emitted by 2µl of air entrainment sample of Adzuki bean, Borno-brown bean Black-eyed bean (mean ± SD).

Compounds	Adzuki bean	Borno-brown bean	Black-eyed bean	RI
LIMONENE	18.589±0.365 a	17.598±0.067 a	17.840±0.374 ab	1030
BENZYL ALCOHOL	17.722±0.089 b	17.003±0.124 b	17.156±0.257 abc	1036
NONANAL	17.670±0.287 b	16.920±0.060 b	18.387±1.472 a	1104
BENZALDEHYDE	17.288±0.020 bc	16.656±0.167 b	17.081±0.218 abc	962
3-CARENE	16.681±0.247 cd	16.183±0.079 c	16.534±0.193 bcde	1011
PROPANOIC ACID, 2-METHLY, -3-HYDROXYL-2,2,4-TRIMETHYL PENTHYLL ESTER	16.526±0.294 cde	16.085±0.247 c	16.710±0.005 bcd	1380
1-HEXANOL, 2- ETHYL	16.075±0.423 def	15.536±0.230 d	16.469±1.446 bcde	1030
PENTANEDOIC ACID, DIMETHYL ETHANE	16.054±0.001 def	15.069±0.170 e	15.459±0.179 defg	1135
1- NONANOL	15.908±0.013 def	16.798±0.242 b	17.043±0.026 abc	1173
2,2,4-TRIMETHYL-1,3-PENTANEDIOL DIISOBUTYRATE	15.861±0.503 ef	15.419±0.256 de	16.184±0.011 cdef	1580
O-XYLENE	15.635±0.021 f	nd	15.689±0.002 cdefg	887
NAPTHALENE	15.563±0.156 f	16.088±0.048 c	15.292±0.027 defg	1182
P - CYMENE	14.601±0.426 g	16.005±0.032 c	14.133±0.299 ghi	1116
BENZENE, 1,2,3,4-TETRAMETHYL	13.455±0.213 h	14.583±0.010 f	12.275±0.129 j	1146
HEXANAL	13.055±0.163 h	13.009±0.162 h	13.589±0.065 hij	800
p - XYLENE	12.895±0.274 h	13.105±0.040 h	13.257±0.255 ij	836
NAPHTHALENE, 1,5-DIMETHYL	nd	14.015±0.107 g	15.030±0.029 efgh	1440
2,4 - DIMETHYL - 1 – HEPTANE	nd	16.950±0.033 b	14.655±0.042 fghi	836

Within the row means with the same letter are not significantly different.

nd: Not detected.

Table 3.3. PCA of the data set on 18 compounds from the 3 bean types showing the scores in the various components

Compounds	PC1	PC2	PC3
BENZALDEHYDE	-0.91023487	-0.245729933	-0.09012856
HEXANAL	1.4380889	-0.033756866	0.25838785
LIMONENE	-5.12804093	0.269890134	0.42957995
3-CARENE	0.08491902	-0.125125986	0.00786
NONANAL	-2.11561307	-0.768813543	-0.0932228
BENZYL ALCOHOL	-1.68662429	0.128700595	-0.07776726
P - CYMENE	0.94728709	0.32004242	-0.18825171
PENTANEDOIC ACID, DIMETHYL ETHANE	0.95888198	-0.026094214	0.2723832
1- NONANOL	-0.54750095	-0.300878349	-0.83991764
1-HEXANOL, 2- ETHYL	0.78541531	0.005844946	0.15925735
NAPTHALENE	0.74072472	0.243973662	-0.16618814
PROPANOIC ACID, 2-METHLY, 3-HYDROXYL-2,2,4-TRIMETHYL PENTHYLL ESTER	0.07930788	-0.317708188	-0.02251188
2,2,4-TRIMETHYL-1,3-PENTANEDIOL DIISOBUTYRATE	0.71286359	-0.235750284	0.125157
O-XYLENE	0.80240137	0.024992176	-0.01063462
2,4 - DIMETHYL - 1 - HEPTANE	-0.38320753	1.191254858	-0.25787768
p - XYLENE	1.44449384	-0.022191908	0.25583615
BENZENE, 1,2,3,4-TETRAMETHYL	1.36629589	0.070432093	0.16968204
NAPHTHALENE, 1,5-DIMETHYL	1.41054205	-0.179081613	0.06835674

Table 3.4. Permanova table

	Df	Sums of Squares	F. Model	Pr(>F)
Compounds	17	4.9813	52.75	0.001 ***
Residual	36	0.2000		

3.4 Discussion

This study identified 18 volatile organic compounds in the three bean types (Borno-brown, black-eyed bean and adzuki bean) examined. Sixteen compounds were shared amongst the 3 cultivars, the black-eyed bean had one (O-xylene) and two (naphthalene, 1,5-dimethyl and 2,4-dimethyl -1- heptane) more compounds compared to Borno-brown and adzuki bean, respectively. Limonene was the major compound, followed by benzyl alcohol and nonanal in adzuki bean; whereas, in Borno-brown, Limonene was also dominant, followed by benzyl alcohol and 2,4-dimethyl-1-heptane. Nonanal was the major compound in black-eye bean, followed by limonene and benzyl alcohol.

The results of the four-choice test indicated that mated females of *C. maculatus* preferred Borno-brown beans to clean air, pinto beans and adzuki beans. The findings suggest that *C. maculatus* females prefer a familiar host when exposed to hosts from different legume cultivars. According to Ignacimuthu *et al*., (2000), the strong response of *C. chinensis* to uninfested cowpea seeds indicates the presence of cowpea derived volatile attractant. My finding is in agreement with the work of Arnold, *et al*., (2012) which showed that *C. maculatus* was strongly attracted to cowpea odour. When the beetle was presented with two choices (clean air vs a bean type), it showed a strong preference for adzuki beans, black-eyed beans and Borno-brown beans, respectively. Surprisingly, the beetle showed no preference for mung beans (an ancestral host) or pinto beans (an unsuitable host) over clean air! This suggests the beetle cannot detect (or respond to) cues from both bean types at a distance. The results of *C. maculatus* attraction to headspace volatile samples showed that they were attracted to the three bean types (Borno-brown bean, black-eyed beans and adzuki beans) tested. These findings confirm the beetle's preference for an alternative host when a familiar or most

preferred host is not presented. It further indicates that the behavioural attraction of the beetle to the samples was induced by chemical stimuli as visual cues were excluded in the study. The roles of visual, taste and olfactory cues in host location and discrimination by other insects have also been reported (Chapman, 2012).

The eighteen candidate volatile compounds associated with the headspace samples from the preferred bean types have been identified in this study, most of the compounds have been reported to elicit attraction in red palm weevil (Gunawardena & Herath, 1995), legume pod borer (Bendera *et al*., 2015; Zhou *et al*., 2015) and cucujid beetles (Mushobozy, *et al*., 1993). For example, Ajayi *et al*., (2015) identified thirty-one volatile compounds from seeds of 3 legume cultivars; whereas, Adhikary *et al*., (2015) reported the presence of 23 compounds from the seeds of four varieties of *Lathyrus sativus*. Although the number and composition of compounds present in each bean type examined varied slightly (which could be due to the differences in the sequence of genes in the bean cultivars (Köllner *et al.*, 2004). Limonene, benzyl alcohol and nonanal dominated the abundance profile of the volatile compounds, and the importance of these compounds in managing agricultural pests has been documented. For example, limonene and benzaldehyde were among the volatile compounds of cowpea that influenced the behaviour of *Maruca vitrata* (Zhou *et al.*, 2015) and the granary pest, *A. obtectus* (Khelfane-Goucem *et al.*, 2014). A synthetic blend of nonanal, Linalool, 1-octanol, 3-octanol and 3-octanone elicited behavioural attraction of *C. maculatus* (Adhikari *et al*., 2015). Benzyl alcohol has been reported to induce the attraction of natural enemies during insect pest infestation (De Moraes *et al*., 1998; Tabata *et al.*, 2011), thus acting as a defensive compound. Also, hexanal has been found to be associated with the VOCs of *Pisum sativum* L. (Ceballos *et al.*, 2015).

In summary, this study has demonstrated (a) that the behaviour of female *C. maculatus* is influenced by odour stimuli associated with Borno-brown beans, black-eyed beans and adzuki beans, (b) limonene, benzyl alcohol and nonanal are potential compounds that could be inducing the beetles' behavioural attraction to the bean types and (c) that volatile compound composition and abundance profiles vary within compounds and among bean types.

CHAPTER FOUR: ATTRACTION of *C. maculatus* TO HEADSPACE VOLATILE SAMPLES FROM DIFFERENT DEVELOPMENTAL STAGES OF COWPEA PODS.

4.1. Introduction

Cowpea, *Vigan unguiculata* is an important source of food protein, and a source of income to cowpea growers and exporting nations. But its production is facing serious pest infestations, especially, from the cowpea bruchid, *C. maculatus*. The gravid females of this storage beetle lay eggs on pods of cowpea plant in the field, and the harvested pods are thrashed and kept in storage where adults emerge and re-infests the beans. Emerging adults leave a hole on the bean's surface and have mined out the internal tissue of the bean, thus damaging its economic value. As a result, farmers use cheap and readily available insecticides to control this pest. However, legislation banning the use of pesticides requires the need for new, safer pest control methods.

Plants release volatile substances that include; sulphuric compounds, terpenoids, fatty acid derivatives and nitrogen-containing compounds (Pare & Tumlinson, 1999). These are mainly lipophilic products with molecular masses less than 300. Most of the substances are emitted from the plant's vegetative parts, whereas a few are emitted from the roots (Steeghs *et al.*, 2004). The most extensively studied vegetative volatile is Isoprene (Sharkey & Yeh, 2001), a thermoregulator which protects plants against heat-stress (Sharkey *et al*., 2008). Other substances are released to protect plants against natural enemies; for example, Takabayashi & Dicke, (1996) observed that spider mites' infestations on lima beans leaves and apple plants attracted predatory mites. The anti-microbial and anti-herbivore functions associated with

flower volatiles protects the floral organs against its natural enemies (Hammer *et al*., 2003), while some attract pollinators (Reinhard *et al*., 2004).

The role of plants' VOCs as a host identification cue by insect pests has gained recent attention, especially in pest management studies. They induce behavioural interactions between organisms, and assist insect pests in locating a suitable host (either for food or oviposition purposes), and in avoiding unsuitable ones (Bruce *et al*., 2011). The quantity and number of the volatile substances emitted by a host influenced by several factors: plants release a smaller amount of VOC under low light conditions. Also, lima bean plants release more VOC under water-stressed conditions (Takabayashi *et al*., 1994). According to Van Wassenhove *et al.*, (1990), the addition of high organic nitrogen fertilizers and minerals reduced the number of volatile substances emitted by celery.

Despite the fact that *C. maculatus* is commonly associated with, and controlled during its infestation of, stored beans field trials have shown that the cowpea, *V. unguiculata,* is susceptible to *C. maculatus* infestations during its pod formation stages (Taylor & Agbaje, 1974) and that adult females also lay eggs on the host pods in the field. These egg-laden pods are harvested, kept in storage where cross infestations and re-infestations continue. The field infestation route is suggested to be triggered by the existence of two different morphs of the beetle, an active (flighted) and a normal morph (flightless) with different life-history strategies (Utida, 1954: Caswell, 1984). The flightless form attacks stored seeds, whereas the active morph (with flight propensity) causes field infestations (Messina & Renwick, 1985), thus an infestation circle is established.

Developing (Umar & Turaki, 2014; Zannou *et al*., 2003) and mature stages of cowpea plants (Caswell,1984) are also susceptible to infestations by the active form

of *C. maculatus* in the field. Ouedraogo & Huignard, (1981) reported that the eggs of *C. maculatus* were found on the seeds and walls of non-dehiscent cowpea pods; and Messina, (1984), states that *C. maculatus* females prefer fully developed pods to younger or mature pods, and were attracted to pods with exposed seeds compared to intact pods. According to Ajayi *et al.*, (2018), *C. maculatus* could infest cowpea plants at the green and ripened pods stages. Other work reported *C. maculatus's* strong preference for cowpea in the field (Utida, 1972; Sanofujii, 1984).

These preferences have been suggested to be triggered by the volatile compounds emitted by the host at that developmental stage. For example, a senesced banana leaf was found to contain 2R, 5S-theaspirane as an active component unlike other developmental stages (Abagale *et al.*, 2019) of banana leaf. Similarly, studies on soybean seeds show variance in VOCs emitted at different developmental stages (Boué *et al.*, 2003). A study by Ajayi, *et al*., (2018) identified benzaldehyde and octanone as key volatile compounds associated with pods of cowpea plants at different growth stages, thus, suggesting there may be important chemical cues correlated with the host's life-history stage.

There is therefore considerable evidence that the beetle interacts with the host plant long before the seeds are stored by farmers (Ouedraogo & Huignard, 1981; Taylor & Agbaje, 1974). This is important for several reasons. Firstly, the focus on chemical control is on storage - where the population has the highest growth potential. Second, any move towards non-chemical control is more likely to succeed if it focuses on a life-history bottleneck that is spatially and temporally restricted to facilitates exploitation by the control method (e.g. attracting gravid females to lures). Analysing the chemical components of cowpea pods at various developmental stages and the examination of the pest's behavioural attraction to such chemicals is important to

predict how the life cycle of the plant influences its vulnerability to infestations by the pest. With this in mind, this chapter examines the response of cowpea beetle to volatiles from the pods of two cowpea varieties at different growth stages, and predicts that odour cues from pods' categories would induce attraction of *C. maculatus.* As a field-to-store pest, this measure is to identify the most vulnerable pod's growth stage to infestation together with the volatile compounds moulding such action.

Given the context above, this chapter is based on the hypothesis that preference for the cowpea plant is mediated by volatile cues from the host pods.

4.1.1. Chapter objectives

This chapter aims to;

- Examine the responses of mated female *C. maculatus* to volatile samples from pods of different age from two cowpea cultivars.
- Identify candidate volatiles compounds associated with the pods.
- Examine the abundance profile of the compounds on each pod category and cowpea cultivars examined.

4.2 Materials and methods

4.2.1. Insect

A wild strain of *C. maculatus* was used in this study. The strain was collected from infested Borno-brown beans in a farmer's field in Taraba State, Nigeria, and cultured in breeding containers (17 x 11.5 cm) containing 200 g of uninfested whole Borno – brown bean. Lids of the containers were perforated to allow for ventilation. The cultures were kept in a laboratory at a temperature of 28 $\pm$ 2 ^{0}C and relative humidity of 30 $\pm$ 5 %.

4.2.2. Growing of cowpea

This was carried out in a greenhouse at AWEC, University of Sheffield, United Kingdom. The greenhouse day and night temperatures were maintained at 27 $\pm$ 1^{0}C, and 22 $\pm$ 1 ^{0}C, respectively. 30 – 60 % RH was used throughout the study. The photoperiod was set at 9 hr light and 15 hr dark. A total of nine pots (30 cm diameter) each, were used for the study. Three clean seeds of Borno brown and California black-eyed cultivars were sown/pot which was later pruned down to a plant stand/pot after one week of germination. The plants were tagged at the onset of flowering (anthesis) to accurately estimate the pods' age (Figure 4.1).

The age classes were based on the number of days after onset of flowering as categorized below;

- 15 – 17 days after onset of flowering (Developing pods)
- 18 – 20 days after onset of flowering (Fully developed pods)
- > 20 days after onset of flowering (mature pods).

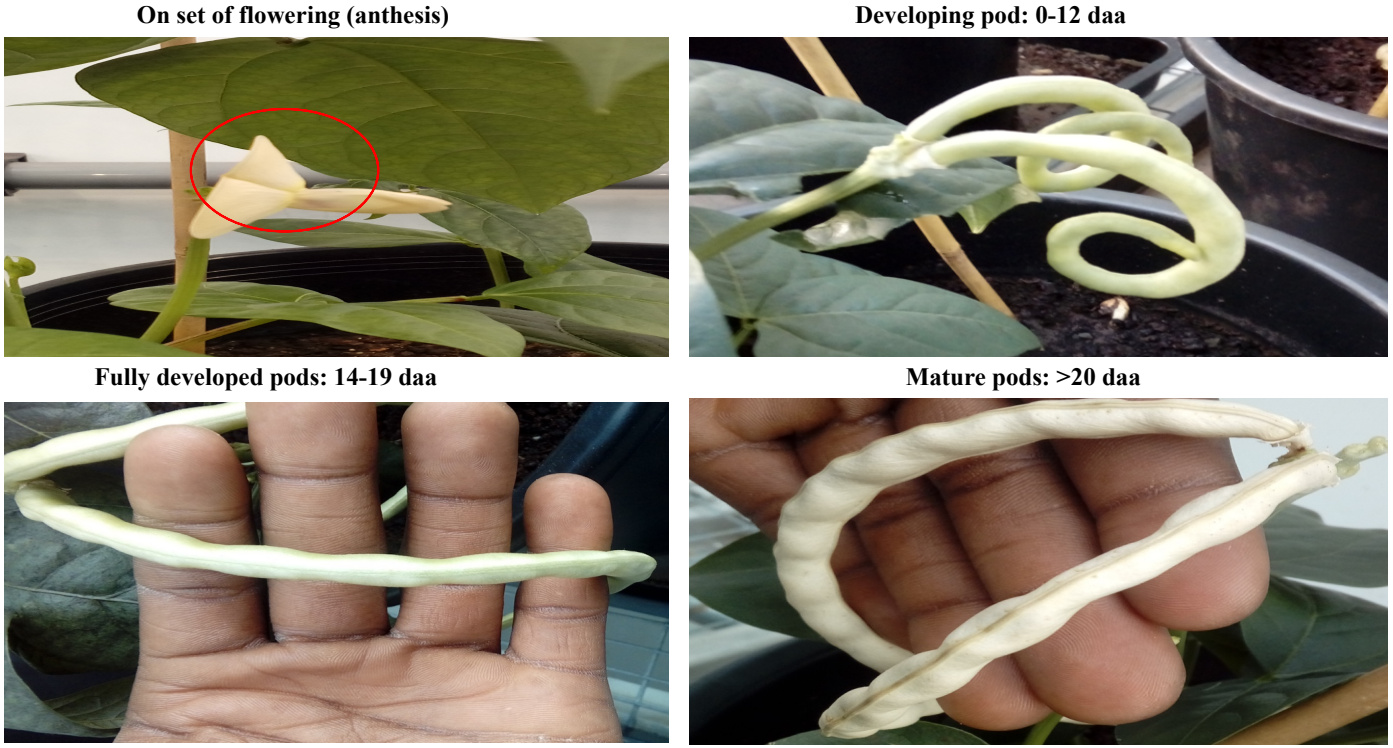


Figure 4. 1. Developmental stages of cowpea pods from anthesis to maturity.

4.2.3. Collection of headspace samples from plant pods

Headspace VOCs of the pods were trapped with air entrapment equipment from the cowpea plants at the defined developmental stages. All equipment was washed with detergent, rinsed with hexane and distilled water, and then dried in an oven at 120 ^{0}C for 15 hr. Transparent oven bags used for the study were also pre-conditioned by heating them in an oven at 120 ^{0}C for 15 hr. Each cowpea plant at various growth stage was enclosed with an oven bag, and charcoal-filtered air passed through a Porapak Q absorbent (Alltech Associates, Lancashire, UK) at a constant rate of 300 ml/ min (Figure 4.2). All the connections were made with PTFE tubing and tape (Supelco, Bellefonte, PA). VOCs absorbed on Porapak Q were eluted with 1 ml of hexane. Extracted samples were further concentrated to 100 µl by a low stream of nitrogen, and stored in glass vials in a freezer at -80 ^{0}C.

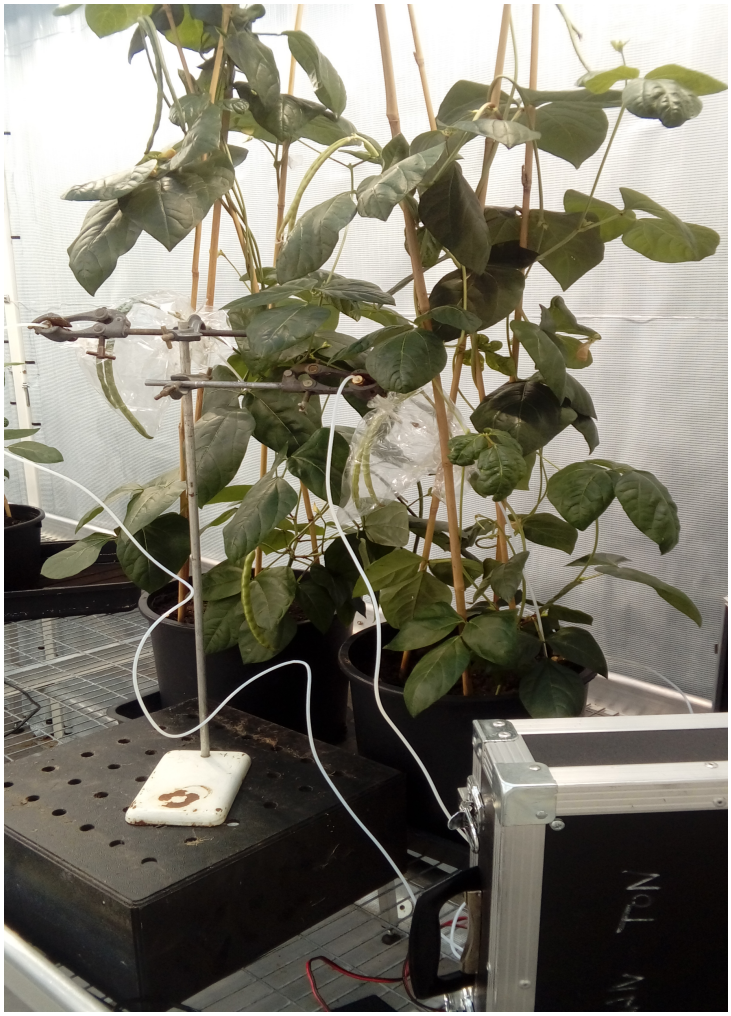


Figure 4. 2. Collection of volatile organic compounds from cowpea pods.

4.2.4. Beetles' response to headspace volatile samples.

A two-arm olfactometer was used to examine the attractiveness of *C. maculatus* to volatile samples collected from the plant pods. The olfactometer consists of three layers representing; the base (floor), the observation layer and the cover clipped together to form an eight-sided shape with a two-arm exposure chamber. Each layer is made of a transparent Perpex with 6 mm thickness. The first layer (floor) was lined with a Whatman filter paper base (110 mm) to provide traction for the weevil. Another layer, the observation arena had a hole (3 mm diameter) drilled from both edges into the two arms to accommodate the odour chambers. Then, a third layer (cover), all of the same size and shape had a hole (4 mm diameter) drilled at the centre. Two 60 ml BD plastiak's (syringes) served as the odour chambers. A Teflon tube (1.5mm ID x 3.2mm OD) was used to connect each of the chambers to both arms of the olfactometer, and the connections were tightened with a PTFE tape. A 60 W light bulb was positioned 1m above the olfactometer to provide uniform illumination.

Twenty microliters (20 µl) of volatiles samples from pods of the plants at the three different growth stages (Developing, fully developed and mature pods) were applied on a piece of filter paper, respectively, and 1 min was allowed for solvent evaporation. The treated filter paper was then put into one of the odour chambers, while the second chamber was used as a control which contained a piece of filter paper treated with 20 µl of hexane. A mated normal female of the beetle was then introduced into the centre of the olfactometer (observation arena). Air was drawn through both arms using a vacuum, and regulated with a flow meter at a rate of 100 ml/min. After introduction, each beetle was given 3 min to settle in the observation arena, and the movement towards both arms was observed for 15 min. Beetles that do not make any choice after

5 min of introduction were regarded as “non-responders” and discarded. All materials used were washed, rinsed with distilled water, and then cleaned with 70 % ethanol.

4.2.5. Coupled gas chromatography – mass spectrometry (GC – MS)

The candidate compounds associated with the volatile samples from the plants’ pods were identified using GC-MS. A 2 μL of the air headspace sample was injected onto a capillary GC column (30 m x 0.25 mm ID, 0.25 μm film thickness), which is directly coupled to a mass spectrometer (PerkinElmer, Clarus® SQ 8T). The carrier gas was Helium with a flow rate of 1.02 ml min^{-1}. Ionization was achieved by electron impact at 70 eV, 230 ^{0}C. The injection port was maintained on a splitless mode. The GC initial oven temperature was maintained at 30 ^{0}C min^{-1}, then ramped at 5 ^{0}C min^{-1} to 240 ^{0}C, and held for 20 min. Mass spectrum acquisition was scanned using a *m/z* range from 35 to 450.

Candidate compounds were identified by comparing the chromatograph retention index and mass spectra with a library database spectra using the National Institute of Standards and Technology (NIST) mass spectra search programme (version 2.2, NIST 14, Gaithersburg, Maryland, USA). The retention index of each compound identified was calculated using a series of straight alkanes (C_8 – C_{20}). The abundance of each identified compound was calculated by integrating the peak areas of the total ion chromatograph and averaged.

4.2.6. Statistical analysis

The two-choice data on the beetles’ responses to VOCs from each pod category was analysed using Chi-square (χ^2) test. Staked bars were used to present the proportion of time spent by the beetles in each arm of the olfactometer. To determine the

similarities or differences among the compounds identified, the chemical analysis data on the abundance of volatile compounds from each pod category was subjected to permanova analysis. The similarities of the compounds were interpreted using cluster analysis (by Ward's method); whereas, principal component analysis (PCA) was used to indicate the ordination of the compounds and their relationships in the first two components. Finally, a Venn diagram was plotted to interpret the number of unique and shared compounds among pods' developmental stages. R statistical software (R Core Team, 2013) was used for all analyses.

4.3 Results

4.3.1 Beetle's response to headspace volatile compounds

Olfactometer bioassays with natural samples of pod volatiles from Borno-brown beans showed that samples from developing pods ($\chi^2 = 0.051$, df = 1, p = 0.820; Figure 4.3) and fully developed pods ($\chi^2 = 0.170$, df = 1, p = 0.679; Figure 4.3) did not elicit responses from mated females of *C. maculatus*. However, there was significant attraction ($\chi^2 = 10.397$, df = 1, p = 0.001; Figure 4.3) to volatiles from mature pods. When the odour sources from pods of black-eyed beans was tested, the results showed that beetles spent significantly more time on arms with fully developed ($\chi^2 = 7.255$, df = 1, p = 0.007; Figure 4.3) and mature pods ($\chi^2 = 5.215$, df = 1, p = 0.022; Figure 4.3) samples. But, when given a choice between volatiles from developing pods and the control, the female did not differentiate between the two treatments ($\chi^2 = 1.849$, df = 1, p = 0.173; Figure 4.3).

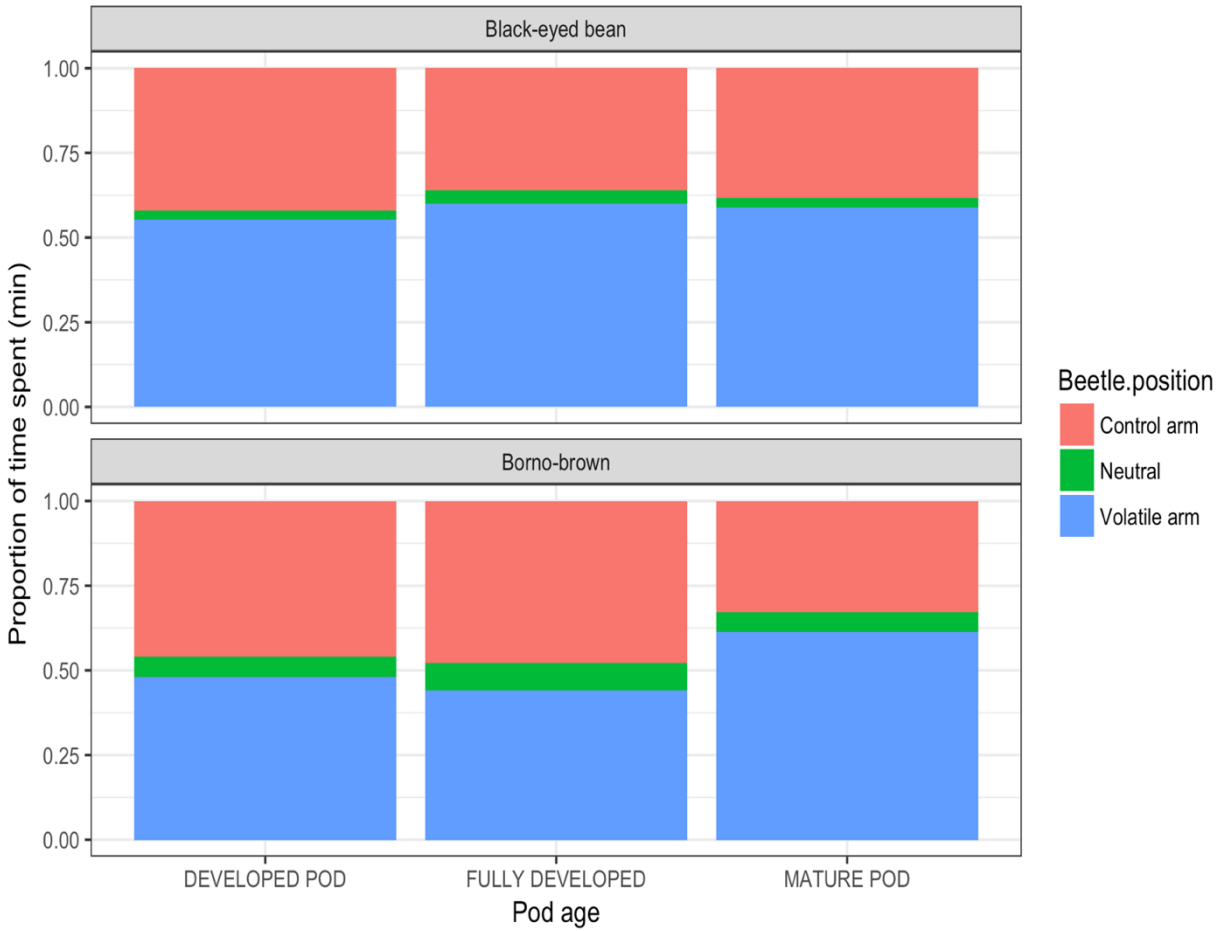


Figure 4. 3. Proportion of time spent by mated female *C. maculatus* in response to volatile stimuli from cowpea pods in a two-arm olfactometer.

4.3.2. Identification and chemical analyses of volatile compounds from pods of black-eye cowpea.

The analysis of the volatiles from the cowpea cultivar revealed a total of 12 compounds, but only eight were detected on the developing and fully developed pods of black-eyed cultivar, whereas, 11 compounds were detected on the mature pods (Table 4.1; Figure 4.6): Each of Hexanal and 3-Hexen-1-ol-acetate was only present on the mature and developing pods of black-eye cowpea, respectively (Table 4.1).

The PCA showed that components, 1 and 2 explained more than 99 % of the variances in the abundance of VOCs examined on pods of black-eye cowpea (Figure 4.5), and the cluster analysis classified the compounds in three cluster; Benzaldehyde and ethanol, 2- (2-butoxyehoxy)-acetate representing cluster 2 have similar abundance profile (Figure 4.4). The PCA biplot and dendrogram fully describe how the other compounds are related.

The chemical analyses of the compounds indicated that they varied significantly on pods of black-eye cowpea ($F = 2351.6$, $df = 11, 35$, $P < 0.01$; Table 4.3). Benzaldehyde, was significantly more abundant, followed by p-xylene and m-xylene on developing pods of black-eye cowpea; whereas, in the fully developed pods, benzaldehyde was more abundant, followed by m-xylene and p-xylene, although, they do not differ significantly (Table 4.1). Similarly, ethanol, 2- (2-butoxyehoxy)-acetate, followed by m-xylene and benzaldehyde were more abundant on mature pods of black-eye cowpea. However, 1-octane-3-ol was the least abundant compounds on developing pods of black-eye cowpea; whereas, Nonanal was the least abundant on the fully developed and mature pods of black-eye cowpea, respectively (Table 4.1).

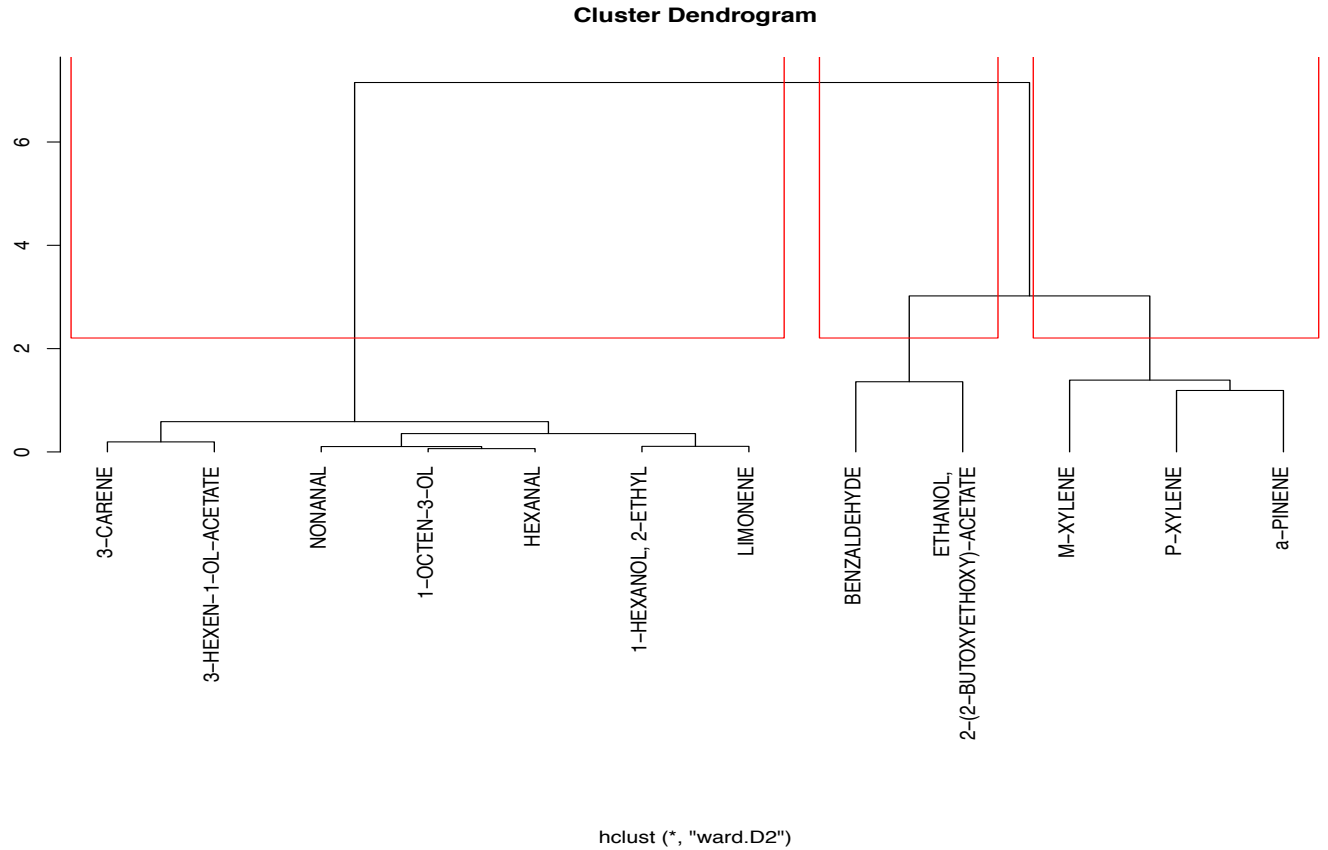


Figure 4. 4. Dendrogram showing relationship among 12 volatile compounds from pods of black-eye cowpea based on their relative abundance. The red rectangular boxes represent each cluster.

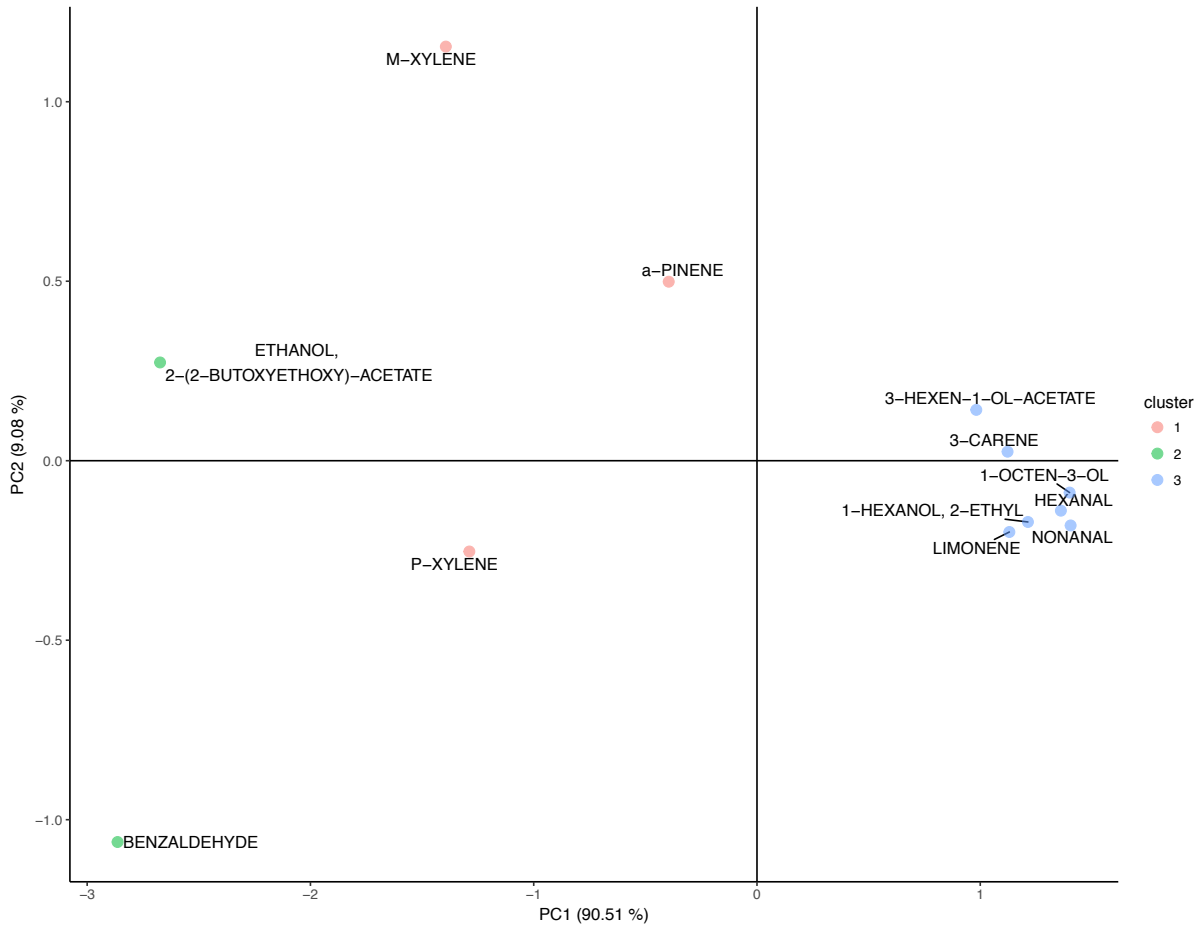


Figure 4. 5. Biplot showing the ordination of the clustered volatile compounds

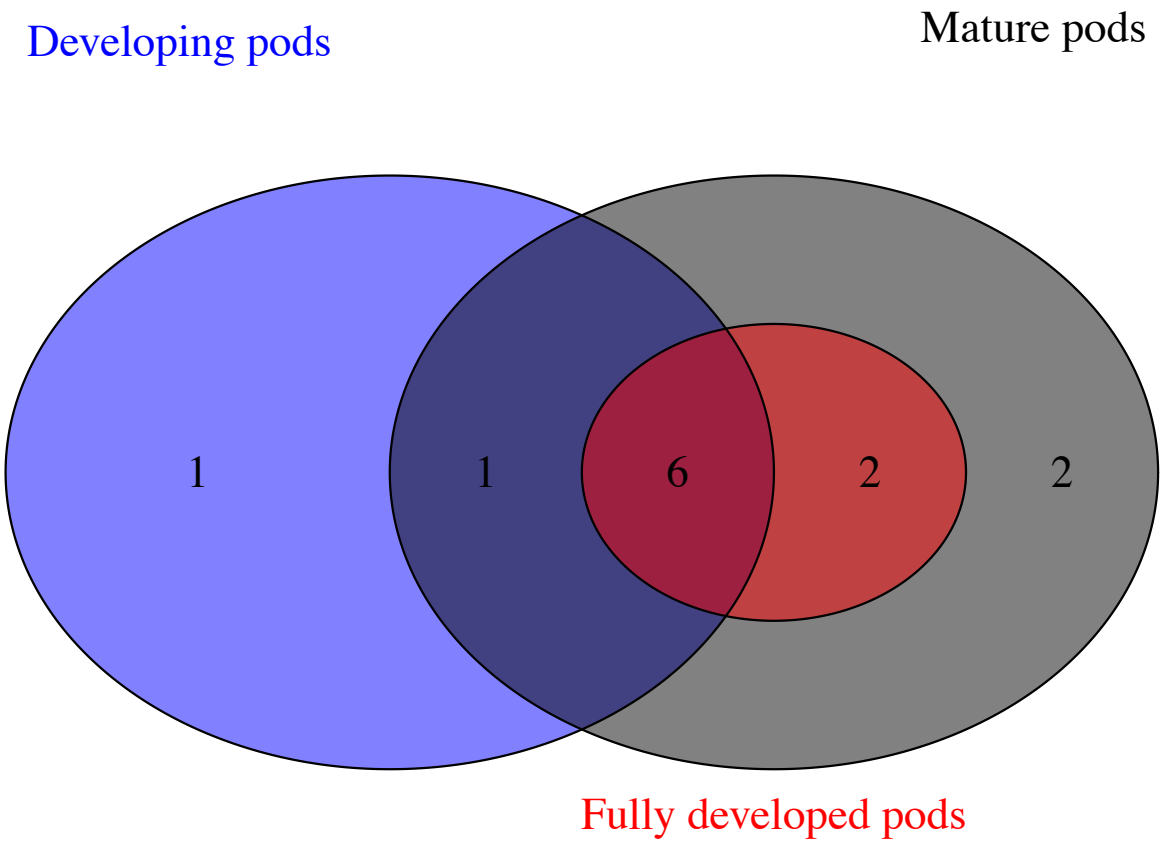


Figure 4. 6. A Venn diagram showing the number of unique and shared compounds from pods of black-eye cowpea.

Table 4.1. GC-MS analysis of volatile organic compounds emitted by 2µl of air entrainment sample of black-eye cowpea pods (mean ± SD).

	Developing pods		Fully developed pods		Mature pods		RI
BENZALDEHYDE	16.790±0.241	a	17.839±0.329	a	18.818±0.071	a	962
P-XYLENE	16.195±0.228	b	17.267±0.191	a	18.684±0.300	a	865
M-XYLENE	15.072±0.103	c	17.633±0.095	a	19.029±0.242	a	866
LIMONENE	14.203±0.249	d	15.592±0.011	b	15.582±0.682	cd	1030
NONANAL	13.375±0.065	e	11.241±0.464	c	13.376±0.391	f	1104
3-HEXEN-1-OL-ACETATE	12.923±0.245	ef	nd		nd		1005
3-CARENE	12.875±0.296	f	15.721±0.106	b	16.669±0.379	b	1011
1-OCTEN-3-OL	11.527±0.029	g	nd		14.811±0.025	de	980
1-HEXANOL, 2-ETHYL	nd		14.996±0.704	b	15.794±0.079	c	1030
a-PINENE	nd		17.083±0.213	a	18.585±0.000	a	937
ETHANOL, 2-(2-BUTOXYETHOXY)-ACETATE	nd		nd		19.167±0.000	a	1366
HEXANAL	nd		nd		14.568±0.004	e	880

Within the row means with the same letter are not significantly different.

nd: Not detected.

Table 4.2. PCA of the data set on 12 compounds from pods of black-eye cowpea showing the scores in the various components

Compounds	PC1	PC2	PC3
P-XYLENE	-1.2882928	-0.2529189	-0.2950193
M-XYLENE	-1.3935467	1.15360301	0.10678603
a-PINENE	-0.3952549	0.49863123	-0.0658959
BENZALDEHYDE	-2.8638032	-1.0621921	0.14525092
3-CARENE	1.1222836	0.02514555	0.06934755
1-HEXANOL, 2-ETHYL	1.2134994	-0.1706581	0.01200817
ETHANOL, 2-(2-BUTOXYETHOXY)-ACETATE	-2.6734279	0.2736915	-0.0121025
LIMONENE	1.1303642	-0.198596	0.07494472
3-HEXEN-1-OL-ACETATE	0.9822323	0.14163713	0.0021194
1-OCTEN-3-OL	1.4002251	-0.0887865	0.00581118
NONANAL	1.4047202	-0.1803734	-0.0494081
HEXANAL	1.3610006	-0.1391834	0.00615786

Table 4.3. Permanova table

	Df	Sums of Sqs	F. Model	Pr(>F)
Compounds	11	4.4381	140.01	0.001 ***
Residuals	24	0.0692		

4.3.3. Identification and chemical analyses of volatile compounds from pods of Borno-brown cultivar.

The analysis of the volatiles from the cowpea cultivars revealed that a total of 9 were emitted from the pods of Borno brown cultivar; all 9 compounds were detected on the developing pods, but longifolene was not present on the fully developed and mature pods (Table 4.4; Figure 4.9).

The PCA showed that components, 1 and 2 explained more than 98 % of the variances in the abundance of VOCs examined on pods of black-eye cowpea (Figure 4.8) and all the 9 compounds showed loadings in the first two components. The cluster analysis classified the compounds in three cluster. 1h-indene, 1-ethyl lindene, representing cluster 2 has no similarity with any other compound, and other compounds in the same cluster have similar abundance profile (Figure 4.7). The PCA biplot and dendrogram fully describe how these compounds are related.

The chemical analyses of the compounds indicated that they varied significantly on pods of Borno-brown cowpea ($F = 100.59$, $df = 8, 26$, $P < 0.01$; Table 4.6). Benzaldehyde, was significantly more abundant, followed by 1h-indene, 1-ethyl lindene and p-xylene on developing pods of Borno-brown cowpea; whereas, in the fully developed pods, benzaldehyde was significantly dominant, followed by 2,2,4-trimethyl-isobutyrate and p-xylene (Table 4.4). On the mature pods, 1h-indene, 1-ethyl lindene was more abundant, followed by 3-pentanediol, diisobutyrate and benzaldehyde. However, (E)-4,8-dimethyl nona-1,3,7-triene was the least abundant compounds on developing and fully developed pods of Borno-brown cowpea; whereas, hexanal was the least abundant on the mature pods (Table 4.4).

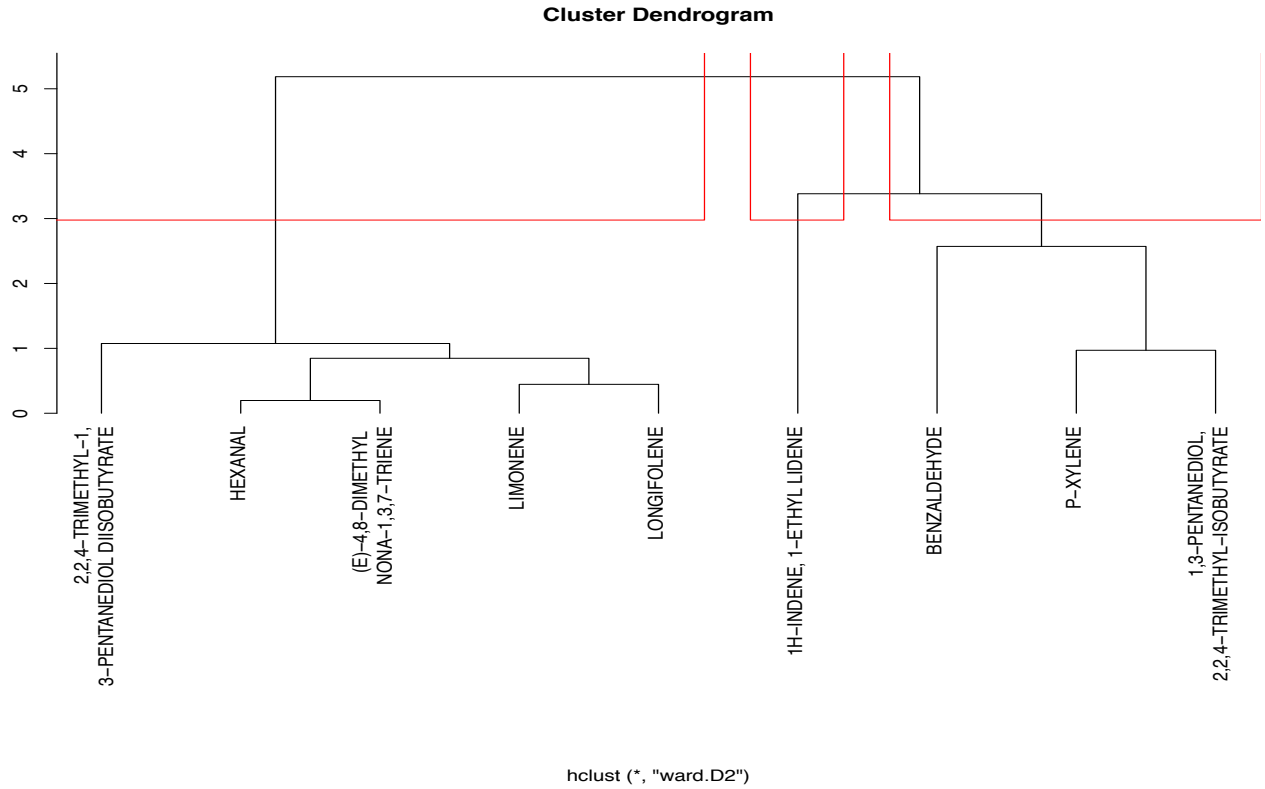


Figure 4. 7. Dendrogram showing relationship among 9 volatile compounds from pods of Borno-brown cowpea based on their relative abundance. The red rectangular boxes represent each cluster.

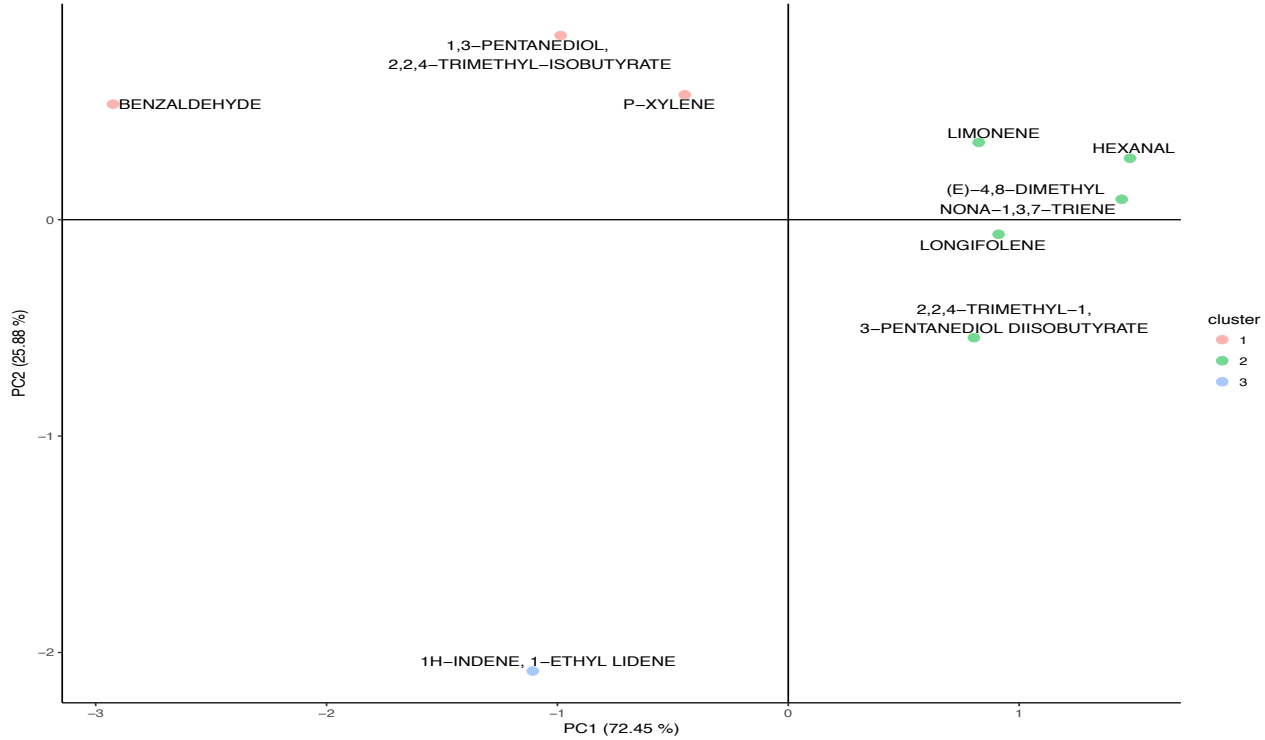


Figure 4. 8. Biplot showing the ordination of the volatile compounds from pods of Borno-brown cultivar.

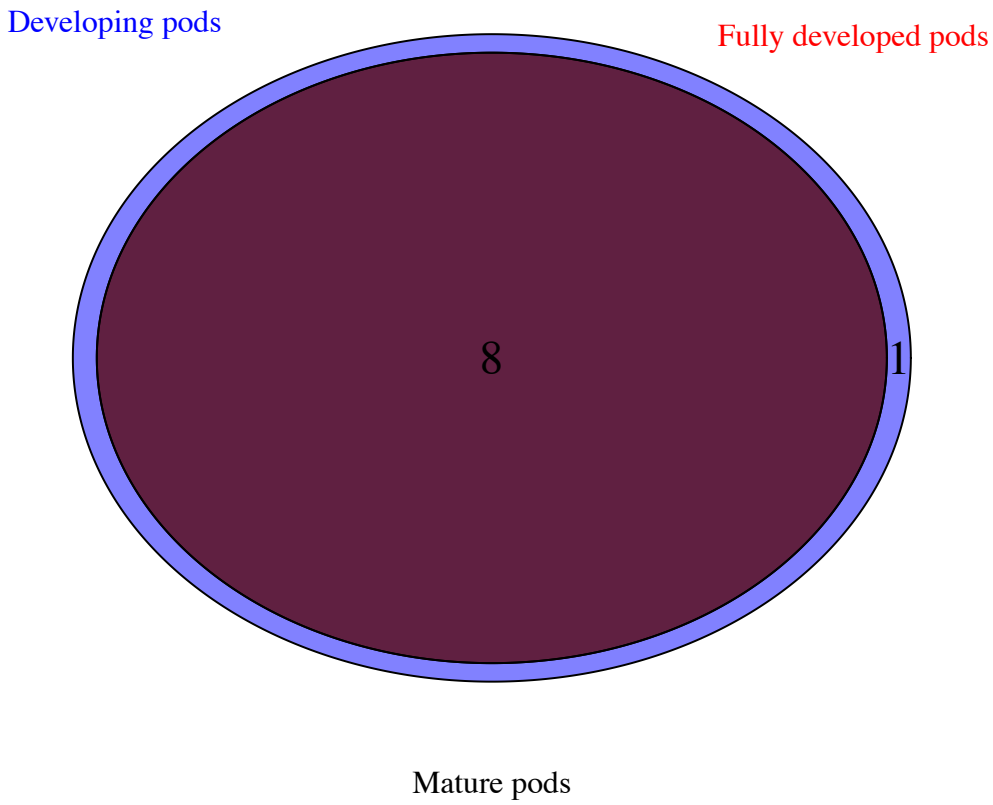


Figure 4. 9. A Venn diagram showing the number of unique and shared volatile compounds from pods of Borno-brown cowpea.

Table. 4.4. GC-MS analysis of volatile organic compounds emitted by 2µl of air entrainment sample of Borno-brown pod (mean ± SD).

	Developing pods		Fully developed pods		Mature pods		RI
BENZALDEHYDE	16.00±0.126	a	17.234±0.088	a	17.601±0.144	abc	962
1H-INDENE, 1-ETHYL LIDENE	15.224±0.235	ab	15.583±0.579	c	18.218±0.261	a	1335
P-XYLENE	15.155±0.721	ab	16.463±0.112	b	16.491±0.274	cd	865
1,3-PENTANEDIOL, - 2,2,4-TRIMETHYL-ISOBUTYRATE	14.375±1.433	abc	16.965±0.397	ab	16.772±0.049	bcd	1380
LIMONENE	14.223±0.061	abc	15.592±0.0134	c	15.744±0.095	d	1030
LONGIFOLENE	13.094±1.723	bc	nd		nd		1405
2,2,4-TRIMETHYL-1, 3-PENTANEDIOL DIISOBUTYRATE	12.965±0.813	bc	15.076±0.014	c	17.955±1.333	ab	1580
HEXANAL	12.416±0.457	c	13.706±0.0126	d	13.873±0.257	e	880
(E)-4,8-DIMETHYL NONA-1,3,7-TRIENE	12.097±0.080	c	12.888±0.244	e	15.599±0.017	d	1116

Within the row means with the same letter are not significantly different.

nd: Not detected.

Table. 4.5. PCA of the data set on 9 compounds from pods of Borno-brown cowpea showing the scores in the various components

Compounds	PC1	PC2	PC3
BENZALDEHYDE	-2.9247424	0.53395161	0.14308114
HEXANAL	1.4804515	0.28392084	0.10658366
P-XYLENE	-0.4480953	0.57735731	0.29599372
LIMONENE	0.8250549	0.35727251	0.08089173
1H-INDENE, 1-ETHYL LIDENE	-1.1065272	-2.08567898	0.02205382
2,2,4-TRIMETHYL-1,3-PENTANEDIOL DIISOBUTYRATE	0.8039782	-0.54510722	-0.2314637
1,3-PENTANEDIOL, 2,2,4-TRIMETHYL-ISOBUTYRATE	-0.9859765	0.85121692	-0.4637878
(E)-4,8-DIMETHYL NONA-1,3,7-TRIENE	1.4445936	0.09429446	0.06990006
LONGIFOLENE	0.9112631	-0.06722745	-0.0232527

Table. 4.6. Permanova table

	Df	Sums of Sqs	F. Model	Pr(>F)
Compounds	8	1.936	20.652	0.001 ***
Residuals	18	0.211		

4.4. Discussion

This study identified 12 volatile organic compounds from the pods of black-eyed cowpea, and 11 compounds were detected on the mature pods, whereas, 8 compounds were identified on the developing and fully developed pods. Of all compounds present, benzaldehyde, was more abundant, followed by p-xylene and m-xylene on developing pods of black-eye cowpea; whereas, in the fully developed pods, benzaldehyde was more abundant, followed by m-xylene and p-xylene, although, they do not differ in quantity. Ethanol, 2- (2-butoxyehoxy)-acetate, followed by m-xylene and benzaldehyde were the major compounds on mature pods of black-eye cowpea. On the pods of Borno-brown cultivar, all 9 compounds identified were present on the developing pods, but longifolene was not present on the fully developed and mature pods. Benzaldehyde, was the key compound, followed by 1h-indene, 1-ethyl lindene and p-xylene on developing pods of Borno-brown cowpea; whereas, in the fully developed pods, benzaldehyde was also dominant, followed by 2,2,4-trimethyl-isobutyrate and p-xylene. On the mature pods, 1h-indene, 1-ethyl lindene was a major compound, followed by 3-pentanediol, diisobutyrate and benzaldehyde.

Results of the headspace volatile samples of the cowpea pods collected at different developmental stages elicited varying behavioural (olfactometer) attraction on mated females of *C. maculatus.* The results show that the beetles moved towards odour samples from the fully developed pods (of black-eyed cultivar) and mature pods (of black-eyed and Borno-brown cultivars), respectively. This suggests that the beetles' attraction to the host plant increases with the pod's age. The preference is likely driven by the fact that the host beans (the primary target) are developing as the pods mature. Another possible reason for the beetle's preference for older pods could be due to the organic compounds associated with the developing seeds in the pods

which may be difficult to detect at an early podding stage. Other work has revealed that cowpea plants with pods attracted more beetles compared to cowpea plants without pods (Zannou *et al.*, 2003). These findings agree with the work of Ouedraogo & Huignard, (1981) on the vulnerability of dry and mature pods of cowpea to *C. maculatus* infestations. Similarly, Abagale *et al.*, (2019) found that the banana weevil (*Cosmopolites sordidus*) was attracted to the odour of senesced banana leaf material.

The variation in attraction to the pods are linked with the differences in the chemical composition of the plant part which affects the abundance and quality of VOCs (Li *et al*., 2016; Shiojiri & Karban, 2006). The results of the GC-MS analyses of the headspace volatile samples from the pods showed that the VOCs profile differed with cowpea cultivar and pod's age. The variation in gene sequence has been suggested to be affecting the chemical composition of plant cultivars or ecotypes, thus, triggering the release of diverse blends of compounds (Köllner *et al.*, 2004). It has been shown that as a plant grows, the ratio of compounds present changes (Najar-Rodriguez *et al.*, 2010; Vallat & Dorn, 2005). Most of the compounds identified (Benzaldehyde, M-xylene, Hexanal, P-xylene, Limonene etc.), are among the common volatile compounds associated with most leguminous plant parts (Blight *et al.*, 1984; Webster *et al.*, 2008). Although they have been only identified as candidate compounds in this study, a probable role in eliciting behavioural attraction in the beetle still remains a strong possibility.

This study has shown a step forward in confirming that volatile compounds in growing plants can drive host identification and selection by *C. maculatus.* The approach presents great potential for the management of the pest using semiochemicals. In summary, I have demonstrated that (a) mated females of *C. maculatus* are attracted to fully developed and mature pods of cowpea, (b) Identified

the volatile compounds that could be inducing the beetles' behavioural attraction to older cowpea pods (c) shown that volatile compounds composition and abundance profile vary between cowpea cultivars at different pods' developmental stage.

CHAPTER FIVE: THE EFFECT OF BEAN PREFERENCE ON LIFE-HISTORY TRAITS IN WILD AND LAB-ADAPTED *C. maculatus*.

5.1. Introduction

C. maculatus has been an important experimental tool in life-history studies as well as studies aimed at understanding the nature of traits underpinning those decisions. For instance, identification of traits driving cost of mating (Crudgington & Siva-Jothy, 2000); variation in population fecundity (Appleby & Credland, 2003); host discrimination (Beck & Blumer, 2014; Boeke *et al.*, 2003; Cope & Fox, 2003); effects of temperature and relative humidity on adult emergence (Howe & Currie, 1964) and the quality of available food on adults fitness (Kawecki & Mery, 2003) have been detected in the beetle. However, stocks/populations of this beetle have been reared in different laboratories and are known to differ in life-history traits such as mortality, reproduction and development rate (Dick & Credland, 1984). Differences in egg-spacing behaviour (Savalli *et al*., 2000) and host-seed damage and larval respiration rate (Guedes *et al*. , 2003) have also been reported between strains.

Despite its substantial representation in the life-history literature (resulting from its adaptations to exploiting stored products and therefore its pest status), few studies have examined this beetle in its pest context: such as host preferences and life-history trajectories in the wild with respect to the beetle's genetic background as well as natural host plants. In the developing world, there is substantial geographic variation in local varieties of host plants affected by the beetle and therefore likely local adaptation by beetle populations. By contrast, most life-history studies use lab-adapted strains fed on commercially available black-eyed peas. As a post-harvest pest

responsible for huge economic losses to near-subsistence communities, there is a need to provide chemical-free sustainable control solutions to cowpea farmers.

Host preference and insect performance have enriched our understanding of the evolution of natural selection and its application in managing pests of economic importance (Dent, 2000). The preference-performance hypothesis assumes that gravid females would prefer hosts that will increase their offspring's fitness, and the forces driving these "mother-knows-best" hypothesis have been suggested in many theories (Craig & Itami, 2008; Jaenike, 1990; Mayhew, 1997). Several studies have examined the interaction between a mother's choice and how it affects her offspring's wellbeing (Thompson, 1988b; Trivers, 1972). Work supporting (Barker & Maczka, 1996; Nylin & Janz, 1993) and contradicting (Faria & Fernandes, 2001; Fritz *et al.*, 2000; Underwood, 1994) the hypothesis have been reported although, there is a general notion that these contradictions can be explained by several factors including ecological and life-history variations (Craigs & Itami, 2008).

Host-quality status and the availability of a preferred host play a key role in the preference-performance relationship (Craigs & Itami, 2008). A plant with marginal diet quality may be preferred because it offers protection against competitors (Wise & Weinberg, 2002) and natural enemies (Björkman *et al.,* 1997). Aggregation of offspring may also affect offspring performance as females that lay eggs in clutches are assumed to have a stronger preference for high-quality hosts compared to females that lay eggs singly (Gripenberg *et al.,* 2010). This is important because choosing the wrong oviposition substrate is a risk for the former, while laying single eggs could be a risk-mitigation strategy by the latter (Hopper, 1999; Mangel, 1987). The preference-performance hypothesis can also be affected by the potential of an insect to feed as an adult. Adequate resource acquisition by developing larvae may be moulding

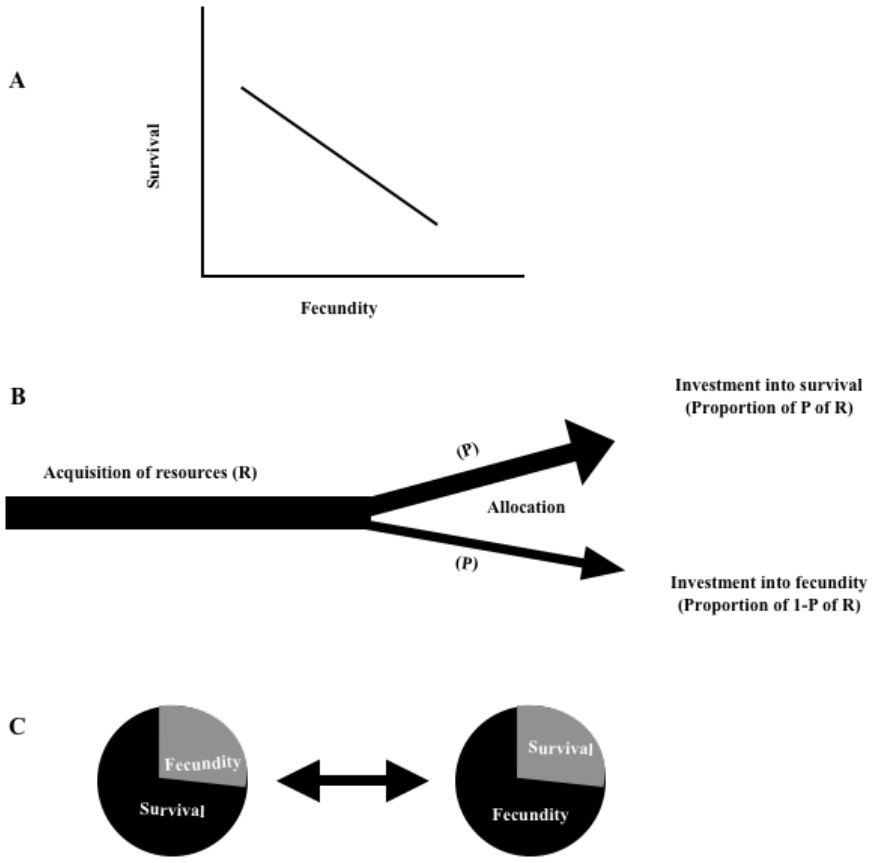
preference in non-adult feeding females, thus a positive linkage between a mother's choice and her offspring's' wellbeing is highly likely. According to Thompson, (1988a), an insect's interaction with an unfamiliar host can also trigger a weak or negative preference-performance relationship.

The avoidance of a host by herbivorous insects has been attributed to resistance genes (Bergelson & Crawley, 1992). Plants use different mechanisms to deter herbivorous insects or suppress their infestation levels (Sarfraz *et al.,* 2006). Reduction in longevity, survival, body mass/weight, reproduction success, development time (which may increase their vulnerability to natural enemies) are some of plant's resistance features (Ebrahimi *et al*., 2008; Sarfraz *et al.,* 2007; Sarfraz *et al.*, 2010; Syed & Abro, 2003). These modalities were summarised by Painter, (1951) as antixeriosis, antibiosis and tolerance in plants. For example, the reproduction and population growth rate of the giant willow aphid, *Tuberolachnus salignus* varied across six willow clones, and the concentration of phenolic glucosides was suggested to be the cause of variation in aphid performance in willow plants (Kendall *et al.*, 1996). The compounds were also used by larvae of chrysomelid beetle, *Phratora vitellinae* as a defensive mechanism against its natural enemies (Rank *et al.,* 1998). Another study on preference-performance showed that the oviposition preference, development rate and reproduction of the diamondback moth, *Plutella xylostella* varied between *Canola sp*. cultivars (Fathi, 2010). The study further proposed the chemical profile of the cultivars tested as the key source of variation. Studies reporting variations in host preference and performance among and within populations have also been documented (Scriber *et al.,* 1991; Jaenike, 1990; Tabashnik *et al.,* 1981). Strain differences are an important feature in an insect-plant relationship and is essential in understanding host selection strategies and the factors that mould them.

Preference and performance studies can be integrated as a component of pest management strategies (Nylin, 2001). This is important in pest management as it gives an understanding of how an insect pest interacts with a host. In most conditions, there is often no clear relationship between host suitability and offspring fitness. However, when a strong correlation tends to exist, it will provide a clue on what drives a female's choice of host. A poor relationship, on the other hand, could mean that the pest prefers a host that has a detrimental effect on offspring fitness. Interestingly, such a host-plant can be used as a trap crop in a mixed cropping system to reduce pest population level. Under conditions where there is no particular preference pattern (weak correlation) it could suggest that such females lay eggs indiscriminately, and is common in insects with a very short life-span (Larsson & Ekbom, 1995).

It is noteworthy that female *C. maculatus* infests other legume cultivars (i.e. alternative hosts), and some substitute hosts result in sub-optimal development of the larvae (Gatehouse *et al*., 1990). Consequently, a female is faced with the challenging task of detecting the right plant, at the right time, and identifying a suitable oviposition substrate that is not already occupied; a critical and complex life-history choice that, if made incorrectly will be detrimental to offspring fitness. For example, because fitness differences exist among individuals in a population (for example, variation in food availability due to competition or other sources of variation in availability for limiting resources) individuals have to differentially allocate limiting resources between key life-history traits in order to optimise fitness (Fabian & Flatt, 2012). During reproduction, allocation of such resources is often affected by the number of offspring produced: For a given resource, fewer offspring will enjoy larger *per capita* energy investment from their parents while, more offspring will have fewer resources *per capita*. The basis of these parental decisions (if offspring number is similar in both

cases, but *per capita* investment is different) will result in parents who invest less into their offspring surviving longer than those investing more (see Figure 5.1).



Source: (after Fabian & Flatt, 2012)

Figure 5. 1. A schematic drawing showing the principles of life-history trade-offs.

(A): Showing a negative relationship between two life-history traits: Fecundity and survival.

(B): A Y- model of resource allocation trade-offs showing how a limited resource like nutrient is acquired and invested into survival at the cost of fecundity.

(C): The use of a finite pie to illustrate resource allocation trade-offs.

Understanding what drives variation in life-history traits amongst individuals and how adaptations to environmental conditions affect individual fitness is also important in pest control; Individuals can be grouped based on similarities in their life-history traits, and such information can be used in identifying the existence of different forms in a population (invasive forms), and to proffer advice on how they can be managed.

In the above context therefore, this chapter examines the bean preference behaviour of two strains of *C. maculatus* and the effect of their choices on the fitness their offspring using five agriculturally important bean types. I hypothesized that the Wild strain would show greater plasticity while choosing an oviposition substrate, and that offspring's fitness between both strains will vary.

5.1.1. Chapter objectives

- To examine the oviposition preferences of lab-adapted and wild strains female *C. maculatus* on five agriculturally important bean types.
- To determine the effect of these choices on the performance (development rate, body weight and longevity) of emerging adult progenies.

5.2 Methods

5.2.1. Insects

Two *C. maculatus* stocks were used: A wild strain (from a farmer's field in Taraba State, Nigeria, maintained in the lab for 3 months) and a lab-adapted strain (maintained in Sheffield for more than 3 decades). Both stocks were cultured by placing individuals from each strain separately into breeding containers (17 x 11.5 cm) containing 200 g of uninfested whole Borno – brown beans. The lids of the containers were perforated for ventilation. The cultures were kept in controlled climate conditions of 28 $\pm$ 2 ^{0}C and relative humidity of 30 $\pm$ 5 %.

5.2.2. Beans

Seeds of five bean types were used in this study; "Borno brown", black-eyed bean (cultivars of *Vigna unguiculata* L. Walper), adzuki bean (*Vigna angularis* Wild), mung bean (*Vigna radiata* L. Wilzek) and pinto bean (*Phaseolus vulgaris* L.). With the exception of "Borno brown" from Nigeria, all were sourced from a local Whole Food Store (in Sheffield). Three kg of each bean type was frozen at -20 ^{0}C for 10 days to ensure they were free of infestation. Then, the beans were equilibrated for 2 weeks at 28 + 2 ^{0}C and 60 + 5 % relative humidity.

5.2.3. Bean preference

I used two different "choice" options to examine how female beetles would allocate their reproductive resources when faced with different ecological situations. In one females had access to several different bean varieties in the same arena ("choice"). In the other, they were presented with only one type of bean (but different replicates were

presented with different beans-“no choice”). My aims were to determine (a) what female’s preference was when presented with several varieties (b) whether females could modulate this preference when presented with only one life-history option (c) to simulate a field (choice) and store (no-choice) conditions and (d) to identify what is possible against what is actual .

5.2.3.1. The “choice” experiment

In this study, ten (10) seeds from each of the five bean varieties were collected and mixed in a Petri-dish (8 cm diameter) and replicated 10 times. Newly emerging females from the wild and lab-adapted adults were collected and paired with a newly emerged males within 24 h of emergence and introduced into each of the Petri-dishes. They were allowed to copulate and lay eggs for 24 h, and the total number of eggs laid was counted (Figure 5.2).

5.2.3.2. The “no-choice” experiment

Here, fifty (50) seeds from each of the five bean varieties were collected and placed in five Petri-dishes (8 cm diameter), respectively and replicated 10 times. Then, newly emerging females from the wild and lab-adapted adults were collected and paired with a newly emerged males within 24 h of emergence and introduced into each of the Petri-dishes. They were allowed to copulate and lay eggs for 24 h, and the total number of eggs laid was counted (Figure 5.2).

5.2.4. Measure of progeny fitness

In order to provide conditions that do not support the emergence of active adults, excess eggs were removed from seeds that bore more than one egg in the oviposition study. This eliminates larval crowding which leads to an increase in bean temperature

due to larval metabolism (Sano, 1967). The seeds were then placed individually in an isolated cell in a grid box (greiner bio-one) to remove bean volume effect (Sano, 1967; Utida, 1954), and to ensure emerging adults did not mate. Within 24 h of emergence, the date, sex and fresh body weight of the emerged adult were recorded. To obtain mated adults, a male and female from each bean type were paired, introduced into the experimental Petri-dishes and allowed to copulate for 24 hr. Virgin adults remained isolated. Longevity of both virgin and mated adults was then examined by daily monitoring of adults from emergence to mortality. The dead body weight of each adult was then determined within 24 hr of mortality.

5.2.5. Statistical analyses

Data collated from both studies was subjected to analyses of variance (ANOVA) and the differences between means separated using Tukey-HSD. R statistical software (R Core Team, 2013) was used to perform all analyses.

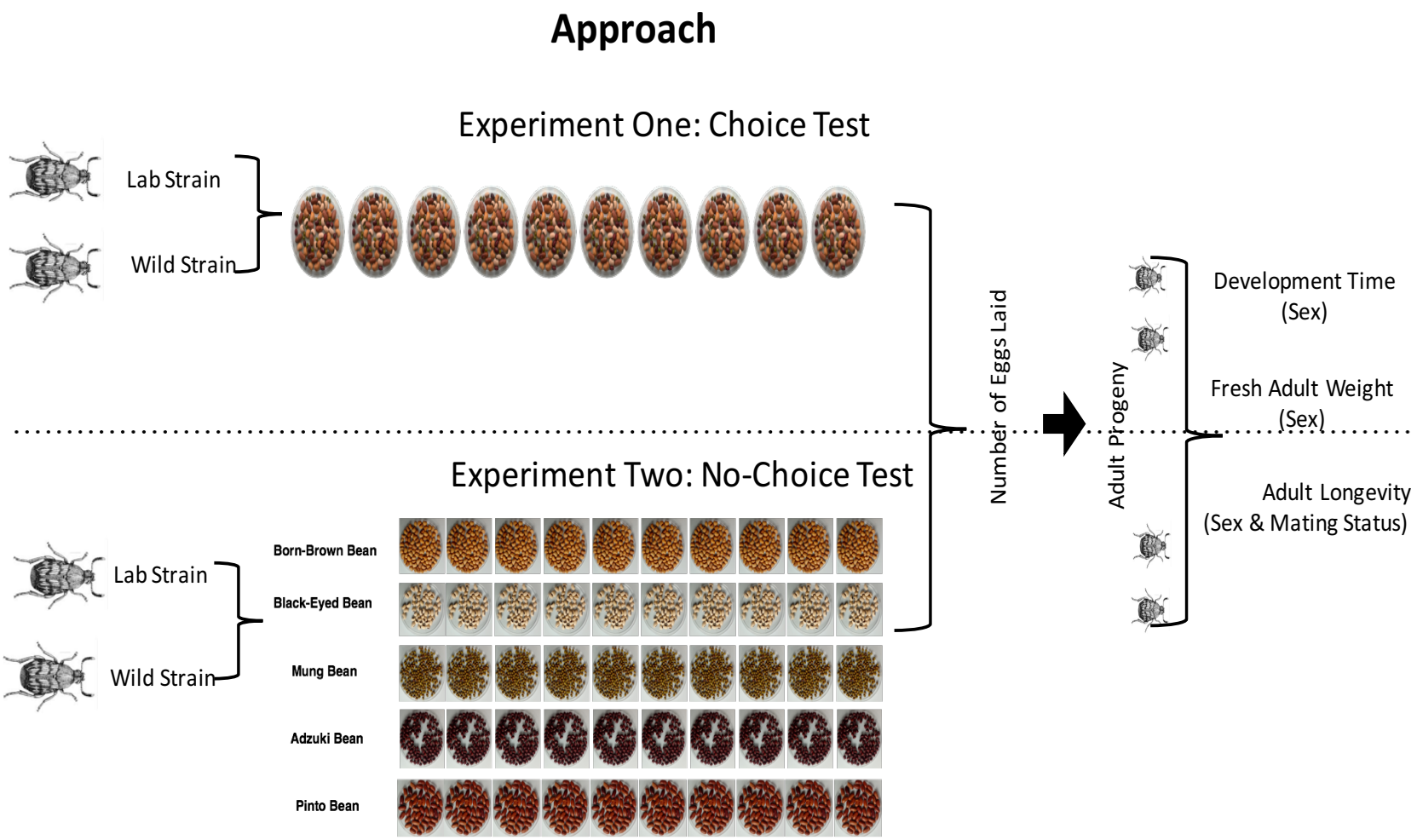


Figure 5. 2. Summary of chapter five experimental procedure.

5.3. Results

5.3.1. Bean preference

In the "no-choice" bean preference test, there was no main effect of bean type ($F_{4, 90}$ = 1.53, P = 0.202; Table 5.2., Figure 5.3) or beetle strain ($F_{1, 90}$ = 3.36, P = 0.070; Table 5.2., Figure 5.3). Similarly, no interaction was detected in the number of eggs laid per bean type by the beetle strains ($F_{4, 90}$ = 0.73, P = 0.576; Table 5.2., Figure 5.3). In the "choice" bean preference test, there was no main effect of bean type ($F_{4, 90}$ = 1.47, P = 0.218; Table 5.1., Figure 5.3) or beetle strain ($F_{1, 90}$ = 1.05, P = 0.309; Table 5.1., Figure 5.3). However, there was a significant interaction between the bean type and the strain: The lab strain exhibited a strong preference for its familiar host (black-eye bean) whilst, the wild-type laid significantly more eggs on pinto beans (unfamiliar host) ($F_{4, 90}$ = 3.10, P = 0.019; Table 5.1., Figure 5.3). Mung beans were the least preferred as oviposition substrate by both beetle strains. Furthermore, both strains did not prefer seeds of any bean type when compared to the cowpea cultivars (Black-eye bean and Borno brown).

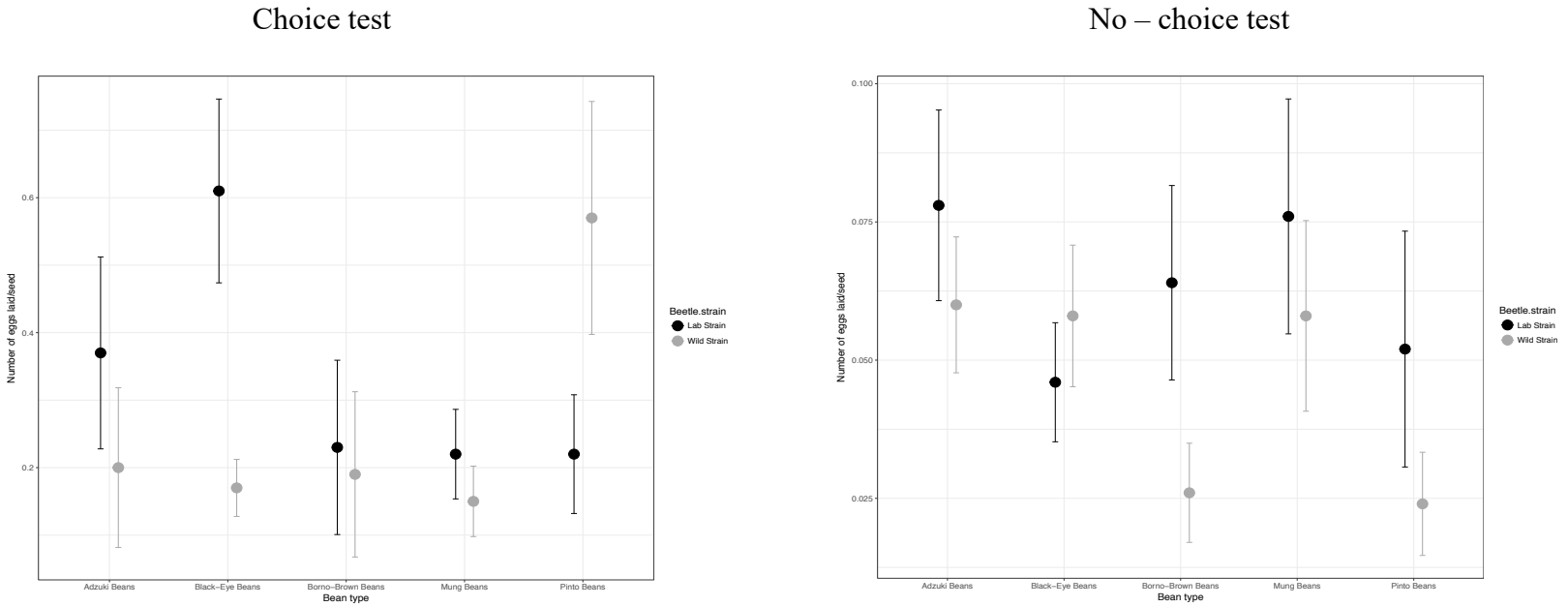


Figure 5. 3. Number of eggs laid by females of lab and wild strains of *C. maculatus* reared on different bean types. (Mean ± S.E.)

5.3.2. Development rate

The development rate of adult *C. maculatus* in both "choice" and "no-choice" experiments was significantly affected by their sex, strain, and bean type, respectively. Adults of the wild strain developed significantly faster than the lab strain in both "choice" ($F_{1, 80}$ = 156.92, P<0.001; Table 5.3., Figure 5.4) and "no-choice" ($F_{1, 80}$ = 106.21, P<0.001; Table 5.4., Figure 5.4) tests. The main effect of sex showed that males developed significantly faster than females in both "choice" ($F_{1, 80}$ = 6.68, P = 0.011; Table 5.3., Figure 5.4) and "no-choice" ($F_{1, 80}$ = 4.94, P = 0.028; Table 5.4., Figure 5.4) tests. The effect of bean type showed that the development of both beetle strains was significantly slower on adzuki beans compared to others in both "choice" ($F_{3, 80}$ = 122.99, P < 0.001; Table 5.3., Figure 5.4) and "no-choice" ($F_{3, 80}$ = 92.02, P < 0.001; Table 5.4., Figure 5.4) experiments.

A significant interaction between beetle sex and strains showed that development rate was faster in males of the wild strain in the "no-choice" test ($F_{1, 80}$ = 4.70, P = 0.032; Table 5.4., Figure 5.4) but, there was no interaction in the "choice" test ($F_{1, 80}$ = 1.78, P = 0.185; Table 5.3., Figure 5.4). A two-way interaction between sex and bean types indicated that adult development rate was significantly faster on male adults reared on black-eye beans, and slower on females from adzuki beans in choice ($F_{3, 80}$ = 6.62, P < 0.001; Table 5.3., Figure 5.4) and no-choice tests ($F_{3, 80}$ = 3.54, P = 0.018; Table 5.4., Figure 5.4). The development rate of the strains was statistically the same across black-eye bean, Borno brown and mung beans. Pinto beans did not support the development of the beetles. There were no differences recorded in the three-way interaction involving the strain, bean type and beetle sex in "choice" ($F_{3, 80}$ = 0.71, P = 0.551; Table 5.3., Figure 5.4), and "no-choice" tests ($F_{3, 80}$ = 0.27, P = 0.848; Table 5.4, Figure 5.4).

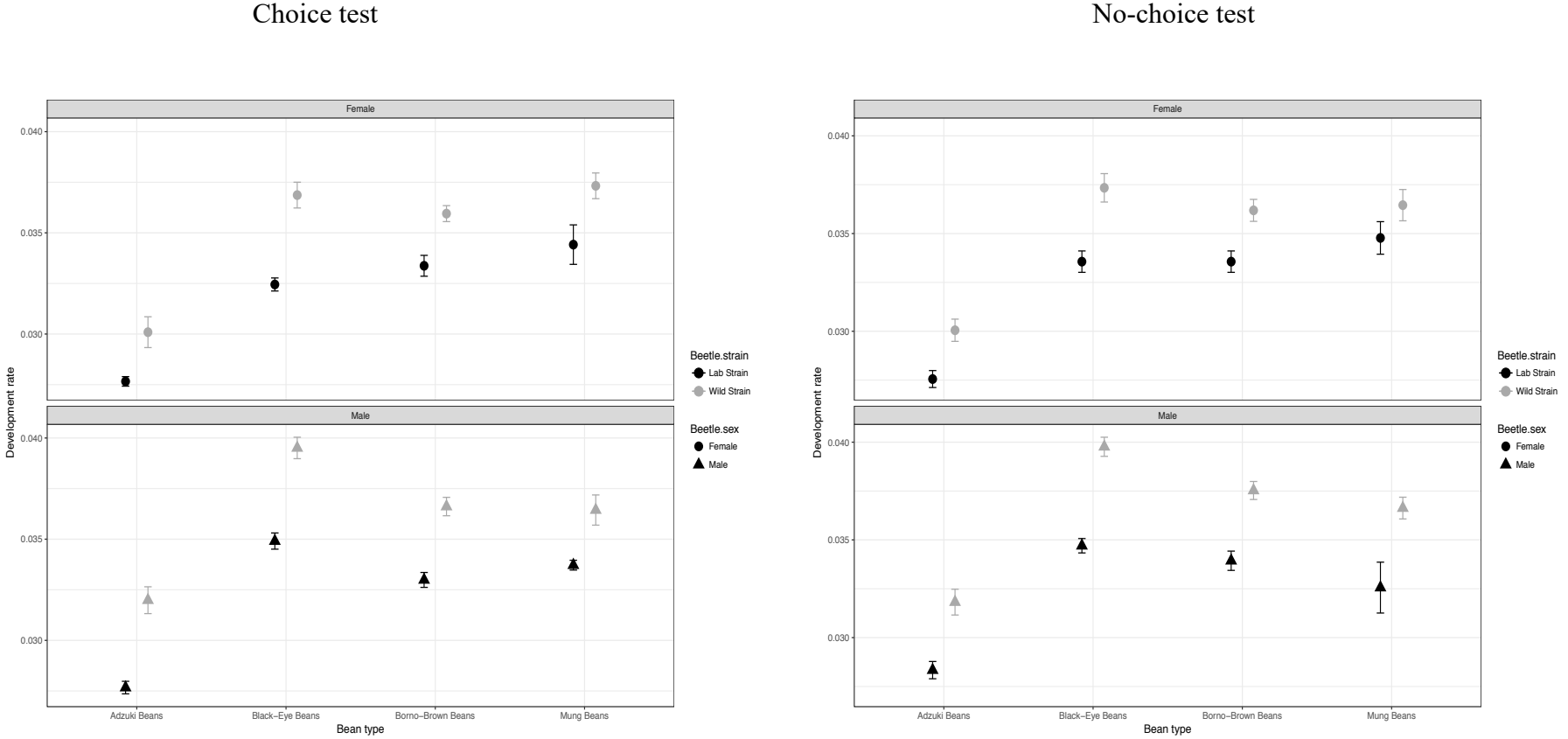


Figure 5. 4. Development rate of males and females of lab and wild strains of *C. maculatus* reared on different bean types (Mean ± S.E.).

5.3.3. Fresh body weight

Beetle strain, sex and bean type, each showed a significant main effect on the beetles' fresh body weight in both tests. Between strain, significantly higher fresh adult body weight was recorded by the lab strain in both "choice" ($F_{1, 80} = 529.47$, $P<0.001$; Table 5.5., Figure 5.5) and "no-choice" tests ($F_{1, 80} = 507.01$, $P<0.001$; Table 5.6., Figure 5.5). Female adults weighed more than males in both "choice" ($F_{1, 80} = 536.80$, $P<0.001$, Table 5.5., Figure 5.5) and "no-choice" (Figure 5.5; $F_{1, 80} = 477.21$, $P<0.001$; Table 5.6., Figure 5.5), tests. The effect of bean type showed that the beetles that emerged from adzuki beans weighed significantly less compared to other bean types in both tests ("choice": $F_{1, 80} = 10.45$, $P<0.001$, Table 5.5., Figure 5.5; "no-choice": $F_{1, 80} = 16.03$, $P<0.001$, Table 5.6., Figure 5.5).

A significant strain and sex interaction showed that females of the lab strain recorded the highest fresh body weight in both "choice" ($F_{1, 80} = 18.56$, $P<0.001$; Table 5.5., Figure 5.5) and "no-choice tests" ($F_{1, 80} = 11.51$, $P<0.001$; Table 5.6., Figure 5.5). A significant interaction between beetle strain and bean type was also detected in the "choice" ($F_{3, 80} = 5.42$, $P = 0.001$; Table 5.5., Figure 5.5) and "no choice" ($F_{3, 80} = 6.76$, $P <0.001$; Table 5.6., Figure 5.5) tests. However, no difference was recorded in a three-way interaction involving beetle strain, beetle sex and bean type in the "choice" ($F_{3, 80} = 1.06$, $P=0.370$; Table 5.5., Figure 5.5) and "no-choice" ($F_{3, 80} = 1.69$, $P = 0.175$; Table 5.6., Figure 5.5) experiments.

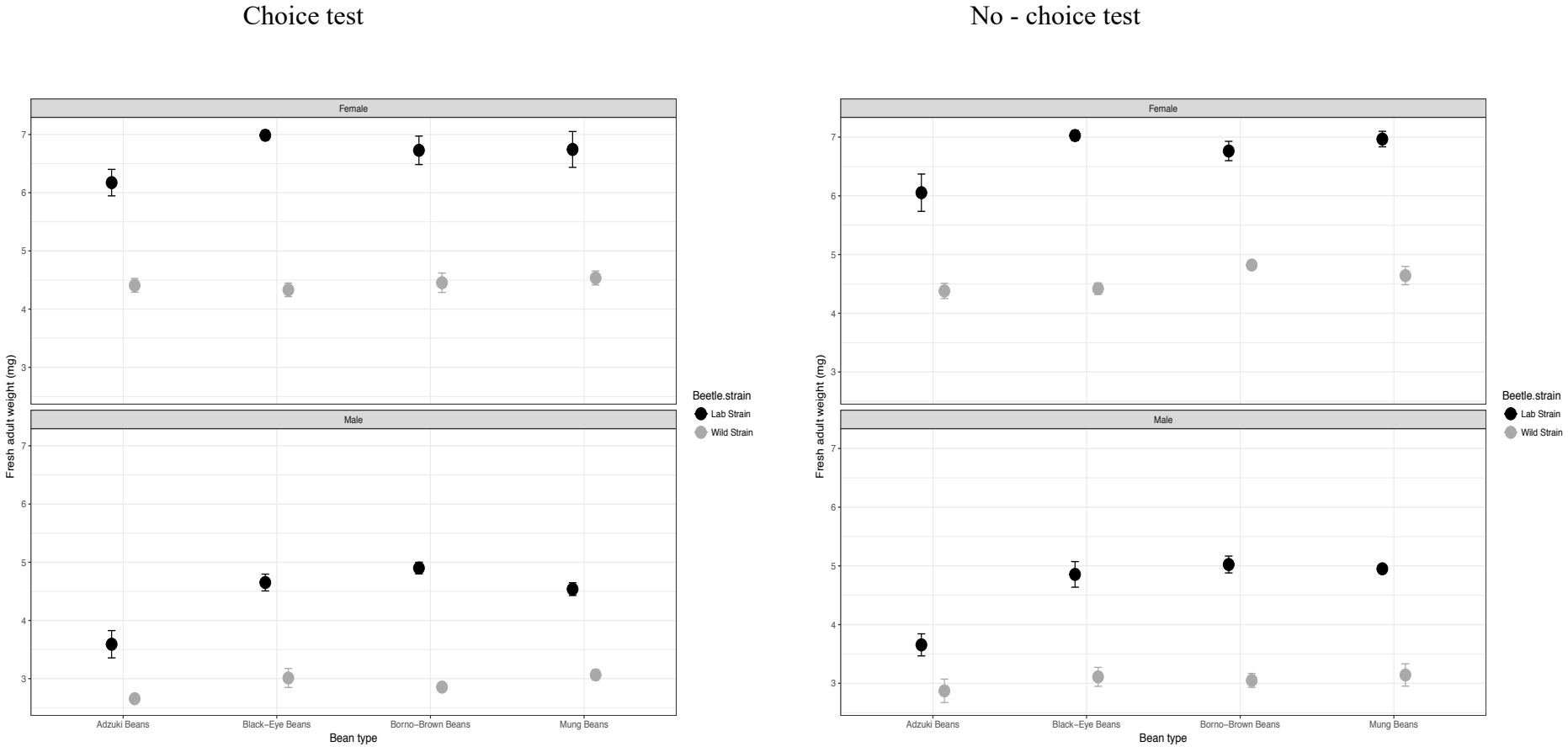


Figure 5. 5. Fresh adult weight of *C. maculatus* strains reared on different bean types. (Mean ± S.E.).

5.3.4. Adult longevity

The longevity of adult *C. maculatus* in the "choice' and 'no-choice" test was significantly affected by main effects of mating status, sex and strain of the beetle, respectively. The results showed that lab strain lived significantly longer than the wild type in both "choice" ($F_{1, 64} = 150.21$, $P<0.001$; Table 5.7., Figure 5.6) and "no-choice" ($F_{1, 64} = 234.56$, $P<0.001$; Table 5.8., Figure 5.6) tests. Virgin adults lived significantly longer than mated adults in both "choice" ($F_{1, 64} = 387.45$, $P<0.001$; Table 5.7., Figure 5.6) and "no-choice" ($F_{1, 64} = 565.44$, $P<0.001$; Table 5.8., Figure 5.6) tests. Similarly, on main effect of sex, the result showed that female adults lived significantly longer than males in both "choice" ($F_{1, 64} = 84.49$, $P < 0.001$; Table 5.7., Figure 5.6) and "no-choice" ($F_{1, 64} = 76.84$, $P < 0.001$; Table 5.8., Figure 5.6) tests. A significant interaction between mating status and strain indicated that longevity was highest in virgin adults of lab strain and lowest in mated adults of wild strain in both "choice' ($F_{1, 64} = 16.69$, $P<0.001$; Table 5.7., Figure 5.6), and "no-choice" ($F_{1, 64} = 36.55$, $P<0.001$; Table 5.8., Figure 5.6) tests.

In both experiments, a significant sex and mating status interaction indicated that sex of both strains was not affected by the longevity of mated adults but, on the virgin adults. Furthermore, longevity was highest in female virgin adults, and lowest in mated male. A four-way significant interaction involving strain, sex, mating status and bean type indicated that virgin females of the lab strain reared on black-eye beans recorded the highest longevity in both "choice" ($F_{3, 64} = 3.88$, $P = 0.013$; Table 5.7., Figure 5.6) and "no-choice" ($F_{1, 64} = 2.83$, $P = 0.045$; Table 5.8., Figure 5.6) tests.

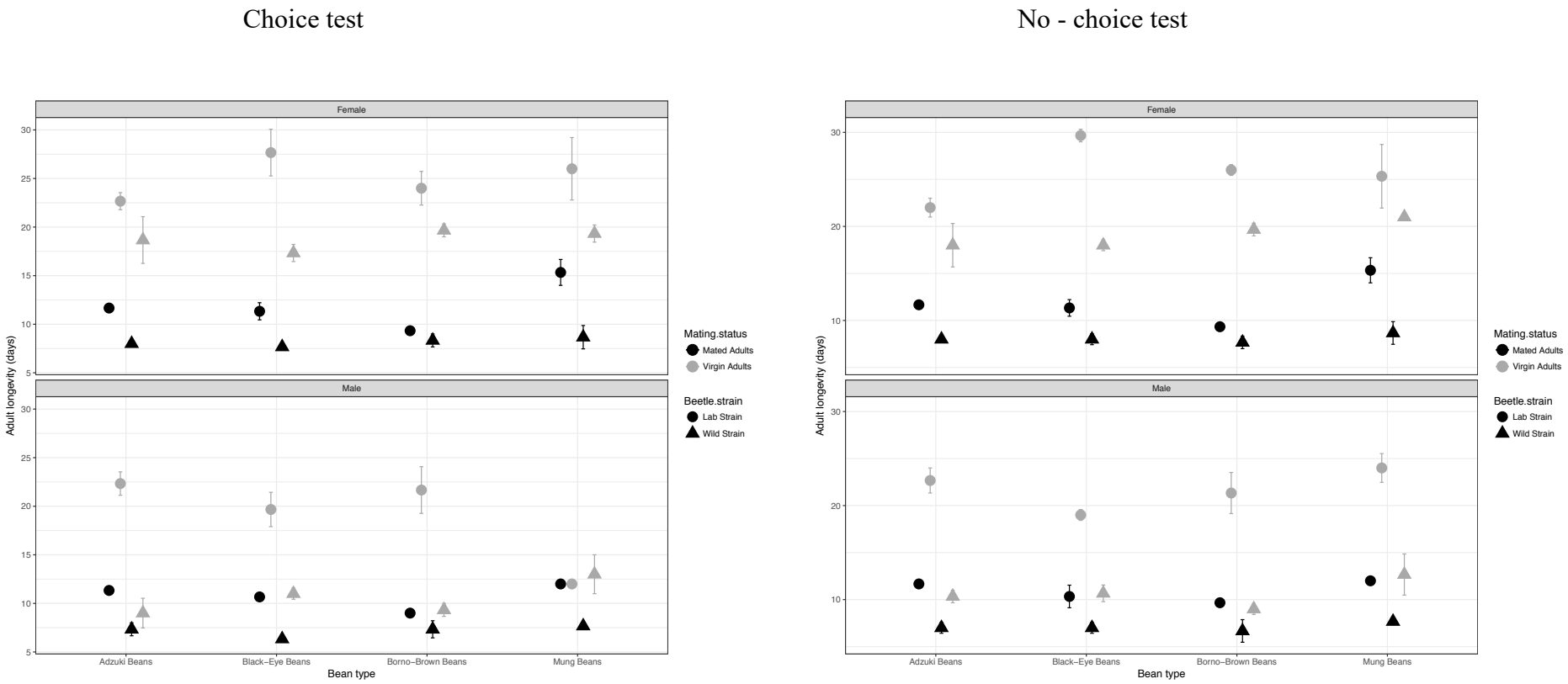


Figure 5. 6. Longevity of mated and virgin adults of *C. maculatus* strains reared on different bean types (Mean ± S.E.).

5.3.5. Dead body weight

The body weight of dead adults in both tests showed a strong significant main effect of beetle strain, beetle sex and bean type. The "choice' ($F_{1, 64}$ = 124.55, P <0.001; Table 5.9, Figure 5.7) and "no-choice" ($F_{1, 64}$ = 162.38, P <0.001; Table 5.9, Figure 5.7) experiments showed that the lab strain weighed significantly more than the wild type. Interestingly, the dead body weights of both strains was not affected by their mating status in both "choice" ($F_{1, 64}$ = 1.86, P = 0.177; Table 5.9, Figure 5.7) and 'no-choice" ($F_{1, 64}$ = 0.017, P = 0.895; Table 5.10, Figure 5.7) tests. However, a significant interaction between beetle sex and mating status was recorded in both tests.

Beetle strain interacted significantly with bean type in both "choice" ($F_{3, 64}$ = 4.89, P = 0.004; Table 5.9, Figure 5.7) and "no-choice" ($F_{3, 64}$ = 7.45, P < 0.001; Table 5.10, Figure 5.7) tests. In a three-way interaction involving sex, strain and mating status, no difference was detected in the "choice" experiment ($F_{1, 64}$ = 1.75, P = 0.191; Table 5.9, Figure 5.7) but, a week interaction was recorded in the "no-choice" test ($F_{1, 64}$ = 4.85, P = 0.031; Table 5.10, Figure 5.7). There was no difference in a four-way interaction involving beetle strains, beetle sex, bean types and mating status in both "choice" ($F_{3, 64}$ = 1.73, P = 0.170; Table 5.9, Figure 5.7) and "no-choice" ($F_{3, 64}$ = 1.72, P = 0.171; Table 5.10, Figure 5.7) tests.

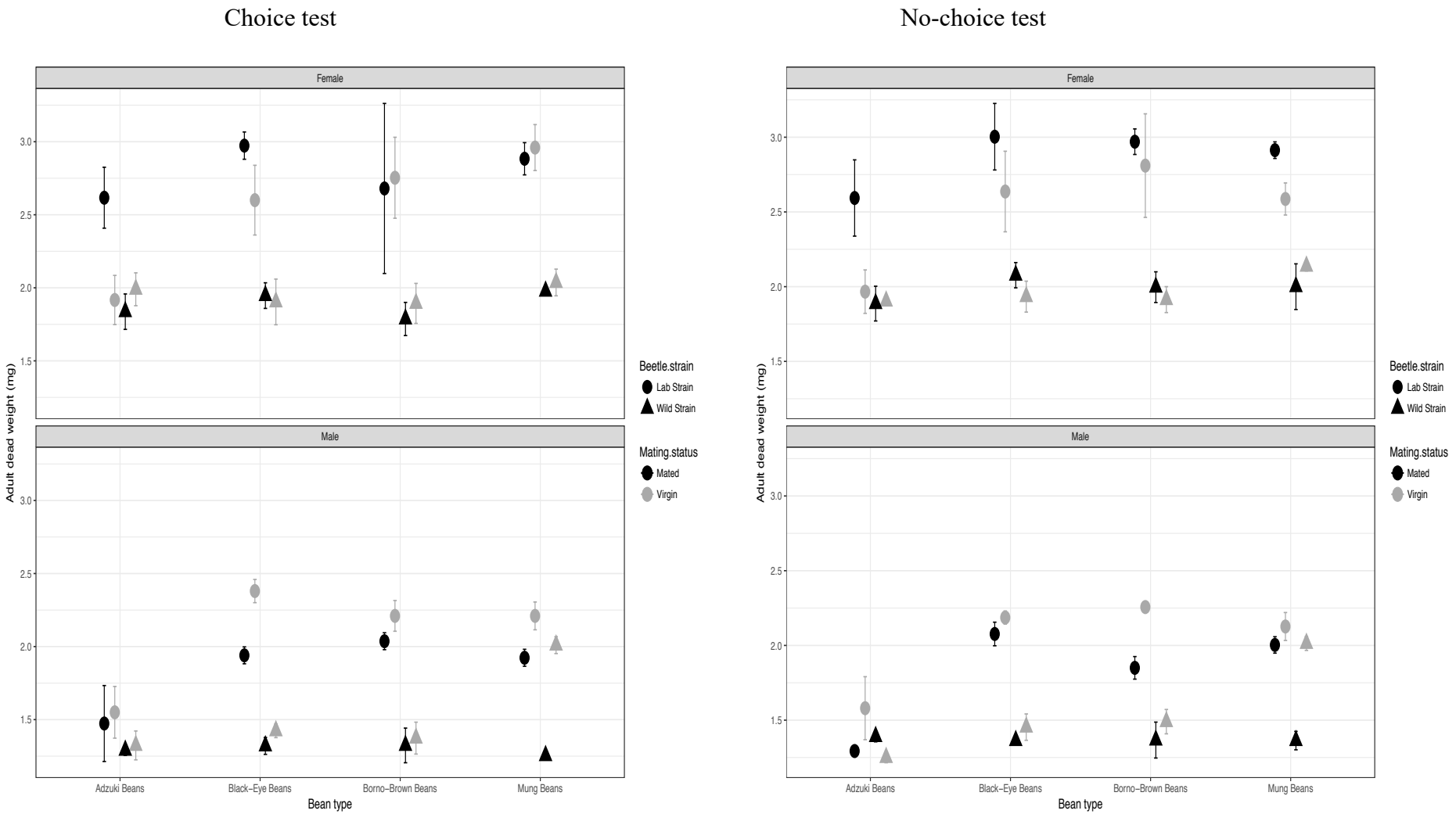


Figure 5. 7. Dead adult body weight of *C. maculatus* strains reared on different bean types (Mean ± S).

Table 5.1. Two - way ANOVA results on number of eggs laid by females of lab and wild strains of *C. maculatus* on different bean types.

	Choice test				
	df	**Sum Squares**	**Mean Square**	**F - value**	**P - value**
Beetle strain	1	0.137	0.1369	1.046	0.3092
Bean type	4	0.769	0.1922	1.468	0.2185
Beetle strain x Bean type	4	1.621	0.4052	3.096	0.0195 *
Residuals	90	11.779	0.1309		

Table 5.2. Two - way ANOVA tables results on number of eggs laid by females of lab and wild strains of *C. maculatus* on different bean types.

	No-choice test				
	df	**Sum Squares**	**Mean Square**	**F - value**	**P - value**
Beetle strain	1	0.0081	0.008100	3.362	0.070 .
Bean type	4	0.0147	0.003674	1.525	0.202
Beetle strain x Bean type	4	0.0070	0.001750	0.726	0.576
Residuals	90	0.2168	0.002409		

Table 5.3 Three - way ANOVA results on development rate of males and females of lab and wild strains of *C. maculatus* reared on different bean types.

Choice test

	Df	Sum Squares	Mean Square	F value	Pr(>F)
Beetle strain	1	0.0002857	2.857e-04	156.917	< 2e-16 ***
Bean type	3	0.0006718	2.239e-04	122.986	< 2e-16 ***
Beetle sex	1	0.0000122	1.217e-05	6.682	0.011558 *
Beetle strain x Bean type	3	0.0000099	3.310e-06	1.816	0.150973
Beetle strain x Beetle sex	1	0.0000032	3.230e-06	1.776	0.186433
Bean type x Beetle sex	3	0.0000362	1.206e-05	6.623	0.000471 ***
Beetle strain x Bean type x Beetle sex	3	0.0000039	1.280e-06	0.705	0.551707
Residuals	80	0.0001457	1.820e-06		

No-choice test

Table 5.4. Three - way ANOVA results on development rate of males and females of lab and wild strains of *C. maculatus* reared on different bean types.

	Df	Sum Squares	Mean Square	F value	Pr(>F)
Beetle strain	1	0.0002690	2.690e-04	106.209	2.46e-16 ***
Bean type	3	0.0007005	2.335e-04	92.203	< 2e-16 ***
Beetle sex	1	0.0000125	1.251e-05	4.942	0.0290 *
Beetle strain x Bean type	3	0.0000094	3.120e-06	1.233	0.3031
Bean strain x Beetle sex	1	0.0000119	1.189e-05	4.697	0.0332 *
Bean type x Beetle sex	3	0.0000269	8.960e-06	3.540	0.0183 *
Beetle strain x Bean type x Beetle sex	3	0.0000020	6.800e-07	0.267	0.8487
Residuals	80	0.0002026	2.530e-06		

Table 5.5. Three – way ANOVA results on adult fresh body weight of lab and wild strains of *C. maculatus* reared on different bean types.

		"Choice" test			
	df	Sum Squares	Mean Square	F - value	P - value
Beetle strain	1	84.36	84.36	529.472	< 2e-16 ***
Bean type	3	4.99	1.66	10.445	7.14e-06 ***
Beetle sex	1	85.41	85.41	536.082	< 2e-16 ***
Beetle strain x Bean type	3	2.59	0.86	5.415	0.00192 **
Beetle strain x Beetle sex	1	2.96	2.96	18.563	4.64e-05 ***
Bean type x Beetle sex	3	0.69	0.23	1.441	0.23692
Beetle strain x Bean type x Beetle sex	3	0.51	0.17	1.060	0.37082
Residuals	80	12.75	0.16		

Table 5.6. Three – way ANOVA results on adult fresh body weight of lab and wild strains of *C. maculatus* reared on different bean types.

		"No-choice" test			
	df	Sum Squares	Mean Square	F - value	P - value
Beetle strain	1	82.79	82.79	507.013	< 2e-16 ***
Bean type	3	7.85	2.62	16.029	3e-08 ***
Beetle sex	1	77.92	77.92	477.209	< 2e-16 ***
Beetle strain x Bean type	3	3.31	1.10	6.755	0.000405 ***
Beetle strain x Beetle sex	1	1.88	1.88	11.506	0.001081 **
Bean type x Beetle sex	3	0.18	0.06	0.377	0.769787
Beetle strain x Bean type x Beetle sex	3	0.83	0.28	1.693	0.175162
Residuals	80	13.06	0.16		

Table 5.7. Four – way ANOVA results on longevity of mated and virgin male and female adults of *C. maculatus* strains reared on different bean types.

		"Choice" test			
	Df	Sum Squares	Mean Square	F - value	P - value
Beetle strain	1	726.0	726.0	150.207	< 2e-16 ***
Bean type	3	5.4	1.8	0.374	0.772344
Beetle sex	1	408.4	408.4	84.491	2.62e-13 ***
Mating status	1	1872.7	1872.7	387.448	< 2e-16 ***
Beetle strain x Bean type	3	26.1	8.7	1.799	0.156311
Beetle strain x Beetle sex	1	5.0	5.0	1.043	0.310946
Bean type x Beetle sex	3	38.7	12.9	2.670	0.054951 .
Beetle strain x Mating status	1	80.7	80.7	16.690	0.000125 ***
Bean type x Mating status	3	46.3	15.4	3.190	0.029475 *
Beetle sex x Mating status	1	222.0	222.0	45.940	4.56e-09 ***
Beetle strain x Bean type x Beetle sex	3	96.0	32.0	6.624	0.000575 ***
Beetle strain x Bean type x Mating status	3	83.9	28.0	5.787	0.001450 **
Beetle strain x Beetle sex x Mating status	1	7.0	7.0	1.457	0.231867
Bean type x Beetle sex x Mating status	3	9.5	3.2	0.658	0.580885
Beetle strain x Bean type x Beetle sex x Mating status	3	56.2	18.7	3.876	0.013053 *
Residuals	64	309.3	4.8		

Table 5.8. Four – way ANOVA results on longevity of mated and virgin male and female adults of *C. maculatus* strains reared on different bean types.

	"No-choice" test				
	df	Sum Squares	Mean Square	F - value	P - value
Beetle strain	1	962.7	962.7	234.558	< 2e-16 ***
Bean type	3	68.3	22.8	5.550	0.00189 **
Beetle sex	1	315.4	315.4	76.843	1.45e-12 ***
Mating status	1	2320.7	2320.	7 565.442	< 2e-16 ***
Beetle strain x Bean type	3	3.0	1.0	0.244	0.86554
Beetle strain x Beetle sex	1	30.4	30.4	7.401	0.00838 **
Bean type x Beetle sex	3	28.1	9.4	2.284	0.08732 .
Beetle strain x Mating status	1	150.0	150.0	36.548	8.47e-08 ***
Bean type x Mating status	3	13.0	4.3	1.056	0.37415
Beetle sex x Mating status	1	165.4	165.4	40.294	2.55e-08 ***
Beetle strain x Bean type x Beetle sex	3	34.8	11.6	2.826	0.04556 *
Beetle strain x Bean type x Mating status	3	22.3	7.4	1.814	0.15353
Beetle strain x Beetle sex x Mating status	1	30.4	30.4	7.401	0.00838 **
Bean type x Beetle sex x Mating status	3	35.5	11.8	2.880	0.04270 *
Beetle strain x Bean type x Beetle sex x Mating status	3	34.8	11.6	2.826	0.04556 *
Residuals	64	262.7	4.1		

Table 5.9. Four – way ANOVA results on dead adult body weight of males and females of lab and wild strains of *C.maculatus* reared on seeds of different bean types.

	"Choice" test				
	df	Sum Squares	Mean Square	F - value	P - value
Beetle strain	1	10.179	10.179	124.549	< 2e-16 ***
Bean type	3	2.180	0.727	8.892	5.22e-05 ***
Beetle sex	1	8.845	8.845	108.229	2.17e-15 ***
Mating status	1	0.152	0.152	1.860	0.17741
Beetle strain x Bean type	3	1.198	0.399	4.886	0.00403 **
Beetle strain x Beetle sex	1	0.242	0.242	2.961	0.09012 .
Bean type x Beetle sex	3	0.060	0.020	0.244	0.86541
Beetle strain x Mating status	1	0.128	0.128	1.561	0.21602
Bean type x Mating status	3	0.509	0.170	2.075	0.11232
Beetle sex x Mating status	1	0.618	0.618	7.557	0.00776 **
Beetle strain x Bean type x Beetle sex	3	0.160	0.053	0.653	0.58382
Beetle strain x Bean type x Mating status	3	0.199	0.066	0.810	0.49309
Beetle strain x Beetle sex x Mating status	1	0.143	0.143	1.745	0.19123
Bean type x Beetle sex x Mating status	3	0.199	0.066	0.810	0.49283
Beetle strain x Bean type x Beetle sex x Mating status	3	0.424	0.141	1.728	0.17018
Residuals	64	5.231	0.082		

Table 5.10. Four – way ANOVA results on dead adult body weight of males and females of lab and wild strains of *C.maculatus* reared on seeds of different bean types.

	"No-choice" test				
	df	Sum Squares	Mean Square	F - value	P - value
Beetle strain	1	8.138	8.138	162.375	< 2e-16 ***
Bean type	3	2.548	0.849	16.946	3.28e-08 ***
Beetle sex	1	9.875	9.875	197.049	< 2e-16 ***
Mating status	1	0.001	0.001	0.017	0.895230
Beetle strain x Bean type	3	1.120	0.373	7.451	0.000235 ***
Beetle strain x Beetle sex	1	0.356	0.356	7.113	0.009679 **
Bean type x Beetle sex	3	0.108	0.036	0.721	0.542945
Beetle strain x Mating status	1	0.136	0.136	2.709	0.104701
Bean type x Mating status	3	0.274	0.091	1.823	0.151859
Beetle sex x Mating status	1	0.962	0.962	19.196	4.47e-05 ***
Beetle strain x Bean type x Beetle sex	3	0.048	0.016	0.318	0.812302
Beetle strain x Bean type x Mating status	3	0.281	0.094	1.868	0.143832
Beetle strain x Beetle sex x Mating status	1	0.243	0.243	4.849	0.031273 *
Bean type x Beetle sex x Mating status	3	0.014	0.005	0.093	0.963536
Beetle strain x Bean type x Beetle sex x Mating status	3	0.259	0.086	1.720	0.171684
Residuals	64	3.207	0.050		

5.4. Discussion.

This study revealed variation in oviposition behaviour and life-history performance between the wild and lab-adapted strains of *C. maculatus*; beetles from the wild developed faster, weighed less and lived shorter. The study also indicated that mung bean was the least preferred as an oviposition substrate, whereas, adzuki bean delayed the beetles' development and reduced their body weight. However, the fitness (total eggs laid) of both strains was the same in both experimental set-ups: this could have been driven by the constraints imposed by a restricted opportunity to copulate.

Results of the "no-choice" test showed that both strains of *C. maculatus* laid eggs equally on the various bean types. This finding agrees with the result of Ofuya & Credland (1996), which reported that *Bruchidius atrolineatus* (Pic) laid an equal number of eggs in a no-choice experiment. Similarly, no difference was found in oviposition by B. *atrolineatus* on seeds of various varieties of cowpea. My findings showed that *C. maculatus* like other bruchids such as B. *atrolineatus* (Ofuya & Credland, 1996), *B. incarnatus* (Metwally, 1990), *Zabrotes subfasciatus* Boh. (Meik & Dobie, 1986), would lay eggs on bean types when beetles have no alternatives.

When beetles were presented with a mixture of bean types in the "choice" test, the lab strain showed a preference for black-eyed beans (its familiar host), whilst the wild-type laid more eggs on pinto beans but, did not prefer it to Borno-brown beans (a familiar host). This is in line with earlier results of choice studies conducted between bean species which showed that *Callosobruchus* spp. prefer cowpea cultivars to other legume cultivars (Cope & Fox, 2003; Kawecki & Mery, 2003; Mainali *et al*., 2015; Messina, 2004; Paukku & Kotiaho, 2008). The strong preference for black-eyed beans displayed by the lab adapted strain may be due to selection in the lab that affected behavioural and physiological traits influencing host choice. It may also

reveal a short association history with other bean types (Ofuya & Credland, 1996). The oviposition choice displayed by the wild-type could be due to reported evidence of decreasing preference for cowpea by bruchids from West Africa to Asia (Kawecki & Mery, 2003). It could also be due to the new environment (laboratory condition) in which the beetles were reared as it differs from host selection conditions in the wild.

In a mixture of all bean types, my findings also showed that the number of eggs laid by both strains was lowest on mung bean. Earlier studies have revealed that choice of oviposition substrate is influenced by the difference in seed size (Cope & Fox, 2003; Kawecki & Mery, 2003) and seed surface area (Bhattacharya & Banerjee, 2001). Furthermore, use of chemical cues (Credland & Wright, 1989), sensory receptors on maxillary palps (Messina *et al*., 1987), or experience (Chiu & Messina, 1994), have also been reported to affect oviposition and discrimination among host species.

Findings from this study showed that Borno-brown beans, black-eyed beans, adzuki beans and mung beans, all supported the successful development of *C. maculatus*, but pinto beans did not. However, it is worth noting that neither strain avoided pinto beans as a choice of oviposition substrate even though it was unsuitable for offspring development, indicating the beetles' inability to detect an unsupportive host. The toxicity of seeds of *Phaseolus vulgaris* on bruchid larvae had been reported many years ago. Toxicity exhibited by pinto beans on the strains of *C. maculatus* could be due to the presence of phytohaemagglutinin, a lectin present in most varieties of *P. vulgaris*. Non-protein and protein antimetabolites in legume seeds have been shown to have insecticidal properties against bruchids of economic importance (Gatehouse *et al.*, 1990). Further studies have also shown α-amylase inhibitor to be toxic to bruchids (Huesing *et al*., 1991). The presence of toxic compounds within the testa

(Simmonds *et al*., 1989), and hardness of the testa (Thiéry *et al.*, 1994), have been found to prevent bruchids from penetrating seeds of most legumes.

The effects of bean type on larval development rate have also been reported (Boeke *et al*., 2003). The Borno-brown beans, black-eyed beans and mung beans had a similar effect on the development rate of the beetles when compared to adzuki bean which delayed their development in both tests. This agrees with the work of Mainali *et al.*, (2015), which observed *C. chinensis* developing longer in adzuki bean when compared with cowpea and mung bean. Generally, the development rate was faster on wild strain compared to the lab type. Differences in development rates of both strains could be due to variation in their genetic make-up as they were originally collected from two separate continents with different climatic conditions which make similarities in their life-history pattern not expected. Their faster development could also be a survival mechanism against natural enemies in the wild.

The freshly emerged virgin adults from the lab strain weighed more than the wild-type, and females from both strains weighed more than the males. The findings are likely related to the longer development rate and longer longevity recorded by the lab strains against the wild-type, and the virgin females against the males, respectively. The fresh body weight of the lab strain suggests they are reared in a favourable condition, unlike the wild-type which is faced with challenges in the wild.

The longevity of each strain was not affected by the different bean types. This is contrary to the findings of Mainali *et al.*, (2015), which reported that longevity was higher on adzuki beans in a choice test. The lab strain lived longer than the wild-type suggesting there could be a trade-off between fresh body weight, development rate and longevity in a host-specific situation. This further explains that the ability of the wild-type to develop faster than the lab strain could be a fitness cost in their reduced

longevity. According to Rolff *et al*., (2004), a decline in immune function investment is associated with a faster development rate. Fresh body mass has been found to have a positive correlate with body condition (fitness) suggesting why the lab strain and females lived longer than the wild-type and males, respectively. Variation in insect body condition has been linked with their rearing and/or environmental conditions. Insects with better body condition have increased longevity (Petersen, 2003). Barone & Frank, (2003) reported that body condition can also be used as a yardstick for habitat quality. As the strain from the wild is exposed to several natural enemies including threats from extreme environmental conditions, a contrary life – history parameters with a lab adapted strain as reported in this study is inevitable. Variations in the life-history of both strains could also be due to genetically transferred traits in developing larvae which supports the prevailing rearing conditions.

The consequences of mating have been studied in insects (Crudgington & Siva-Jothy, 2000; Kotiaho & Simmons, 2003; Rolff & Siva-Jothy, 2002). Mating status of the strains was affected by their longevity as virgin adults lived longer than mated adults in both tests. The ability of the virgin adults to live longer than mated ones could be due to the trade-off between mating and immunity as reported by Rolff & Siva-Jothy, (2002), in mealworm beetle (*Tenebrio molitor*). A trade-off between mating and longevity in *C. maculatus* have also been studied (Paukku & Kotiaho, 2005). Early death recorded by mated adults could be due to the damage caused by male genitalia to the female genitalia during copulation, and the repeated kicks given to the male by the female as an act of defence (Crudgington & Siva-Jothy, 2000). The Longevity of virgin adults was affected by the beetle sex, but not on mated adults. On virgin adults, females lived longer than males which suggest there could be differences in resource acquisition during larval development.

The body weight of dead adults was higher in lab strain when compared with the wild type. However, their body weight was not affected by their mating status. The former could be as a result of the higher fresh body weights recorded by the lab strain at emergence. When compared with beetle sex and mating status in the choice experiment, dead body weight of virgin females was statistically the same with their mated females. This suggests that males may be providing females with polyandry, a nutrient which enhances female fitness during copulation (Arnqvist & Nilsson, 2000). According to Savalli & Fox, (1999), the primary reason for re-mating in females is to acquire nutrients from the seminal fluid.

The bean types selected by both strains as oviposition substrate in the "choice" experiment had varying effects on the fitness of their progeny. Between strain, progeny development rate was slow on adzuki beans despite been next to the most preferred substrate by the beetles. Interestingly, the least preferred bean type (mung beans) together with the familiar substrates (Borno-brown and black-eyed beans) supported faster development of progeny. On the beetles' fresh body weight, again, adults that emerged from adzuki beans weighed less compared to other bean types, but, the longevity of the adult progenies was not affected by any of the bean varieties. The performance (slower development rate and reduced body weight) recorded by the beetles that emerged from adzuki beans may indicate resistance against *C. maculatus,* and could suggest why it was not strongly preferred by both strains. Studies have shown that plants can cause a reduction in longevity, body mass and reproduction in adult progenies or indirectly increase an insect's risk to attacks by natural enemies *via* delayed emergence or developmental time (Syed & Abro, 2003; Sarfraz *et al*., 2007, 2010, Ebrahimi *et al.*, 2008). According to Sarfraz *et al*., (2006), plants that express antibiosis can suppress the pest population from causing economic damage.

Consequently, the performance of *C. maculatus* adults on adzuki bean could result in reduced infestation levels indicating its potential as a pest control tool, although further study is required to fully explore these findings. Furthermore, the ability of *C. maculatus* to oviposit, develop and survive on unfamiliar hosts as revealed in this study agrees with earlier findings that *C. maculatus* quickly modifies its egg-laying behaviour when presented with novel hosts (Fox, 1993; Wasserman & Futuyma, 1981). This could explain why the beetle successfully infests different bean types.

In summary, this study revealed that (a) lab-adapted beetles showed a strong preference for black-eyed beans, whereas, the wild-type indicated a preference for pinto beans when presented with different bean choices, (b) the beetles laid eggs equally on all bean types in a no-choice, (c) the wild strain developed faster, weighed less and lived shorter compared to the lab-adapted type (d) a mother's choice of oviposition substrate affects the fitness of her progeny.

CHAPTER SIX: GENERAL DISCUSSION

6.1. Thesis summary

This thesis aims to improve the body of knowledge regarding host detection, host finding, host preference and host utilisation in the context of management of *C. maculatus*. The project defines the beetle's touch, smell and taste organs, examines how the beetle utilizes these senses in choosing a host (pods and seeds of cowpea) and finally investigates the relationship between hosts and measures of fitness.

6.1.1. Major findings

I started by defining the sensory anatomy of host detection in *C. maculatus* (using two strains) with focus on the antennae and genitalia sensilla (Chapter 2).

The findings show that the sensory systems that respond to mechanical and chemical stimuli in *C. maculatus* are similar to many other insects. The morphology of the antennae does not differ between strain and sex. However, there is sexual dimorphism in antennal anatomy (Chapter 2): The type V antennal sensilla occur in higher density in males. The differences suggest the males also need to detect hosts in the wild, and/or need to be responsive to female pheromones (Mbata, *et al*., 1999, 2000) when not in a stored-product environment (where females are plentiful). The abundance of touch and taste sensilla on the female genitalia suggests the importance of these senses during copulation and oviposition.

After defining the host detection sensilla, I investigated how female *C. maculatus* use their sense of smell to identify a host (Chapter 3). By using four-choice and two-choice olfactometers, I measured the attraction of beetles to bean odours. In a mixture of different beans (four-choice), the beetles preferred the familiar host to

other bean types. When given the choice between clean air and bean odour in a two-choice test, they showed preference for unfamiliar host types. This suggests *C. maculatus* has distinct but plastic preferences for bean types, and confirms the beetle's ability to switch to alternative hosts in the absence of a preferred one.

I then examined the attraction of female *C. maculatus* to pods of two cowpea cultivars grown in a controlled condition (Chapter 4). This is relevant because it is likely that this beetle is a field-to-store pest. The pods' ages were categorised as; "developing", "fully developed" and "mature", and the headspace volatile organic compounds of each age category were collected, and their attractiveness tested using a two-choice olfactometer. With this protocol, I was able to ascertain the pods' developmental stage that is most susceptible to infestation by the pest. The data showed that the attractiveness of pods increased with the pod's age. This could mean that *C. maculatus* is not only attracted to stored dry beans but also on freshly matured beans in the field, during the growing cycle of the plant. No research has been conducted on this aspect of the insect-plant relationship and it might provide some opportunity for novel control strategies.

In Chapter 5, the beetle's ability to identify a preferred host as an oviposition substrate was tested following the results from the sense of smell studies (Chapter 3 and 4). A choice (a mixture of different bean types) and a no-choice (one bean type) experimental set-ups were presented to a wild and lab-adapted strains of the beetle. Then, the consequences of a mother's choice of a substrate on her progeny's performance (development rate, body weight and longevity) were examined.

In the mixture of all bean types, the lab-adapted strain displayed a strong preference for its familiar host, but the wild-type surprisingly preferred an unfamiliar bean type (that is actually unsuitable for larvae development!), although the preferred

host shares a similar colour with the familiar host. These findings suggest there may be a host colour effect as a mechanism that influences host selection, and confirms the inability of females to detect the toxic substances associated with an unsuitable host. It also shows that the lab-adapted strains of *C. maculatus* do not behave like the strains that infest the crop in the wild. This is an important result, since most work on *C. maculatus* (and is used to inform control approaches) is done on lab-reared populations. Following the variation in behaviour and life-history traits between the beetle strains, my work recommends that future projects aimed at managing the pests should be on systems collected from the wild as it will provide contextualised knowledge of the beetle's behaviours, and its life-history trajectories. The life-history results revealed that a mother's choice of bean does not necessarily reflect her offspring's' fitness (there was no clear correlation between preference and performance). This finding has been reported as a norm in most insects (Nylin, 2001), and could indicate that females are laying eggs indiscriminately (a potentially important strategy in insects with high fecundity, Nylin, 2001) .

Finally, key life-history parameters such as strain, sex, mating status, development rate and body weight were investigated as predictors of longevity in the beetle (Appendix 3). Of all parameters measured, only strain, sex and mating status were significant predictors of longevity in *C. maculatus*.

6.2. Discussions arising from the thesis

6.2.1. Gender differences

The life-history of an organism is shaped by factors such as natural selection, adaptation and constraints (Stearns, 1992) which often result to fitness differences among individuals or conspecifics: Habitat quality (Barone & Frank, 2003) and rearing conditions (Petersen, 2003) are some of the mechanisms that drive behavioural differences among individuals. In many insects, females invest more in immune mechanisms than males (Siva-Jothy *et al*., 2008). Specific responsibilities such as locating a mate and laying eggs are not unrelated to their sensory abilities and may account for the differences observed on the antennal sensilla of both sexes (Chapter 2).

6.2.2. Strain differences

I have shown differences in the behaviour and life-history performance of lab-adapted and wild strains of *C. maculatus*. The wild-type developed faster (spent less time acquiring resources), weighed less and died early compared to the lab-adapted strain (Chapter 5). This is predictable, given that lab-adaptation usually means the insect is living in a surfeit of resources and a lack of predation and parasitism – it can therefore maximise traits that enhance reproductive output (such as body size, longevity, etc)

6.2.3. Host odour recognition

An insect's ability to detect a preferred host from a distance is usually guided by chemical cues emitted by the host (Bruce *et al*., 2005; Adams, 2007), which could be from the vegetative part of a plant or its seeds (Steeghs *et al*., 2004). The chemicals are mainly organic compounds with low molecular mass such as terpenoids, isoprene, fatty acid derivatives.

I found a consistent response from female *C. maculatus* towards odour from the beans and pods of cowpea. The beetles were attracted to odour stimuli from adzuki beans, Borno-brown beans, black-eyed beans, fully developed pods and mature pods, respectively (Chapter 3 and 4). This confirms the notion that beetles' can recognise the odour of a preferred host from a distance.

6.2.4. Choice of host-bean and effect on progeny fitness

The overall wellbeing and behaviour of an organism are largely influenced by its developmental conditions which are associated with the nutrients acquired. This effect is more pronounced when the developing organism has no choice of diet and rather depends on the food choice of the mother. In *C. maculatus*, developing larvae rely on the nutritional composition of the host chosen by the mother for survival. Consequently, different host types are likely to have a varying effect on progeny fitness. This "preference and performance" strategy (Thompson, 1988a) is important in making pest management decisions (Nylin, 2001).

In Chapter 5, I found varying effects of host bean quality on the fitness of individuals examined. The beans mostly preferred by female *C. maculatus* had comparable life-history effect with the least preferred ones. This result could mean that the mother is more interested in mating success and/or high reproductive rate of

her progeny. As female adults do not feed, it further suggests that the choice of a suitable host may be strongly related to the host's physical properties (host size, colour and surface texture). Also, the weak relationship between a female's choice of host and progeny fitness could mean there are higher chances of increased infestation when cowpea is cultivated or stored close to other leguminous cultivars.

6.3. What next?

Integrating these key findings into designing future work that will bring us closer to achieving a sustainable management strategy is the way forward. Consequently, a phase-by-phase implementation strategy is my ideal approach.

With this in mind, the proposed future work will be carried out in 4 phases so as to capture the original aim of the project.

The first phase will involve taking the findings to the wild. As a field project, one of the challenges will be to get the local farmers (especially those from the northern part of Nigeria) to understand the relevance and prospects of the research, and the need to withdraw from using chemicals in controlling pests. This initial approach will be difficult as there is no immediate alternative control method to present to the farmers. However, educating them on some of the findings from this thesis (such as an estimated time of pods infestation in the field, and the possibilities of the pest switching to an alternative host), and integrating it with safer pesticide application techniques will be a good way to start. For example, a one-time chemical spray at the late stage of podding (the most vulnerable stage – Chapter 4) will greatly reduce the dangers associated with overapplication of the chemicals. And, notifying the farmers on the importance of avoiding cultivating cowpea next to other legume farms or using the same facility to store their produce (cultivated legumes) will help

in reducing cross-infestation as the females have been reported to lay eggs indiscriminately (Chapter 5).

The second phase will focus on establishing a field trial. As a field-to-store pest, the field design will aim at understanding if sensitivity in detecting host in the field is dependent on the beetle's gender. This work has reported that female *C. maculatus* infests host-pods in the field at maturity stage (Chapter 4), and have suggested a pool of VOC that could be inducing such interaction. It will be interesting to observe what happens in the field: If both male and female identify the host at the same time. To achieve this, I will grow cowpea in the field, and monitor and sample *C. maculatus* at different targeted developmental stages.

After establishing the infestation threshold, the third phase will centre on collecting headspace samples from each developmental stage for electroantennographic analysis. This approach will isolate any active VOC compound in the samples. Furthermore, how the beetle's life-history traits (such as body mass and development rate) relate to its sensitivity to the active compounds will also be tested using the olfactometer designed in this thesis.

After this is achieved, the fourth phase will focus on using the active compounds identified to formulate a trap that can be used to lure the pest in the field. This will involve repeating the field trial and using the knowledge from earlier findings to investigate the efficacy of the trap-device. If successful, such traps will be commercialised, and advise on how and when it should be used will be given to the farmers to ensure efficacy. Also, the compounds identified to be actively inducing behavioural activities in the pest can be used to create a barrier such that the beetles' are lured to a trap-crop which does not support its larvae development, thus, any eggs laid will not survive. Following advances in plant breeding and molecular biological

studies, such a trap-crop can be bred to release these compounds that are useful in manipulating the pest behaviour.

By the time the future plan is concluded following the implementation of each project phase, a replication of the entire project in another region (southern Nigeria) will be considered. This is necessary because the development of *C. maculatus* is affected by changes in climate, and the northern and southern parts of Nigeria are regions that differ in these conditions. This will provide bases for comparison and would have contributed immensely to the actualisation of the original objective when completed.

6.4. General conclusion

In this work, I have demonstrated that the life-history performance of *C. maculatus* differ within strains; populations from the wild showed varying behavioural activities and fitness compared to lab-adapted stocks. Also, I identified gender differences in the distribution of key sensory organs in *C. maculatus*, and the cowpea pods' developmental stages that are most susceptible to infestation by the pest.

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APPENDICES:

APPENDIX 1: ANALYSIS OF BEETLES' ANTENNAL LENGTH

In order to correct for body mass effect, the antennae of both beetle strains relative to their fresh body weight were analysed and compared with the beetles' sex.

The beetles' were reared, fixed and examined using the similar protocol as described in Chapter Two.

A *post-hoc* result showed that the lengths of the antennae on males and females from both strains respectively, are not different. However, males antennae are longer than females (Table A1.1, Figure A1.1).

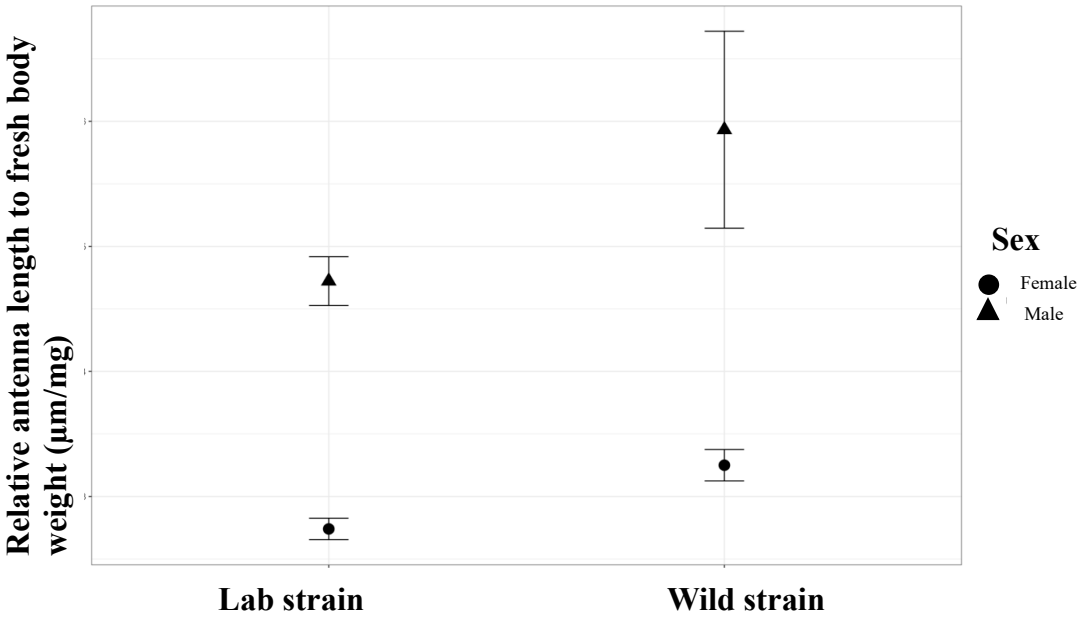

Figure A1. 1. Antenna length of *C. maculatus* strains

Table A1.1. Summary of post-hoc analysis (TukeyHSD)

Main effects	**P - value**
Beetle strain	0.0407
Beetle sex	1.76e-05
Interactions	
Wild strain female x Lab strain female	0.728
Lab strain male x Lab strain female	0.008
Wild strain male x Lab strain female	0.000
Lab strain male x Wild strain female	0.056
Wild strain male x Wild strain female	0.000
Wild strain male x Lab strain male	0.166

Raw p – values are presented with significant effects highlighted in bold.

APPENDIX 2: MEASUREMENT OF COWPEA PODS' GROWTH

The days to flowering and the growth rate of pods from plants of two cowpea cultivars (Borno-brown and California black-eyed beans) as shown in Chapter 4 are explained in the table and figure below.

Table A2.1. Days to flowering and 50% flowering of two cowpea cultivars

Cowpea varieties	Days to 1st flowering	Days to 50% flowering
Borno brown beans	45	51
California black-eyed beans	43	49

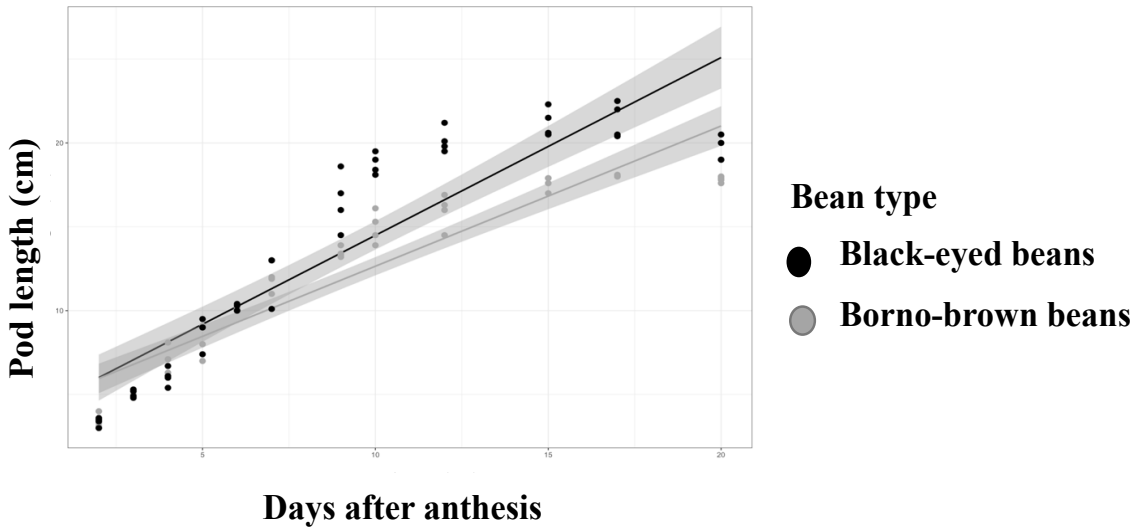

Figure A2. 1. Pods length of two cowpea cultivars from anthesis to maturity.

APPENDIX 3: PREDICTOR MODEL FOR LONGEVITY IN *C. maculatus*.

A3.1. Introduction

The lifespan of an organism vary among species, as behaviour, and life-history traits also vary within populations (Messina, 1990). Differences in inherited traits (Tatar & Carey, 1994), copulation, mating status, population difference are factors that influence the longevity of an organism. For example, in insects, virgin adults live longer than mated adults (Crudgington & Siva-Jothy, 2000; Rolff & Siva-Jothy, 2002). Differences in body sizes also account for the natural variance in lifespan though, there are contradictory reports on the relationship between body size and lifespan. Miller *et al*., (2000) reported that small body size is associated with increased longevity, while Moller *et al.*, (1989) reported the opposite.

These series of inconsistencies across studies has formed part of the reasons there is a paucity of understanding of the key predictors of longevity in insects.

In Chapter 5, I compared bean preferences and life-history fitness of two strains of *C. maculatus* using two experimental setups (choice and no choice). In order to further understand how these key parameters relate in the work, this study tested the hypothesis that at least one of; beetle strain, beetle sex, mating status, development rate and body mass (weight) is a predictor of longevity (lifespan) in *C. maculatus.*

A3.2. Method

A3.2.1. Correlation

To examine how the variables (beetle strain, beetle sex, mating status, development time, body mass (weight) and longevity) are related, a simple correlation test was performed.

A3.2.2. Regression

The outcome of the correlation test was further subjected to a regression analysis to determine if the independent variables (beetle strain, beetle sex, mating status, development time and body mass (weight) are predictors of the dependent variable (longevity). To achieve this, a multivariate linear regression analysis was performed using the following model;

$Y = \beta_0 + \beta_1 x_1 + \ldots + \beta_n x_n + \varepsilon$
Y= dependent variable (outcome)
X1 …. Xn = independent variables (Predictors)
β_0 = Intercept
β_1 = Slope

Three categorical variables (beetle strain, beetle sex and mating status) in the predictor variable were analysed as binary predictors as shown below;

Beetle strain:
Lab strain = 0
Wild strain = 1

Beetle sex:
Male = 0
Female = 1

Mating status:
Virgin = 0
Mated = 1

A3.2.3. Statistical Analyses

A correlation test and multivariate linear regression were used to test the hypothesis. R (R Core Team, 2013) statistical software was used for all analyses.

A3. Result

A3.1. Correlation

A correlation analysis showed there are significant relationships between longevity and all the predictor variables (beetle strain, beetle sex, mating status, development rate and fresh body weight). The respective correlation values for each predictor is shown in Table A1.1.

Table A3.1. Correlations between dependent and independent variables

	Longevity	P	N
Beetle strain	-0.445	.000	192
Beetle sex	0.291	.000	192
Mating status	-0.702	.000	192
Development rate	-0.240	.000	192
Fresh body weight	0.435	.000	192

A.3.2. Regression

A multivariate linear regression result showed that beetle strain, sex and mating status are significant predictors of longevity but, development rate and fresh body weight are not (Table A3.2). However, the overall model is significant ($F_{5,186}$ = 131.944, $p < 0.001$), and explains 77.4% of the variance in longevity in *C. maculatus* adults with only 22.6% unexplained.

Using the binary codes assigned to the categorical variables, the results further explain that;

- Being a female ($\beta = 5.572$, $t = 5.456$, $p < 0.001$) , a virgin ($\beta = -9.231$, $t = -20.048$, $p < 0.001$) and a Lab adapted strain ($\beta = -7.817$, $t = -6.870$, $p < 0.001$) are significant predictors of increased longevity in C. maculatus.

Therefore, the regression model equation is thus;

Longevity = 21.761 + 5.572 sex – 7.817 strain– 9.231 mating status.

Table A3.2. Multiple linear regression model results of individual predictor variable

Variables	B	T	SE	P
Constant	21.761	8.017	2.714	.000
Beetle strain	-7.817	-6.870	1.138	.000
Beetle sex	5.572	5.456	1.021	.000
Mating status	-9.231	-20.048	0.460	.000
Development rate	68.174	0.840	81.179	.402
Fresh body weight	-0.894	-1.857	0.482	.065
	R = 0.883	R^2 = 0.780	R ADJ = 0.774	p < 0.000

A3.3. Discussion

Correlation between dependent and independent variables showed that all predictors tested were significantly related to longevity. A negative correlation of longevity with beetle strain (wild strain), mating status (mated adults) and development rate was recorded. But, beetle sex (female) and fresh body weight related positively with longevity. However, only beetle strain, beetle sex and mating status were significant predictors of longevity in the multivariate regression analyses.