STUDIES ON THE AGGREGATION PHEROMONE OF ADULT MALAGASY MIGRATORY LOCUST, *LOCUSTA MIGRATORIA CAPITO* (SAUSSURE, 1884) AND ITS EFFECTS ON CONSPECIFIC NYMPHAL STAGES.

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Studies on the aggregation pheromone of adult Malagasy migratory locust, *Locusta migratoria capito* (Saussure, 1884) and its effects on conspecific nymphal stages.

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A Thesis submitted in fulfillment for the degree of Doctor of Philosophy in Zoology in the Jomo Kenyatta University of Agriculture and Technology.

2010

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

To my late mother, to my father, to my sister Narisoa and her husband Lobo, to my two little nephews Anjara and Aina and my new born niece Arintsoa, to my little brother Jaime, to my entire family.

To all my friends and especially to my dear and close friend Ms. Patricia Sandra.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANAE	Association Nationale d'Actions Environnementales
A. I.	Aggregation Index
ANOVA	Analysis of Variance
APB	Adult Pheromone Blend
ARS	Agricultural Research Section
BCED	Behavioural and Chemical Ecology Department
CIRAD	Centre de Coopération Internationale en Recherche Agronomique pour le Développement
CNA	Centre National Anti-acridien
DA	Area of Densification
DF	Degrees of Freedom
DDT	Dichloro Diphenyl Trichloroethane
EAD	Electro-Antennography Detector
FID	Flame Ionization Detector
FMG	Malagasy Franc currency
FOFIFA	Foibe Fikarohana momban'ny Fambolena
GABA	γ-amino butyric acid
GC	Gas Chromatography
GC-EAD	Gas Chromatography coupled Electro-Antennography Detector
GIS	Geographic Information System
GC-MS	Gas Chromatography tandem Mass Spectrometry
HPLC	High Performance Liquid Chromatography

icipe	International Centre of Insect Physiology and Ecology
IMA	Initial Multiplication Area
IGR	Insect Growth Regulator
IPM	Integrated Pest Management
I. S.	Internal Standard
JH	Juvenile Hormone
JHA	Juvenile Hormone Analogues
JKUAT	Jomo Kenyatta University of Agriculture and Technology
Meq	Male equivalent
NPB	Nymphal Pheromone Blend
PAN	Phenylacetonitrile
P. O.	Paraffin oil
RT	Retention time
SAS	Statistical Analysis Systems
SE	Standard Error
SNK	Student –Newman Keuls
TMA	Transitory Multiplication Area
RH	Relative Humidity
US$	United States Dollar currency

ABSTRACT

Locusta migratoria capito is the major agricultural pest to cereal production in Madagascar. Unlike other members of this species (e.g. *L. migratoria migratorioides and L. migratoria manilensis*), hardly any study has been done on its chemical ecology. This project sought to characterize the aggregation pheromone of the adult *L. m. capito* and to assess the role of its components on the behaviour of conspecifics under laboratory conditions. To assess responses of adult locusts to their own volatiles, bioassays were conducted in a single chamber olfactometer that allowed for dual-choice between air, enriched with volatiles *vs.* clean air. To identify the composition of the volatiles, emanations from the locusts were collected using Super-Q adsorbent and then analysed using Gas chromatography (GC), coupled Gas chromatography-electroantennographic detection (GC-EAD) and coupled Gas chromatography-mass spectrometric (GC-MS) techniques. The identified electroantennographically-active components were assessed for their behavioural and solitarizing effects. Among the identified compounds, phenylacetonitrile (PAN) was further tested for its role in aggregation and anemotaxis on fifth instars nymphs and adults of the Madagascar locust, *L. m. capito.*

Like in the other locust species that have been studied previously, olfactory cues from body and faecal volatiles might play a role in modulating the aggregation behaviour of *L. m. capito*. Body volatiles from mature female locusts elicited moderate (A. I = 29 ± 9%) aggregation responses in both sexes of the adults, whereas only mature males significantly responded to their own volatiles with the highest aggregation index (A. I = 48 ± 9%) recorded in the tests. With regard to faecal

volatiles, only the immature adults of *L. m. capito* showed aggregation responses to the volatiles while the sexually mature adults were repelled.

In chromatographic profiles, no qualitative compositional differences were observed in the body volatile that were produced by mature male and female locusts. However, there were quantitative differences between amounts of components in volatiles from locusts of the two sexes. This was more evident in the production of a high amount of phenylacetonitrile in mature males compared to their female counterparts (0.38 ± 0.04 ng/male *vs.* 0.04 ± 0.003 ng/female).

In GC-EAD and GC-MS analyses, a total of nine electroantennographically active compounds (3-penten-1-ol, anisole, benzyl alcohol, veratrole, n-decanoic acid, (Z) 6-pentadecen-1-ol, palmitic acid, (Z) 9-octadecanoic acid methyl ester and an unidentified C_{27} or C_{28}) were identified in the body volatiles of the adult locusts while five compounds (anisole, benzyl alcohol, guaiacol and beta-ionone and an unidentified compound) were identified in their faecal volatiles. Those from the body comprised of nine compounds of which five were of relatively high volatility and four of high molecular weights. The high volatility compounds included anisole, benzyl alcohol, veratrole and other two compounds whose identity was not fully confirmed (3-penten-1-ol and n-decanoic acid), whereas among the heavy ones ((Z) 6-pentadecen-1-ol, palmitic acid, (Z) 9-octadecanoic acid methyl ester and an unidentified C_{27} or C_{28}), only palmitic acid was fully confirmed. For the body volatiles, female locusts were found to release about twice the amount of highly volatile compounds as their male conspecifics while the heavy compounds were produced in higher amounts by the mature males. There were five components in

faecal of which four were identified to be anisole, benzyl alcohol, guaiacol and beta-ionone. The remaining compound was not identified.

Aggregation bioassays using synthetic standards of the identified single compounds showed that all the compounds tested except benzyl alcohol elicited varying levels of aggregation responses in male and female adult *L. m. capito*. Blends of these synthetic compounds elicited similar patterns of responses as obtained previously with the crude body and faecal odours eliciting slightly lower responses. These compounds were also tested for their primer effects on phase shifts in conspecific nymphal locust. Exposure of fifth instar nymph to each of the compounds, with the exception of benzyl alcohol and palmitic acid, and also to blends of the identified components, induced the appearance of solitarious locust characters with regard to colour and morphometric changes in the emerging fledglings.

Phenylacetonitrile tested at various concentrations elicited no clear pattern in aggregation and anemotactic responses of fifth instars nymphs and adults, *L. m. capito,* as opposed to the desert locust in which it plays a key role as an aggregant for adults. Further investigations to determine the precise role played by this compound in the biology and behaviour of this locust are strongly recommended prior to its use as a behaviour modifying agent for its management.

In locust control, the use of pheromone by controlling the aggregation of locusts appears to be an important strategy for the integrated locust management. This would target the reduction of expansiveness of the areas infested with hoppers and swarms of adult locusts. Consequently, this would lead to minimizing of the quantities of deleterious chemical insecticide that are sprayed an thus, reduction of yield losses of

agricultural products. Results of this study provide an insight into the understanding of the chemical ecology of *L. m. capito* and are also a contribution to the search for an environmentally safe pheromone-based strategy for locust management.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Madagascar is an internationally recognized biodiversity hotspot due to its unique flora and fauna. Agriculture and tourism constitute the backbone of the country's economy. Several cultivated crops, mainly cereals (rice and maize), are staples. Their cultivation is permanently under threat from numerous pests. During years of locust outbreak, spraying of chemical insecticides is heavily practiced by farmers. This exposes the country to inevitable environmental pollution as well as additional monetary expenses for their purchases. Thus, ensuring food sufficiency by controlling pests, and at the same time conserving the country's biodiversity, represents a major challenge to the Malagasy Government.

Two locust species are a threat to cereal crop production and their control presents a source of pollution to the environment with regard to chemical pesticides. These are the Red locust, *Nomadacris septemfasciata* Serville (1838) and the Malagasy migratory locust, *Locusta migratoria capito* Saussure (1884). The former has rarely reached the real gregarious status, unlike *L. m. capito* which aggregates very often, causing significant yield losses, in particular on cereals, mainly rice and maize (World Bank, 1998).

The largest locust plague in Madagascar lasted for 18 years (1939 to 1957) with crop losses amounting approximately US$ 35 million at the current exchange rate

(Randriamanantsoa, 1999). At the peak of the outbreak in 1945, it destroyed 20,000 tons of rice in Marovoay plain of Mahajanga (Anonymous, 2000). Since 1992, there has been resurgence of the locust outbreaks, though at smaller magnitudes (Bergeron, 2001). In 1999, the indirect economic losses due to monitoring and management were about US$ 1.7 million (Zehrer, 1999).

There are various methods that are used for the control of locust outbreaks. Insecticides, mainly organochlorides such as DDT, Dieldrin, hexachlorohexane (HCH), Lindane; organophosphates such as chlorpyriphos-ethyl, diazinon, dichlorvos, fenitrothion, fenthion, malathion, pyrimophos, sumithion; pyrethroids e.g. α-cypermethrin, β-cylfuthrin, cyalothrin, cypermethrin, deltamethrin, esfenvalerate, fluvalinate, λ-cyalothrin and tralomethrin; phenylpyrazole (mainly fipronil®) (Rakotoarimanana, 1997; Randriamanantsoa, 1998) and many others are heavily used to control locust upsurges.

During the locust recession periods, an average of about 80,000 hectares of affected land must be treated per annum (Zehrer, 1999). However, when there are locust invasions, a total of up to two million hectares, covering three quarters of the island are sprayed (Anonymous, 2000). During the upsurges of *L. m. capito* that occurred in the late 1990s and the recent plague of 1999-2001; *circa* 340,000 litres of insecticides, mainly fipronil, was used for the treatment of an estimated 1.3 million hectares of hopper bands and about 96,000 hectares of swarms (World Bank, 1998). The aerial spraying of large quantities of pesticides is not only very expensive, but also pollutes the environment. The Malagasy Government has to provide US$ 3

million each year for locust control. During the invasion of locusts in 1997 - 2000, the Malagasy Government spent US$ 35 million from its reserves and US$ 17.5 million of assistance, totalling to about US$ 52 million to combat the locust menace (Zehrer, 1999; Thomas *et al.*, 2000; Lecoq, 2001).

Since 1928, many studies have been conducted in order to understand the dynamics of phase change and the factors that promote gregarization of the locusts (Lecoq, 1972, 1975; Andrianasolo, 1979; Duranton *et al*., 1979; Scherer, 1999). However, most of these studies have essentially focused on the biology of the locust, eco-climatic factors and on their population dynamics (Launois, 1974; Andrianasolo, 1979). These studies have enabled the delineation of outbreak areas of the Malagasy migratory locust (ANAE, 2004). The recession, breeding and outbreak areas are mainly in South-South West of the island, where some of the major parameters that promote gregarization, including optimum temperatures (25 – 30ºC), rainfall (50 – 200 mm), and also the seasonal south west wind stream are prevalent (Lecoq, 1975; Andrianasolo, 1979).

In locust control, the ultimate goal is the reduction of use of insecticides. Hence, an Integrated Pest Management (IPM) approach is the sound option. Semiochemicals could be a major component of such an IPM approach. An indepth study of the role played by semiochemicals in the biology and phase dynamics of *L. m. capito*, in particular, the factors leading to the outbreaks is important, so that, locusts can be controlled at the very early stages of population build up. Research carried out at the International Center of Insect Physiology and Ecology (*icipe*) on the desert locust,

Schistocerca gregaria Forskål (1775) and the African migratory locust, *Locusta migratoria migratorioides* Reiche and Fairmaire (1850) during the last fifteen years has demonstrated the possible use of adult locust aggregation pheromone for the control of hopper bands of these pests (Niassy *et al*., 1998; Kane, 2004).

In the gregarious desert locust, *S. gregaria*, nymphs and adults have aggregation pheromones of different composition whereby each stage only responds to its own pheromone (Obeng'Ofori *et al*., 1993; Torto *et al*., 1994; 1996). In contrast, solitarious locusts of this species do not produce the aggregation pheromone, but adult locusts are equally responsive to the pheromone of their conspecific adult gregarious locusts (Njagi *et al*., 1996). Furthermore, treatment of gregarious fifth instar nymphs (in field cages and hopper bands in the field) with phenylacetonitrile (PAN), a major component of the conspecific adult locust aggregation pheromone, disrupts their grouping behaviour, and they become more susceptible to sub lethal doses of insecticides and low doses of *Metharizium anisopliae* var *acridium* Metschnikoff (1878) Sorokin (Kane, 2004).

Kane (2004) suggested that, during the subsequent disintegration of bands, nymphs suffer high predation by birds. On the other hand, treatment of the gravid adult females with the nymphal pheromone blend which comprises of 10 compounds (four aliphatic aldehydes: hexanal, octanal, nonanal, decanal, their corresponding acids and two faecal phenols) (Torto *et al.*, 1996) interfered with the communal egg-laying behaviour, leading to random distribution of egg pods (Bashir *et al.*, 2000a; Kane, 2004). Hence, incorporation of semiochemicals in the management of the desert

locust outbreaks may lead to drastic reduction of amounts of insecticides and mycopesticides used, thus minimizing the hazardous effects on the environment and non-target organisms and, greatly reducing the cost of control.

Such information on the role of semiochemicals on the biology and behaviour of *L. m. capito* is virtually lacking. Therefore, this study sought to characterize the aggregation pheromone of adult *L. m. capito* and to evaluate effects of this pheromone on the aggregation behaviour and the solitarising effects on the nymphal conspecific stages of this locust.

1.2 Literature review

1.2.1 Ancient world distribution of *Locusta migratoria spp*

The migratory locust, *L. migratoria* has several subspecies which have colonized and evolved within different ecosystems and environments across the ancient world. These subspecies include: *Locusta migratoria* Linnaeus (1758) in Western Europe and Northern Asia; the African migratory locust, *Locusta migratoria migratorioides* in the whole of Africa South of Sahara and off-shore Atlantic islands; the oriental migratory locust, *L. m. manilensis*, Meyen, (1835) in eastern and south China, S.E. Asia and Pacific region; and *L. m. burmana*, Ramme, (1951) found in upper Burma, Tibet and west China. There are other *Locusta* subspecies that are scattered over India, Middle East and Australia (Figure 1.1).

Locusta migratoria capito is one of the most devastating pests that has been assumed to be isolated off the mainland Africa for many centuries. This species has become a

"distinct island population" probably because of the close resemblance with the Africa mainland species *L. m. migratorioides*. Indeed, the two species display very minor morphological differences and in their morphometrics (Wintrebert, 1970). However, there is no evidence of interchange between the island population and the one on mainland Africa. *Locusta m. capito* only evolves on Madagascar Island and in the Mascareign islands. Furthermore, its behaviour presents some controversial aspects compared to the mainland species.

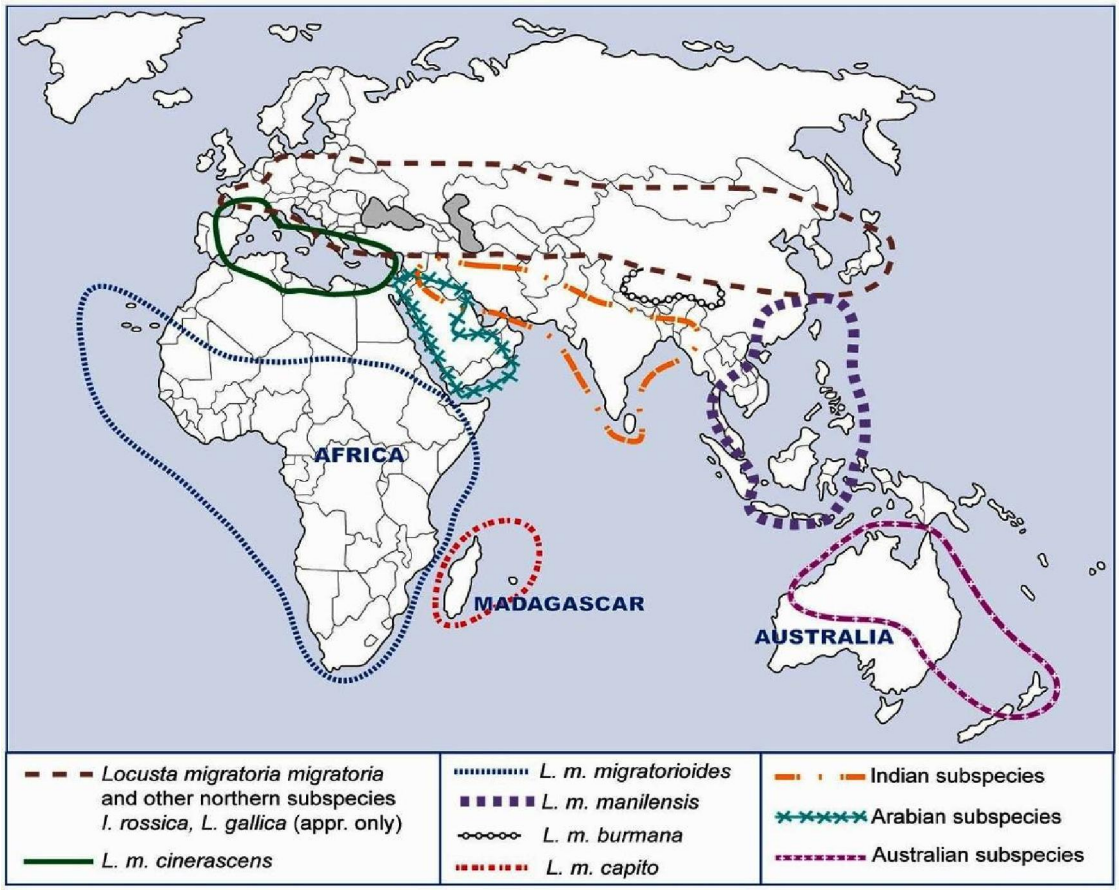

Figure 1.1Worldwide distribution of *Locusta migratoria spp* Linnaeus, 1758, and subspecies (Source: COPR, 1982).

1.2.2 *Locusta migratoria migratorioides* in Africa and *Locusta migratoria capito* in Madagascar

The migratory locust, *L. migratoria* and the desert locust, *S. gregaria*, are highly destructive to crops and are ranked among the notorious and most threatening locust plagues worldwide (Uvarov, 1966, 1977). Their invasions cause total destruction of crops that at times that have led to local or regional famine, making these locusts to be among the most dreaded crop pests. Indeed, an adult locust may consume an equivalent of its own weight (average of 2g) of vegetation material per day. In swarms and migrating formations comprising of millions of individuals, they become a huge threat to rangeland pastures and crop cultivations.

In mainland Africa, *L. m. migratorioides* is prevalent. Though sporadic, occurring invasions inflict considerable crop losses and their control devastate the economies of affected countries (Figure 1.2). For example, in Kenya during the severe infestation by this species in early 1930's, 40% to 50 % of wheat and maize crops were destroyed in its peaks in 1931. In addition, 3% of dicotyledonous crop production were affected, although this locust prefers feeding on grasses and graminaceous plants (Launois-Luong, 1975; COPR, 1982). *Locusta m. migratorioides* was reported to have fully destroyed maize crops that were 3-5 inches high including those attacked at the time of flowering. Further a yield loss of 20% was incurred in plants that were attacked after the grain had formed, but which was still unripe (Bullen, 1969).

In Madagascar, the first report of locust invasions was by the missionary R.P Azevedo on 23rd May 1617 (Rakotobe, 1999). *Locusta m. capito* is a highly ranked pest due to its high potential of crop destruction. In addition, the harmful environmental and ecological impacts due to dusting and spraying of chemical insecticides during control operations are immeasurable.

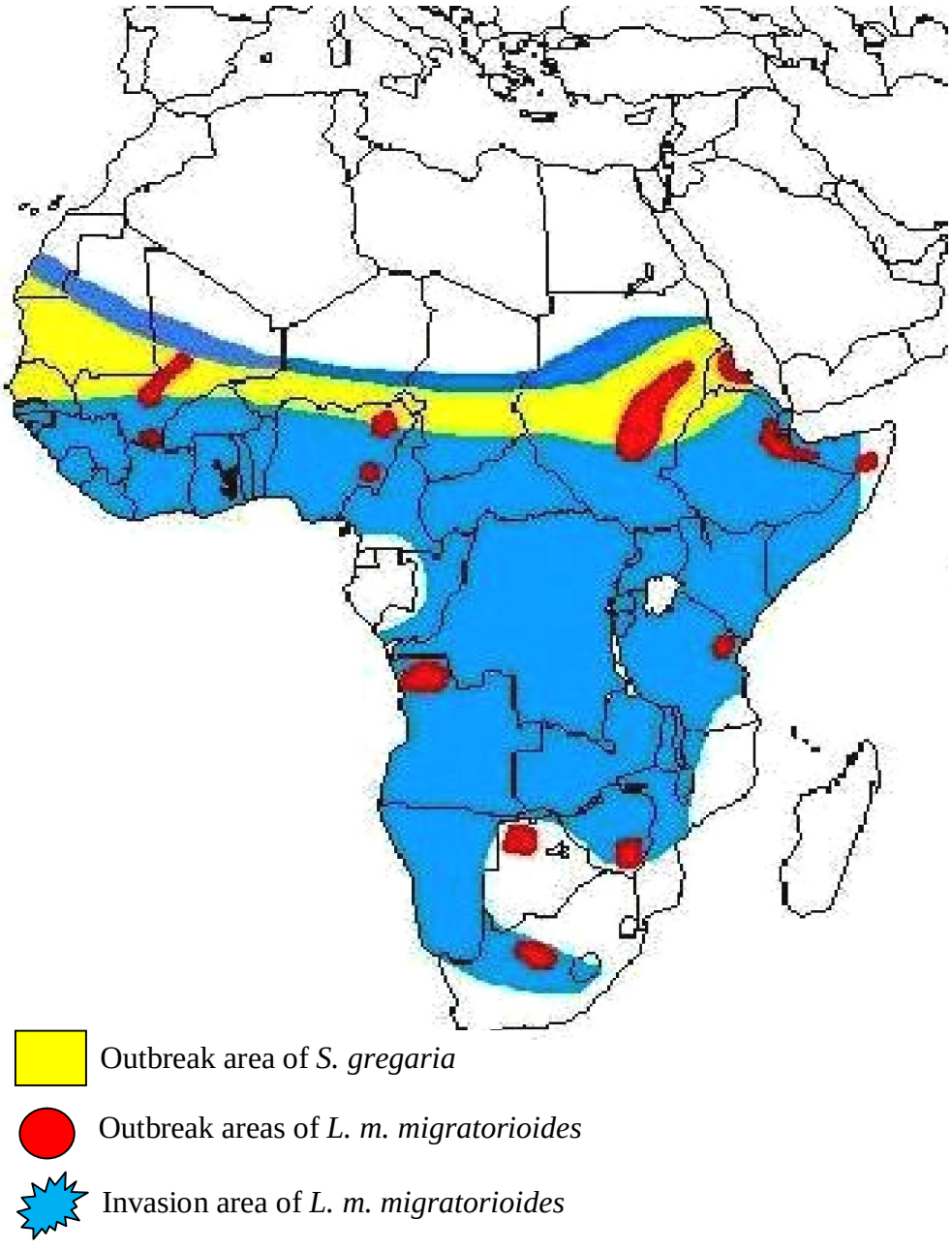

Figure 1.2 Outbreak and invasion areas of *Locusta migratoria migratorioides* and *Schistocerca gregaria* in mainland Africa (Source: Showler, 1996).

Records show that, Madagascar experienced no less than 51 locust plagues during a period of 80 years (1882 to 1962). The longest and most devastating plague lasted for 18 years (1939 - 1957) and led to losses of about US$ 50 million (Randriamanantsoa, 1999; Bergeron, 2001) (Figure 1.3).

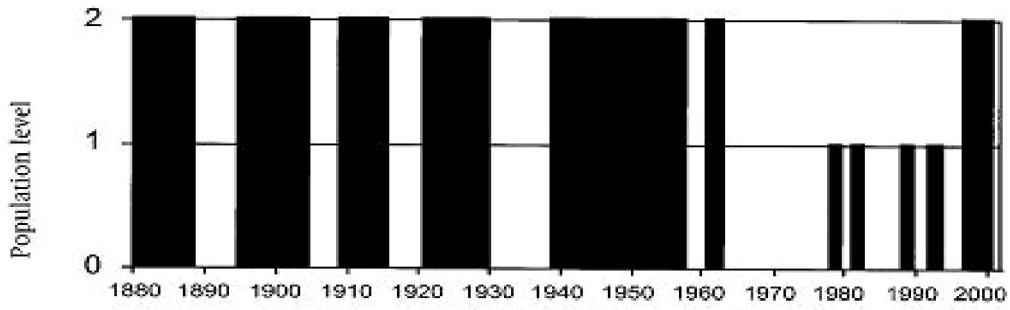

Figure 1.3 Figure 1.3 Outbreak occurrences of *Locusta migratoria capito* in Madagascar Island during the last century. Vertical scale: population levels during (0), Recession; (1) Outbreaks and (2) Invasion periods (Source: Lecoq, 2000).

Substantial amounts of resources amounting to about US$ 3 million per annum is still required to control this pest. Of this total amount, 80% is allocated and spent on the purchase of pesticides (Zehrer, 1999). An uncontrolled locust outbreak may spread and cover up to two thirds of the country, requiring a huge amount of chemical pesticides and deployment of tremendous human and material resources (Duranton *et al.,* 2000; Figure 1.4).

Resurgent invasions occur regularly due to cyclic suitable and favourable eco-climatic conditions probably linked to global climate phenomena and also as a

consequence of lack of funds for sustained locust control (Dinham, 2000; Franc *et al.*, 2003).

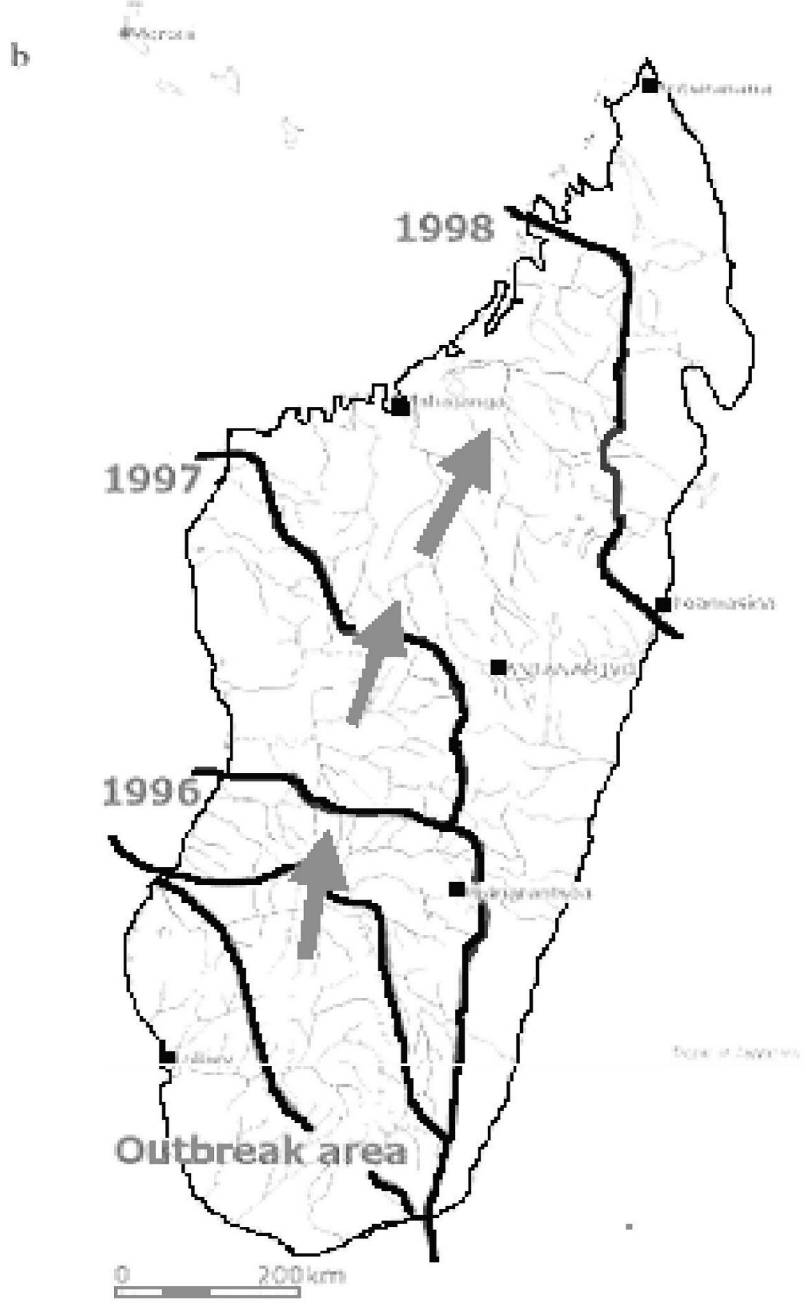

Figure 1.4 Spread and migration (arrows) of the invasion of the latest locust plague in 1996 – 2000 from the outbreak area in the Southwest of Madagascar (Source: Duranton *et al.*, 2000).

1.2.3 General morphology of *Locusta migratoria capito*

The Malagasy migratory locust, *L. m. capito* presents a general morphology similar to other subspecies of the *L. migratoria* family. Their typical shape is defined by the Orthopteroid morphological characteristics. At first view, this species elicits sexual dimorphism in size ranging between 42 – 55mm lengths for males while females have 54 – 72 mm lengths (Figure 1.5) (CIRAD, http://locust.cirad.fr, 2004). The general aspect presents three main body parts.

- **The cephalon** bears most of the sense organs namely a pair of filiform antenna, ocelli and a pair of compound eyes, also the mouth parts bearing the different types of palpi (Chapman, 2003). Antenna and palpi represent the chemosensory organ parts recognized to be involved in food recognition and digestive related functions also the pheromone olfactory detectors (Chapman, 2002).

- **The Thorax** forms the middle part of the body constituted by succession of three main segments with which the pronotum appears the most prominent. Each segment bears two pairs of locomotor organs with adapted motor functions. Articulated and attached to the mesonotum, one pair of membranous wings and a pair of tiny locomotor legs is prevalent. In contrast, massive and prominent hind legs typical to acridids are articulated to the metanotum. The first body part undergoing morphological changes during phase shift comprises the dominant pronotum segment with a saddle shape that gradually acquires morphological variations. Indeed, the pronotum with a convex shape in solitary phase shifts to a concave shape in the gregarious phase locusts (Uvarov, 1966; Pener, 1991).

- **The Abdomen** comprises ten tagmata; each segment is formed by an upper tergum, symmetrical lateral pleurite plates and ventrally the sclerite plate articulated to each other by thin inter-membranes. At its terminal tip, the abdomen bears the genitalia elements formed by differentiated plates, valves and cerci in females whereas in males these later are submitted to constrictions and reduced in size. Valves and plates display on their surfaces numerous setae and sensilla which are acknowledged to serve as chemosensory elements involved in some well defined sexual activities and oviposition processes in females.

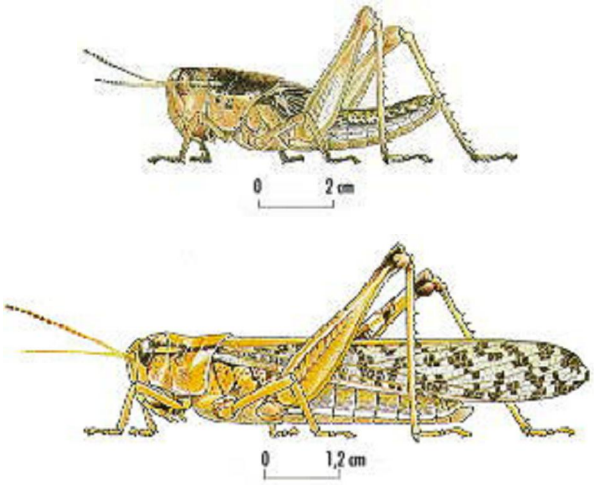

Figure 1.5 Fifth instar gregarious nymph (above) and gregarious adult male (below) of *Locusta migratoria capito* in Les criquets ravageurs (Source: CIRAD, http://locust.cirad.fr, 2004)

1.2.4 Breeding and outbreak areas of *Locusta migratoria capito*

The recession and outbreak areas of *L. m. capito* are located in South - South West of Madagascar (Wintrebert, 1976), covering a total surface area of about 110,000 Km^2 (Figure 1.6) (ANAE, 2004). This is approximatively one fifth of the total area of the

island offering suitable conditions for locust breeding, with temperatures of 25 – 30ºC and 40 - 60% relative humidity (R.H).

This whole area has been subdivided into three biotopes outlined below:

1.2.4.1 Initial Multiplication Area – IMA: This is an area that occupies an estimated 52,000 km^2 which serves essentially as the locust pool. It has been estimated that about 30 to 80 million locust individuals scattered and with generally uniform distribution pass through the area each year and a dynamic population that has a bimodal distribution (Cirad-Gerdat-Prifas, 1997). The biotope is dominated by open savanna and tall grasses.

1.2.4.2 Transitory Multiplication Area – TMA: This biotope has a surface area of 47,000Km^2. Locusts concentrate in this area due to migration of locusts from IMA and rapid multiplication of the local population. It leads to progressive increase in the density of locusts. Usually, the number of locusts in the population is estimated at around 100 to 140 million per annum with unimodal model (Cirad-Gerdat-Prifas, 1997).

1.2.4.3 Area of Densification – DA: This is a narrow band with a surface area of 18,000Km^2 located in the littoral zone. The biotope has a closed formation of bushy forest with many open spaces termed “Clairières Mahafaly” i.e. fallows within the Mahafaly territory. The open areas have savanna type vegetation that is dominated by patches of grass, which facilitate forced aggregation resulting in clumped distribution of the locusts. The number of locusts may be as high 70 - 140

million per year (Cirad-Gerdat-Prifas, 1997). Tremendous and sudden increase in population density may lead to the onset of an outbreak.

The sequence of events of population build up in these biotopes underlies the processes of gregarization of *L. m. capito.* Through the season, the physiognomy of the three biotopes evolves and gradually varies considerably depending on seasonal changes and meteorological factors, mainly rainfall and temperatures (Launois and Duranton, 1997).

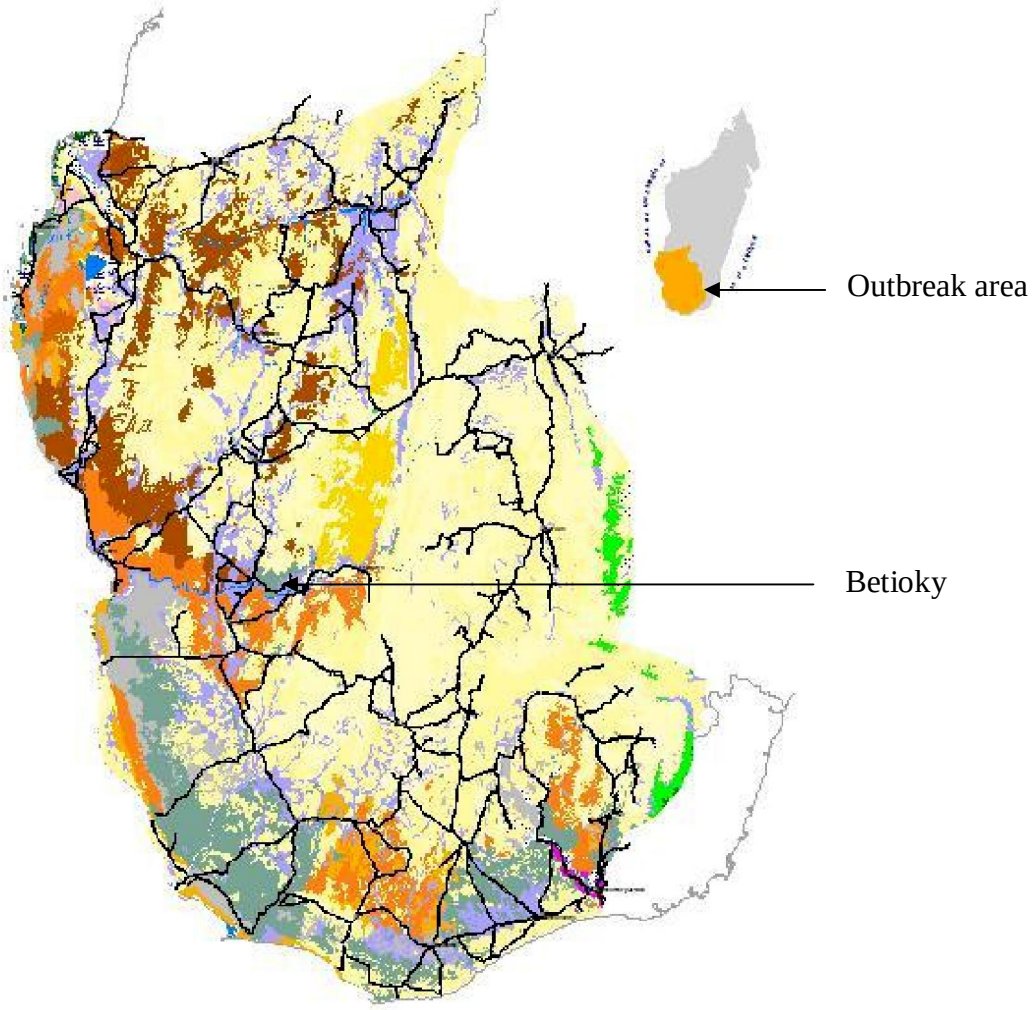

Figure 1.6 South - South Western outbreak area of *Locusta migratoria capito* in Madagascar. A, map of Madagascar island showing location of the area and B, details of the area showing vegetation types and topography (Source: ANAE, 2004)

1.2.5 Biology of *Locusta migratoria capito*

1.2.5.1 Life cycle and cycle duration

Similar to other insects and classified within the class of Orthoptera, *L. m. capito* undergoes different developmental stages steps during its life cycle. From incubated eggs laid in the soil and enclosed in a membranous pod, newly hatched nymphs develop through five to six instars then emerge as sexually immature adults (Scherer, 1999; Lecoq, 2004). These different instars are inter-spaced by several moultings until their fledging into adult locusts. Hemimetabolous, nymphs have a body shape that closely resembles that of the adults with differences only in the presence of wings that characterise the latter. Wing buds on the nymphal instars develop fully into the actual wings on adult locusts. The adult stage comprises the sexually immature locusts that develop and mature sexually upon which male locusts are capable of fertilizing receptive mature females which afterwards, lay eggs after mating (Lecoq, 2004) (Figure 1.7). The complete life cycle of *L. m. capito* ranges between 44 - 66 days in natural conditions, 10 - 14 days of egg incubation, 24 - 38 days of nymph life duration and 10 - 14 days for adults to reach the sexual maturation (Scherer, 1998).

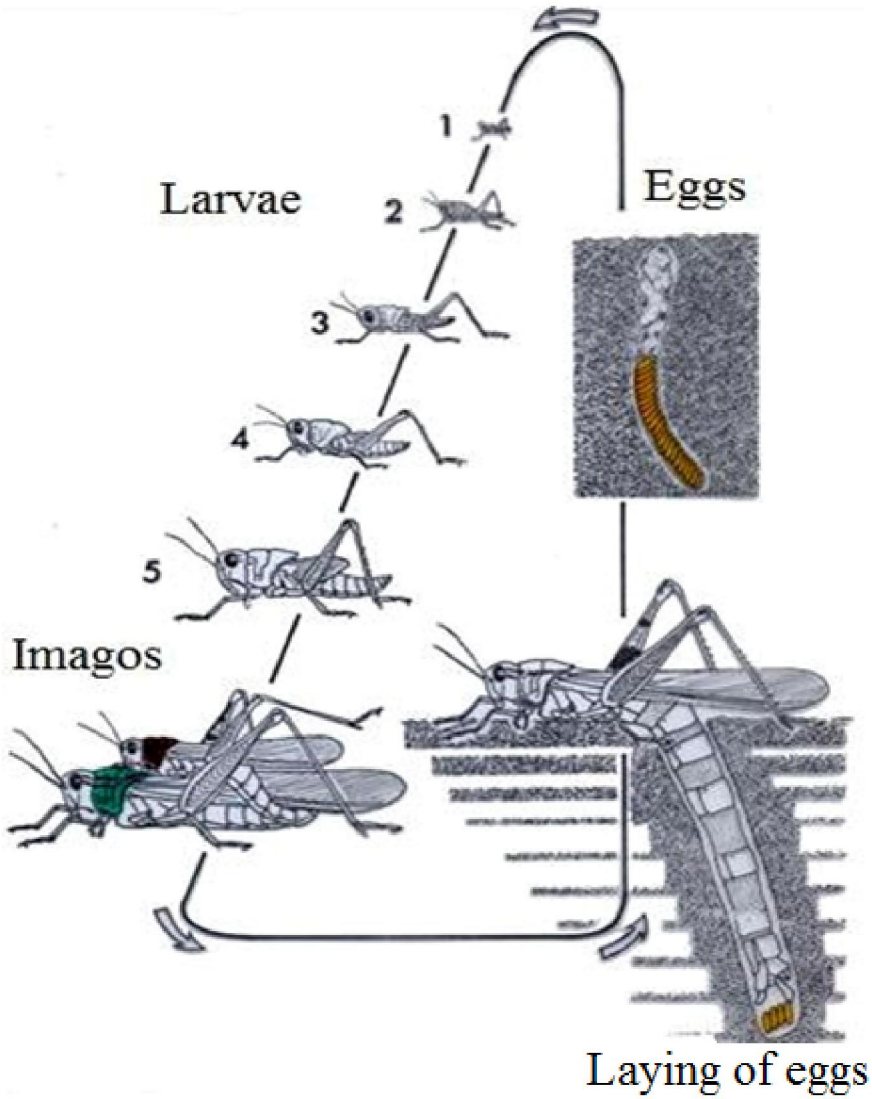

Figure 1.7 Life cycle of *Locusta migratoria capito* with five (1-5) nymphal instars (Source: Duranton *et al.*, 1982).

1.2.5.2 Displacements and number of generation per annum

In contrast to different strains of migratory locusts that are prevalent in different latitudes and which manifest diapause adaptations across the seasons (Kang *et al.*, 1989; Kang and Chen, 1991; Tanaka, 1994), *L. m. capito* belongs to the non-diapausing tropical strain group (Duranton *et al.*, 1982). Annually, four to five

generations have been recorded, presenting differences only in the durations of their cycle throughout the year depending on the seasonal variations (Launois, 1974; Duranton *et al.*, 1982; Lecoq, 2004). The five generations comprise successively the first generation (R1) occurring earlier with the onset of the rainy season and usually in scattered population formation throughout the wide Initial Multiplication Area (IMA). The second generation (R2) coming after the R1 start to migrate and are clumped within the restrained Transitory Multiplication Area (TMA). This latter is followed by the third generation (R3) of locusts that are gathered and confined by convergent winds as well as the restricted suitable environmental conditions prevalent only within the Area of Densation (AD). The gradual increase of population during these migrations and clumping process within the TMA mark the onset of the gradual phase shift. This is amplified by the forced grouped conditions prevalent in the AD that trigger and promote the settlement of psycho-somatic character changes towards the gregarious form within the locust population. Gregarious population of the R4 and/or R5 following the R3 migrate from the Area of Densation to the remaining unoccupied country's territory leading to declared generalised invasion that may cover the whole island if there is no intervention (Launois, 1974). Population fluctuations and displacements within the outbreak area depend on eco-climatic sequential events that promote and lead progressively to gregarization of the locusts (Duranton *et al.*, 1979).

1.2.6 Phase polymorphism and gregariousness

Introduced by Uvarov (1966), the phase polymorphism theory with regard to locusts was proposed to define the ability to a biological breeding adaptation according to the level of population density. This trait of the ability of the locusts to shift reversibly between two quite different morphs, from the *solitaria* to the *gregaria* form passing through intermediate graded levels of differentiations, termed as *transiens*, characterises the main features of the locust taxonomic group. Phase polymorphism manifestations occur in different biological aspects in insect characters namely the morphology and general shape, the morphometrics (Plate 1.1), the physiology and behaviour, affecting even the genetic aspect of its biology (Ellis, 1959; Kennedy, 1961; Uvarov, 1966; Albrecht, 1967; Rowell, 1971).

The acquisition of gregariousness in locusts is gradual and cumulative throughout their successive generations. Indeed, in the two different density population conditions, locusts display quite different shapes and colours in addition to the cryptic behaviour and their tendency to avoid one another thus called "*Solitaria*" in a prevalent low density population. In contrast, a gradual or rapid increase of individual numbers, "density shock", when reaching the threshold number of 2000 to 2500 individuals/ha for *L. migratoria* and 500 individuals/ha for *S. gregaria* leads to a gradual shift toward the gregarious form, denoted as "*Gregaria*", (Uvarov, 1966; Boughdad, 1991). This latter exhibits dense grouping tendency, synchronised movements and high level of interaction between individuals in a population. The

gregarious characteristics lead to the formation of hopper bands in nymphal stages and migrating swarms, in adult locusts.

In different species of locusts, though gregariousness is generally common and a common phenomenon, specie-specific gregarious characters are evident. For some species such as the desert locust, *S. gregaria* the behavioural trait is first acquired after a few hours of increasing population density (Roessingh and Simpson, 1994). However, while in the different subspecies of migratory locusts, morphological and colour changes are more pronounced and noticeable. Latent characters are also affected. In gregarising locusts, the prominent pronotum segment undergoes shape modifications and variations passing from the convex shape in its solitarious phase to concave, and becoming saddle-shaped when shifting to the gregarious phase (Pener, 1991).

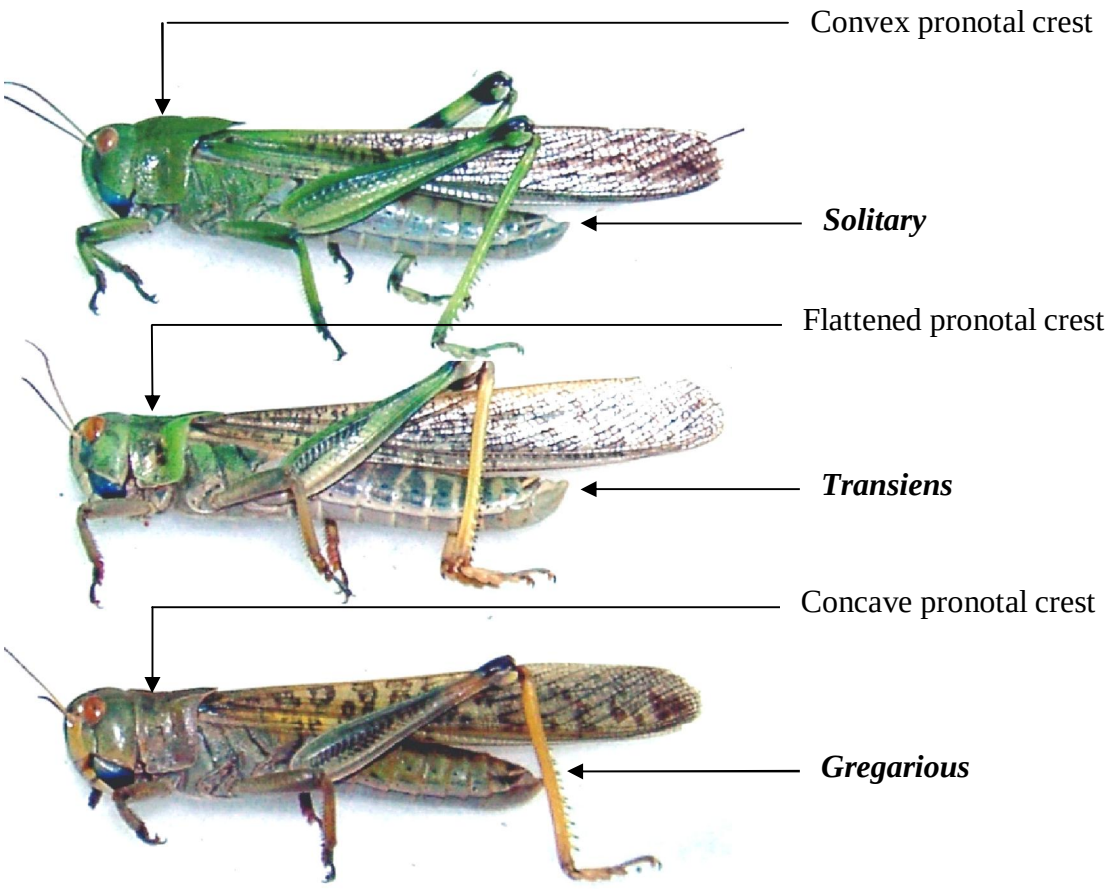

Plate 1.1 Gradual morphological and colour changes from solitaria to gregaria in *Locusta migratoria spp.* (Source: Tanaka and Zhu, 2005)

1.2.7 Biological changes and phase shift adaptations

Solitary and gregarious locusts present a set of characters that differentiate them from one another, ranging from genetic (Nolte, 1967; Kang *et al*., 2004), body shape and biometry of some parts of the body (Uvarov, 1966; Wintrebert, 1970; Uvarov, 1977; Roessingh and Simpson, 1994, Bouaichi *et al.*, 1995; Roessingh *et al.*, 1998), chemistry and biochemistry (Nolte, 1963; Pener, 1991; Deng, 2001), metabolism and hormonal balance (Dale and Tobe, 1990; Breuer *et al.*, 2003). Other traits include their developmental physiology and behavioural trait adaptations (Uvarov, 1966, 1977; Wilson, 2000; Despland, 2004). However, the behavioural labile characters are the first undergo alteration and are visible after only a few hours of crowding of the locusts (Roessingh and Simpson, 1994; Despland and Simpson, 2000). This appears to be a physiological shift from the cryptic, solitarious locusts are shy and have a tendency to avoid one another towards the highly inter-attractive gregarizing locusts that form cohesive groupings that display synchronous behaviour (Bouaichi *et al.*, 1995; Simpson *et al.*, 2001). In addition, gregarious locusts undergo only five nymphal instars while the solitarious counterparts have six (Scherer, 1998).

1.2.8 Gregarisation process and phase plasticity in locusts

Gregariousness in locusts is a fundamentally induced and an adaptation reaction in response to a gradual increase of density and crowded population conditions (Roffey and Popov, 1968). Reversible transformation from the *solitariform* to the *gregariform* through several grades forms of *transiens* occurs during gregarisation and/or solitarisation shifts in locusts. Different factors underlie the phase shift

processes and group cohesion such as the ability of maternal transmission of their gregarious characters to their progeny (Mousseau and Dingle, 1991; Hagele *et al*., 1998; McCaffery *et al*., 1998; Tanaka, 2006). Also, changes in hormonal balance induces phase change and plays preponderant role (Tawfik and Sehnal, 2003; Maeno *et al*., 2004; De Loof *et al.*, 2006). Eco-climatic and environmental cues aggravate the phenomenon through visual interactions (Kennedy, 1939; Volkonsky, 1942; Ellis and Pearce, 1962; Roessingh *et al.*, 1998), tactile excitations (Chauvin, 1941; Ellis, 1952, 1962, Simpson *et al*., 2001; Rogers *et al*., 2003), dietary regime (Jackson *et al.*, 1978, Simpson *et al*., 2001), and the olfactory signals (Ferenz, 1990; Pener, 1991, Obeng'Ofori *et al*., 1993; Torto *et al.*, 1994; Pener and Yerushalmi, 1998). However, controversies still abound with regard to the well-defined roles of these extrinsic and intrinsic factors individually and/or in combination (Despland, 2001; Simpson *et al*., 2001; Matheson *et al*., 2004).

1.2.9 Management and control of locust

1.2.9.1 Traditional methods

Crushing: The oldest method in Madagascar for destruction of gregarious locust used by the Malagasy people consists of beating and crushing locusts with cut tree branches. It is labour intensive and less effective, especially when used on flying adult locust in swarms (Randriamanantsoa, 1999).

Burning: In Madagascar, burning of roosting locusts has also been used for destruction of *L. m. capito* (Randriamanantsoa, 1999). This method requires flame-

throwers but remains very dangerous and hazardous to the environment and also to those involved due to the possible uncontrollable spread of the fire.

Barrier traps: An improved metal sheet barrier-trap was developed by Zolotarevsky in 1927 (Duranton *et al.,* 1982; Randriamanantsoa, 1999). These are placed across paths of wandering hopper bands and the trapped hoppers are either crushed or harvested for food (Randriamanantsoa, 1999). Locusts are also harvested early in the morning or at night or during mating and laying of eggs when their mobility is reduced. This method requires cooperation of many people to be able to capture large numbers of locusts.

1.2.9.2 Chemical control

For the last eight decades, synthetic insecticides have formed the main arsenal for control locusts. The onset of the chemical industry era in early 1940s - 1950s, marked the rise of domination of locust control by the massive use of synthetic insecticides that provided a broad range of chemical compounds with effective acridicidal properties (Rakotoarimanana, 1997). Various chemical acridicides have been used and sprayed in different formulations and concentrations.

Organochlorines: Organochlorines have been heavily used to control locust outbreaks and invasions. Since early times, and like many countries facing locust problems, Madagascar has poured and sprayed huge quantities of DDT, the principal chemical weapon offering a reasonable cost-effective option that justified its heavy use (Rakotoarimanana, 1997). Locust control has followed the development of the

chemical industry discoveries with the rise of different new organochlorine compounds namely: dieldrin, hexachlorohexane (HCH) and Lindane. However, the general issues with regard to these organochlorides are the harmful effects on health of humans and animals because of their bio-accumulation induced effects in addition to being carcinogenic after long term exposure. These, at first, led to the withdrawal and ban of spraying of DDT in agricultural usage in 1989, followed thereafter by the ban of use of all organochlorine-based insecticides in locust control (Rakotoarimanana, 1997).

Organophosphates and carbamates: Due to health effects of organochlorines, organophosphate insecticides have been used as an alternative for locust control starting the 1970s. A wide range of organophosphate compounds have followed the era of organochlorines, and were tested and sprayed in different formulations and various ranges of concentrations to contain the locust menace. The most commonly used organophosphates acridicides are: chlorpyriphos and Malathion, diazinon, dichlorvos, fenitrothion, fenthion, pyrimophos and sumithion.

Besides, carbamates with toxicological effects almost similar to those of organophosphates have also been used. These represent a group comprising several representatives of carbamic acid esters and are considered to be strongly toxic and persistent compounds (Matolcsy *et al.,* 1988; Van Emden and Service, 2004). Although effective, there are health issues with regard to human neurotoxicity, environmental pollution and development of resistance by the locusts.

Pyrethroids: Synthetic pyrethroids are highly toxic to insects but have low toxicity to mammals. They are stable and are sprayed as residual contact poisons. Their activity targets the physicochemical processes on the nerve membrane, similar to DDT.

Deltamethrin and permethrin have been found to be effective on locusts. However, because of their non-specificity, they are very harmful to beneficials leading to imbalance of the ecological and biodiversity climax, thus, leading to resurgence of the pest. They are also toxic to aquatic fauna and other beneficial insects (Van Emden and Service, 2004).

Phenylpyrazole: The 5-amino-1-[2, 6-dichloro-4-(trifluoromethyl)phenyl]-4-[(1-R,S) (trifluoromethyl)sulfinil]-1-pyrazole-3-carbonitrile, commonly called fipronil constitutes a powerful chemical agent in locust control in full coverage sprays as well as in barrier treatments to-date. Its uptake is by contact and oral ingestion and acts through the nervous system γ-Amino Butyric Acid (GABA) blockage (Cole *et al.*, 1993; Hamon *et al*., 1996), thus, interfering with the passage of chloride ions by the channel under control of γ-amino butyric acid. This induces blockage of the regulatory system and translates into hyper-excitations.

Often used as last resort arsenal, the harmful impacts of fipronil on commercial insects and non-target entomofauna have led to progressive withdrawal and eventual ban of its intensive use in locust control (Raveloson, 2000; Rakotondravelo, 2001; Razafindraleva, 2001).

1.2.9.3 Insect Growth Regulators (IGRs) and Juvenile Hormone Analogues (JHAs)

IGRs and JHAs such as Imidacloprid, Dimilin® and Alystin® have been presented as very promising synthetic control agents for locust control because of their specificity to insect metabolism deregulation of hormonal balance during post-embryonic development and also the inhibition of cuticle chitin synthesis (Scherer, 1999a). Chemically and biologically, JHAs are intermediates, derivatives or homologs of the juvenile hormone, whereas, IGRs display growth and moulting hormone-like properties deregulating the insect's normal hormonal balance during the moulting process, hence, inflicting organ abnormalities and malformations during metamorphosis and ecdysis of insects.

Their molecular stability leading to long persistence property under field conditions drastically reduces the number of sprays in the environment thereby making them suitable for application as barrier treatment agents for the control of hopper bands (Rakotoarimanana, 1999). However, their toxicology effects on other groups of insect and their environmental impact require additional studies to be conducted. Further, aspects that limit their use include the timing restrictions in the application because of their activity on very defined stages of the target insects (only effective on nymphal stages) and their effects on a broad range of non-target insects. The use of these agents also requires an early detection of hopper bands. Furthermore, possibilities of locusts developing resistance to them and their high cost minimizes their use for large scale locust control.

From the foregoing, although chemical control tactics are efficient on one hand, on the other, they are harmful to humans and the environment including non-target fauna and flora. Hence, this has led to the withdrawal of several harmful compounds in the control of locusts. Alternative methods for combating the locust menace that have been advocated for are outlined below (Ferenz *et al.*, 1994; World Bank, 1998).

1.2.9.4 Biological control

Natural enemies: Numerous parasites, predators and parasitoids that attack different developmental stages of *L. m. capito*, from eggs to the adult stage have been identified. They include birds (*Milvus migrans, Falco concolor*; *Falco newtoni*; *Merops superciliousus*), lizards and reptiles (*Chalarondon madagascariensis*, Iguanidae, Sauria), insectivorous mammals, spiders and other insects (*Microstylum magnum*, Asilidae, Diptera). Endo-exoparasites such as hymenoptera (*Scelio spp*), diptera and acari (*Trombidium sp* and *Euthrom bidium*) are also known to inflict considerable reduction in locust populations (Raholijaona, 1999; Razafindratiana, 1999).

Plant bioactive compounds: Since early times, insecticidal and acridicidal properties of neem tree, *Azadiracta indica* A. Juss (Nimo) and *Melia azedarach* (Voandelaka) (Meliaceae) were acknowledged by Malagasy planters and used as plant protection methods. Various traditional methods using different parts of the plants were able to provide applicable formulations that elicit insecticidal effects in addition to the anti-feedant and repellent properties observed (Fanantenana and Scherer, 1999). Various improved preparation methods were used thereafter that

permitted the extraction and identification of the main active ingredient, namely, the C-*seco*-limonoid group of triterpenoid: azadirachtin.

Plant bio-active components offer numerous beneficial properties, such as their easy handling and their innocuity to human and domestic animals. However, mass production is not yet ready to cope with the locust control needs (Volkonsky, 1937; Wilps *et al*., 1992; Kang *et al*., 1993; Mordue *et al*., 1993; Linton *et al*., 1997).

Fungi: Currently, entomopathogenic fungi offer a very promising future for the anti-locust control due to their specificity to locusts, especially in the prevention method to control of hopper bands (Lomer *et al.,* 2001). An indigenous strain of Metharizium anisopliae var acridium (SP9 Strain), only specific to locusts was identified in Madagascar in the mid-1990s (Delgado *et al*., 1997). Preliminary tests on their efficacy and efficiency for control of hopper bands of L. m. capito have been ongoing in the field and have produced promising results (Delgado *et al*., 1997)**.**

Pheromones as new tactic in the control of locust: Semiochemicals including pheromones are gradually being integrated as new biotechnology control agents to insects in various domains. Because they are species-specific and their mode of action mainly involves induction and/or alteration of well-defined behaviour during a specific period in the lifespan of a given insect, these tools are environmentally friendly and efficient and have made significant impact on management of plant pests and disease vectors (Knights, 2010; Vacas *et al.,* 2010; Campos and Thomas, 2010).

Use of semiochemicals comprising of pheromones, have been advocated and offered a new trend in locust control because of their species specificity and also their potential effects at very low doses of applications, hence, offering a very environment friendly tool (Ferenz *et al.*, 1994). The integration of these active bio-molecules in the arsenal of controlling locusts has already been demonstrated on their effects on the desert locust, *S. gregaria* (Bashir *et al*., 2000b). The possibility of their use on different locust species such as the Malagasy migratory locust may offer a new method that broadens the locust control capacity for the country with cost-effective and environment friendly tools.

1.2.10 Semiochemicals and pheromones in insects and locusts

A "'pheromone'" is any chemical or set of chemical compounds produced and released by a living organism that transmits a message to another member of the same species. The term "pheromone" was introduced by Karlson and Luscher in 1959 (Thomas, 2003). Chemical communication in insects is highly developed. Under normal physiological conditions they can detect and respond to remarkably small quantities of the biologically active chemicals (Cork *et al.*, 1990). Pheromones were defined later as all info-chemicals that mediate an interaction between two organisms of the same species whereby the benefit is the origin-related organism, to the receiver or both (Dicke and Sabelis, 1988; Jervis, 2005). Communication in insects utilize complex mechanisms involving combination of visual, acoustic and olfactory signals (Prestwich, 1985), though olfaction is the most acute sense in

insects and roles of olfactory cues are the basis of various behaviours and interactions (Anton *et al.,* 2006).

1.2.10.1 Pheromones in species of locusts

Pheromones play a key role in the cohesion and aggregation behaviour of locusts (Uvarov, 1921; Byers, 1991). Further, Volkonsky (1942) provided the evidence of olfactory attraction of *S. gregaria* hoppers to each other in the field, but without sufficient details. Observations on the association of isolated locusts with crowded locusts provided the evidence for mediation of pheromones in airborne volatiles of *S. gregaria* hopper faeces (Nolte *et al.,* 1963; Nolte, 1970). 5-ethylguaiacol named "Locustol" and presumed to be the major volatile component of crowded *L. migratoria* hopper faeces, was believed to be the aggregation pheromone (Nolte *et al.*, 1973; Nolte, 1976). However, many other follow-up investigations have not been able to detect "Locustol" and the current position is that it was a mis-identification (Fuzeau–Braesch *et al.,* 1988; Whitmann, 1990; Torto *et al.,* 1994; Schmidt, 1997; Ferenz and Seidelmann, 2003; Schmidt and Albutz, 2004).

1.2.10.2 Aggregation pheromones of some species of locusts

In the last decade, re-investigations on the implications of chemical mediation in the gregarious behaviour of *S. gregaria* adults and hoppers using improved bioassays and analytical procedures have provided insight into their chemical ecology. There are two stage-specific and very distinct aggregation pheromone systems; one for the nymphs and the other for adult locusts, and each stage only responds to its own pheromone (Obeng'Ofori *et al*., 1993).

Further, research on adult *S. gregaria* stages showed that only older gregarious males produce the pheromone. The gregarious adult pheromone comprises of six aromatic compounds, *viz* phenylacetonitrile (PAN), guaiacol, anisole, veratrole, benzaldehyde, and phenol (Torto *et al.*, 1994). Phenylacetonitrile comprises about 75 –80% of the volatile blend. Results also confirmed sexual and stage differentiation in the production of pheromones. Individuals of the two phases *solitaria* and *gregaria,* respond equally to the pheromone emitted by older gregarious adults. This may play a role in recruitment of solitaria individuals into the group during the early stages of a locust outbreak (Njagi *et al.*, 1996). In contrast to the desert locust, both male and female adults of *L. m. migratorioides* produce a blend of similar compounds, though in different amounts (Niassy *et al.,* 1998). The pheromone produced interacts as a mutual attractant agent between the two sexes. In addition, volatile emissions are compositionally richer *L. m. migratorioides* than in *S. gregaria.* Fourteen compounds comprising of nine different aliphatic aldehydes and five different alcohol components were detected antennographically in volatiles of *L. m. migratorioides* (Niassy *et al.*, 1998).

With regard to *S. gregaria* hoppers in second to fifth instar nymphs, ten compounds comprising of C_6 – C_8 and C_{10} straight chain aldehydes and acids were identified in their volatiles, but these compounds only evoked moderate aggregation responses. Strong aggregation responses by nymphs were observed when a blend of these compounds in combination with some of their faecal volatiles components comprising guaiacol, phenol and indole (Torto *et al.*, 1996; Hassanali and Torto, 1999). Similarity has been observed in the composition of volatile compounds

produced by hoppers of *S. gregaria* and *L. m. migratorioides* but with lower amounts in the latter. This may explain the cohesion of mixed hopper bands of the two species frequently observed in the field (Niassy *et al.*, 1998).

1.2.10.3 Cross stage pheromonal effects

Nymph and adult volatiles have both dual roles depending on the status of the locust population.

1.2.10.3.1 Effects of nymphal pheromone on adults

In *S. gregaria* and *L. m. migratorioides*, volatiles emitted by late fifth instar nymphs play dual role including the aggregation of the hoppers themselves and as sexual maturation retardant to maturing adults. In contrast, for the immature adult locusts the adult locust pheromone is an accelerant for their sexual maturation and also acts as an aggregant for the adult locust conspecifics (Norris, 1964; Assad *et al.*, 1997).

1.2.10.3.2 Effects of adult pheromone on nymphs

Phenylacetonitrile (PAN) has various effects on nymphs of different ages when they are exposed to it. PAN shows deleterious effects on conspecific nymphs such as: reducing grouping behaviour and the uptake of food. It also induces physiological change in nymphs and enhances mortality caused by sub-lethal doses of insecticides. For example, combination of PAN with fenitrothion enhances the efficacy of the latter by increasing the mortality rate up to slightly over four times more than fenitrothion alone (Kane, 2004). PAN also induces growth disruption effects such as

delaying moulting and causing malformations in fledglings emerging from treated nymphs (Kane, 2004).

1.2.10.4 Possible use of pheromones for monitoring and preventive control in locust population dynamics

Predicting and controlling the onset of hopper band formations as well as swarm build up of adult locust populations at their early stages are critical in the locust survey. Early detection of newly forming hopper bands, prediction of the egg laying locations and aggregative behaviour of adults will facilitate their monitoring and control. These will enable advance preparation in strategy and logistics needed for their control operations. Various parameters of their behaviour can be monitored using semiochemicals, such as the aggregative and reproductive behaviour under control of the aggregation, oviposition and sex pheromones. These different behaviours have each been hypothesized to be under control of specific pheromone in the biology of the locust (Byers, 1991).

1.2.10.4.1 Monitoring of locust population dynamics using aggregation and dispersal control methods

As preventive methods of locust upsurges are currently advocated and promoted rather than curative methods, use of specific, appropriate and efficient tools commencing with the early and less destructive stages of *L. m. capito* are desirable. Implementation of new tactics using specific and environmentally friendly tools that may target hopper band formations and also build-up of swarms are the most advocated for methods and they offer alternative approaches to controlling insect

pests (Ferenz *et al.,* 1994; Cork *et al.,* 2003). Also, aggregation and group cohesion may be monitored with efficient aggregation pheromone aiming to control the dynamics of the gregarious/solitarious population.

1.2.10.4.2 Mating and sexual behaviour control method

Disruption of mate recognition (attraction and repulsion) aiming to desynchronize population sexual maturation by the use of sex pheromone, mat lead to the deregulation of egg laying synchrony that may give rise to asynchronous nymph hatching. Use of sex pheromones may, thus, provide a significant impact in the sizes of newly formed hopper bands and swarms and/or in their early build up (Rosa Paiva, 1997). Pheromone control has been shown to be effective on the cotton pest *Pectinophora gossypiella* Saunders (1843) by the use of pink bollworm sex pheromone (Gossyplure) as a mating disruption agent (Copping and Hewitt, 1998).

1.2.10.4.3 Synchronous and communal oviposition site control method

Implementation of methods that enable monitoring of ovipositing locusts by the use of oviposition pheromone as an orientation stimulus to gravid female locusts toward suitable, controlled and accessible oviposition sites may be implemented (Rosa Paiva, 1997). The method will essentially aim at either control of synchronous oviposition into a delineated sire that is pre-treated with biopesticides that decimate the eggs or, the dispersal of ovipositing female locusts to an expansive area to reduce clumping of emerging hoppers.

1.2.10.4.4 Prediction and survey of forming hopper bands and swarming adults

Marching hoppers may be re-orientated toward biopesticide pre-treated sites by pheromones, thus reducing the sprayed surface of treatments that subsequently reducing spraying of large quantities of pesticides into the environment.

1.2.11 Models of successful innovative and applied pheromone based-method in pest control

Success and efficiency of the semiochemicals require good timing, assessment and accurate localisation of the infestations prior to and during their applications. Assisted by geographic Information Systems (GIS), semiochemicals form a powerful method for control and monitoring of invasive pests. For example, a combination of GIS monitoring and application of semiochemicals are currently being used for monitoring the population dynamics of some rare insects and endangered species of beetles in Sweden (Mattias Larsson, pers comm.). Also, the technique is in use for the survey of the dispersal of the accidentally introduced invasive pest, the gypsy moth in the USA (Liebhold, pers comm.). These two pilot projects demonstrate the versatility and potential of this new method in the survey and monitoring of insects by providing real-time information with regards to the population dynamics and dispersal of the insects. They offer a revolutionary model that may be applied to various conservation and pest management projects (Mattias Larsson, pers comm.) (http://www.sandyliebhold.com and http://da.ento.vt.edu) that may be applicable on the Malagasy migratory locust.

1.3 Statement of the problem

Use of insecticides for control of *L. m. capito* during major outbreaks requires about US$ 50 – 60 million. It is therefore expensive and polluting to the environment. Preventive methods aimed at controlling the locusts prior to formation of hopper bands and swarms in the locust breeding habitats may reduce the amount of chemical pesticides needed. Research and field trials on the desert locust with bio-control agents based on semiochemicals and mycopesticides, or a combination of these two, have demonstrated to be promising tools for the control of this species and far less expensive and non-polluting to the environment.

The chemical ecology of desert locust is now well understood although many of the aspects of the aggregation and gregarization processes are yet to be studied (Hassanali *et al*., 2005). Some work has been initiated on the chemical ecology of African migratory locust. However, to date no study on the chemical ecology of *L. m. capito* has been reported. Characterization of the pheromones of other locust species, such as *L. m. capito*, and elucidation of the roles they play in their gregarization and aggregation behaviour may open up similar useful methods for monitoring the crucial phase of aggregation and phase shifts in this species.

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1.4 Justification

Extensive use of chemical pesticides for control of *L. m. capito* is both a health hazard to humans and other organisms and pollutes the environment. It is also a drain of up to US$ 50 million on the country's revenue during an invasion.

Madagascar has a rich and unique but fragile biodiversity and is one of the ten biodiversity hotspots in the world. Hence, the use of more environment-friendly locust control methods has been advocated (World Bank, 1998). These include use of locust-specific mycopesticides and insect growth regulators (IGRs), mainly benzoylureas, supported by an efficient locust monitoring system.

Recent work at *icipe* on the desert locust has shown, in well-documented cases (Torto *et al*., 1996; 1999a; Hassanali and Torto, 1999), that it may be possible to control locusts using their own conspecific pheromone. In addition, the pheromone enhances the susceptibility of the treated insects to chemical and fungal pesticides. Fractional doses of these pesticides combined with the pheromone lead to levels of control comparable to the full-recommended doses of the pesticides.

1.5 Hypotheses

1 – Adult locusts of *Locusta migratoria capito* utilize semiochemicals derived from body and faecal volatiles for aggregation and phase change;

2 – Nymphal and adult stages of *Locusta migratoria capito* have distinct aggregation pheromone systems; and

3 – Components or blends of components of the aggregation pheromone of *Locusta migratoria capito* have a disruptive effect on the grouping behaviour of their conspecific nymphal stages.

1.6 General objective

To identify and characterize the aggregation pheromone of adult Malagasy migratory locusts, *Locusta migratoria capito* and determine their effects on conspecific nymphal stages.

1.6.1 Specific objectives

i. To determine the role of body and faecal volatiles released by gregarious adult *Locusta migratoria capito* on their aggregation behaviour;

ii. To identify the chemical components in the gregarious adult locust volatiles that constitutes the aggregation pheromone.

iii. To determine the role of single compounds and blends of the identified active compounds in gregarious adult locusts to assess their respective solitarising effects on conspecific nymphal stages;

iv. To determine the effects of the identified component PAN on the aggregation and anemotactic behaviour of both adult and nymphal stages *Locusta migratoria capito* under laboratory conditions.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 *Locusta migratoria capito* strain and rearing

Gregarious adults of *L. m. capito* from a colony raised and maintained in the Biocontainment Unit (BU, quarantine) at the facilities of *icipe* (Nairobi, Kenya) were used. The entire colony was propagated from egg pods of Malagasy migratory locust collected from the outbreak area, in Madagascar, during the onset of the 2006 locust control campaign. The eggpods were packaged in moist sand in metal tubes and sent by courier to *icipe* then incubated under appropriate conditions in the insectary within the Biocontainment Unit.

The mass rearing room measuring 4.5 x 3 x 2 m (Plate 2.1) was maintained under photoperiodic regime of 12L: 12D, temperatures at 30 ± 2ºC and 40 - 60% ambient relative humidity. Atmospheric air in the room was continuously purified by an exhaust fan and duct system that maintained negative pressure. Emerging nymphs and subsequent generations were kept under crowded conditions using aluminium cages (dimension: 50 cm x 50 cm x 50 cm) with clear Perspex front (Plate 2.2). Each cage held an average number of 150 to 300 locusts. Locusts were fed daily with wheat bran and fresh 14 - 21 day-old wheat seedlings (Ochieng'Odero, 1994; Niassy *et al.,* 1998). Nymphs and adult stages were kept separate to eliminate pheromonal interactions and their subsequent effects on the behaviour of the locusts.

Solitary locusts were obtained by singly isolating each of the newly hatched nymphs in separate cages (dimension: 10 x 10 x 24 cm) in another room different from the one used for the parental colony. The room was set at the same conditions as described earlier and the locusts were treated similarly to those in the parental colony. To minimize air saturation of the ambient air with locust volatiles in the room, air was exhausted from above the cages through a vent in the ceiling and into a duct system that had 10 - 25 air changes per hour.

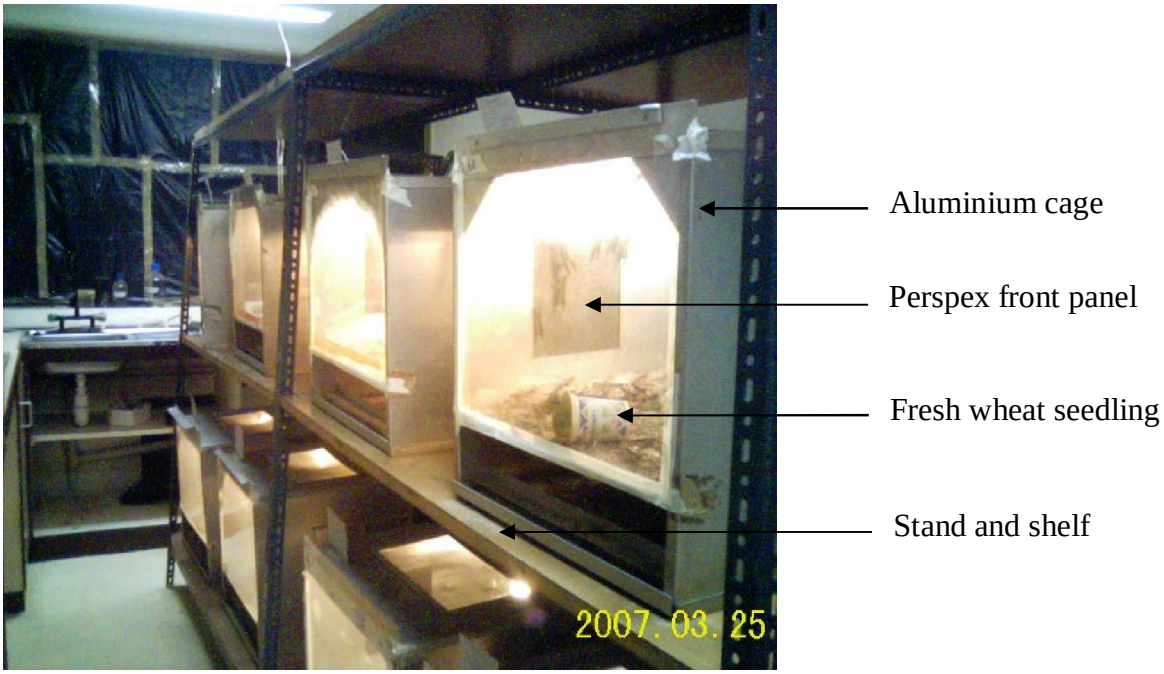

Plate 2.1 Locust mass-rearing room with aluminium cages on shelves.

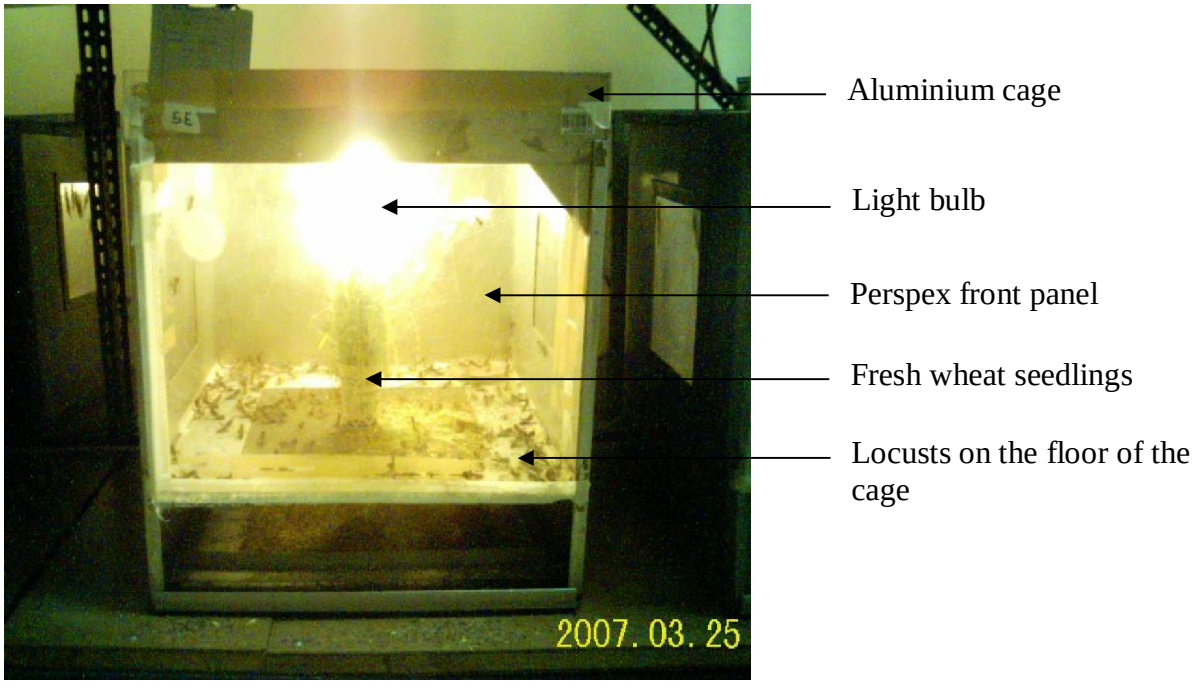

Plate 2.2 Aluminium cage for rearing of locusts.

2.2 Olfactometric apparatus

2.2.1 Single chamber olfactometer for aggregation bioassays

The bioassay device was a single glass chamber (dimension: 60 x 30 x 30cm) with two air inlets through the bottom, one to either side of the chamber as described in detail by Obeng'Ofori *et al.* (1993) (Figure 2.1). The olfactometer was placed under an exhaust hood and kept at a room temperature of 30 ± 2ºC and 40 - 60% R. H. The olfactometer chamber was illuminated by two parallel fluorescent tubes (60W) positioned above it in order to provide uniform lighting within the arena. A removable fine metal wire mesh (1 mm^2) covered the top of the chamber. The base was a galvanized iron sheet with fine holes (2 mm diam.) with a spacing of 1cm, through which the test volatiles and clean air were delivered into the chamber. The source of volatiles was held in a flask while a similar empty flask was used as control (clean air). These flasks were placed below the bench to prevent visual contact between the test (locusts in the arena) and the source locusts (held in one of the flasks). Teflon® tubings were used to connect the flasks to the arena through funnels (base length 28 cm) underneath the base of the chamber. The funnels permitted the delivery of the test volatiles to each half of the chamber. All the connecting joints were sealed with Teflon® thread tape to ensure they were air tight, thus minimizing any losses of the test volatiles that were delivered in charcoal-filtered compressed medical air (BOC gases, Kenya). The two air streams delivering the stimuli were regulated and maintained at a flow rate of 120 ml/min on either side by separate flow meters (Aalborg, Orangeburg, NY, USA) that were connected between the holding flasks and the base of the olfactometer. The flasks and the olfactometer device were

flushed with clean air in between successive replicates to avoid contamination of volatiles. Control and test flasks were regularly switched to avoid positional bias.

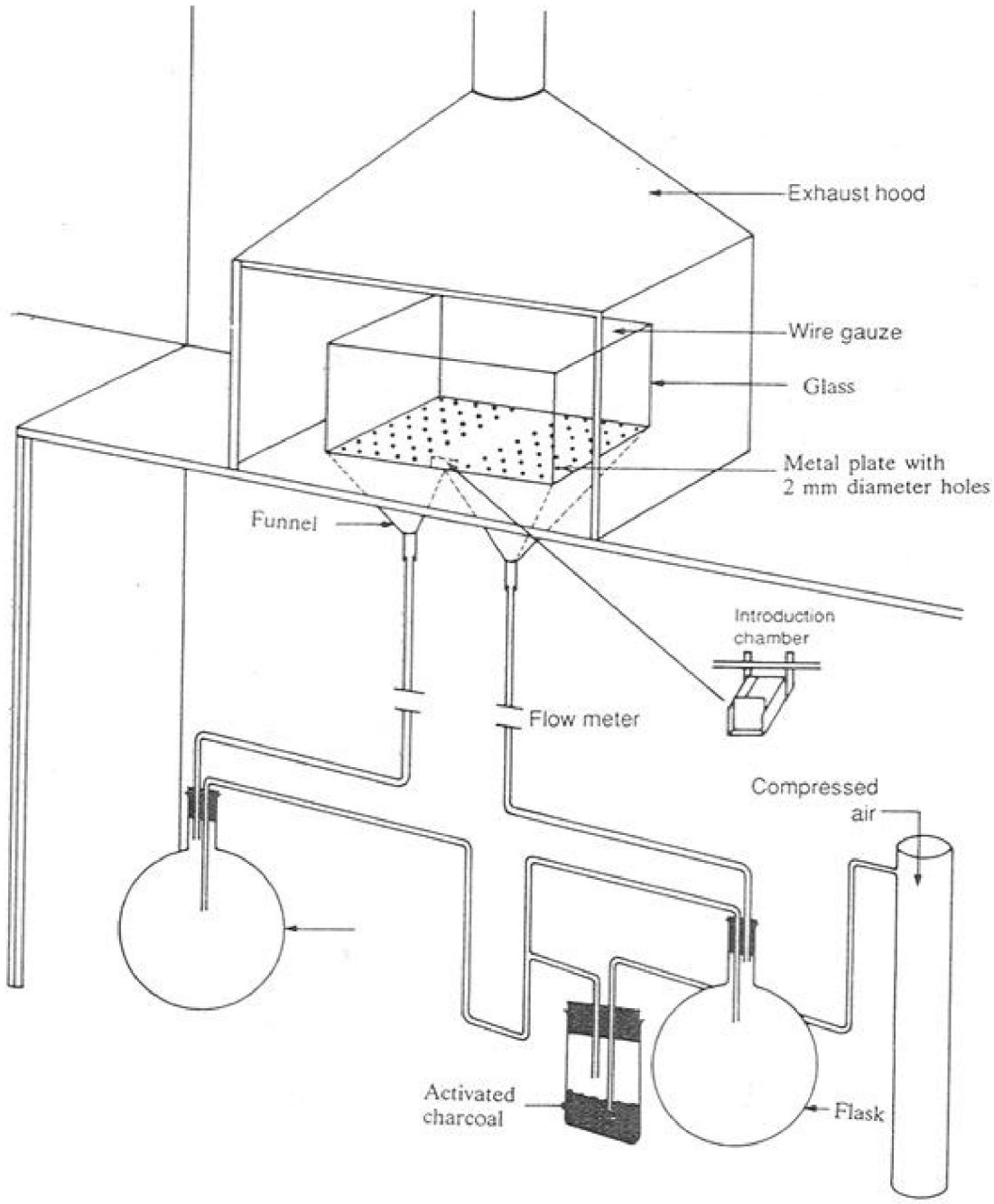

Figure 2.1 Two choice chamber olfactometer device with a vertical air supply system (Source: Obeng'Ofori, 1993)

2.2.2 Wind tunnel for anemotactic bioassays with phenylacetonitrile

The anemotactic responses of the locusts to phenylacetonitrile were evaluated in a two-choice wind tunnel (Figure 2.2) adopted from Rono *et al.* (2008). Only one locust was used for each test. Each locust was introduced into the wind tunnel through an aperture (dimension: 12 x 6 cm) located in the middle part of the horizontal rectangular section (dimension: 84 x 7 x 7 cm) with modified pyramidal funnel ends (dimension: 8 x 7 x 7 cm) to inject the airstreams. PAN vapour was delivered through one inlet of the wind tunnel while clean air was injected through the other inlet. The funnels served as the release points of volatiles within the arena. A vertical vacuum chamber supporting a micro fan (12W; 4 cm diam.) rotating at 2500 rpm was fixed to cover the aperture in the roof of the midsection of the wind tunnel. The bottom of this vacuum chamber was drilled with 4 mm diameter holes uniformly distributed, through which air from the wind tunnel was aspirated and expelled out by the extractor fan, thus, creating horizontal air movement from both ends of the wind tunnel. The base of the tunnel had nylon gauze (40 mesh size) lining to facilitate easy movement of locusts. An average air speed of 0.37 ± 0.01 $m.s^{-1}$ (Solomat, UK)) at the volatile release points was generated by vacuum chamber system and the micro-fan extractor. Separate containers keeping the volatile sources were placed underneath and connected to funnels via Teflon® tubings to the two arms of the wind tunnel. Airstreams from the flasks holding the test materials were regulated by independent flowmeters (Cole Parmer, Chicago, USA) and were maintained at a flow rate of 120 mm/minute. The apparatus was placed under an

operating exhaust hood and experiments were performed under room conditions as previously described (section 2.2.1).

A locust in the working section of the wind tunnel between PAN was provided with a choice between air permeated with PAN and clean air control. A non-response area (34 cm) was set from both sides of the vacuum suction area due to air turbulence created at the centre by the extractor fan. Regular flushing of the wind tunnel with clean air was done at the end of each experiment for 30 to 45 minutes to minimize any contamination by the test compounds. Control and test flasks were regularly switched to avoid any positional bias.

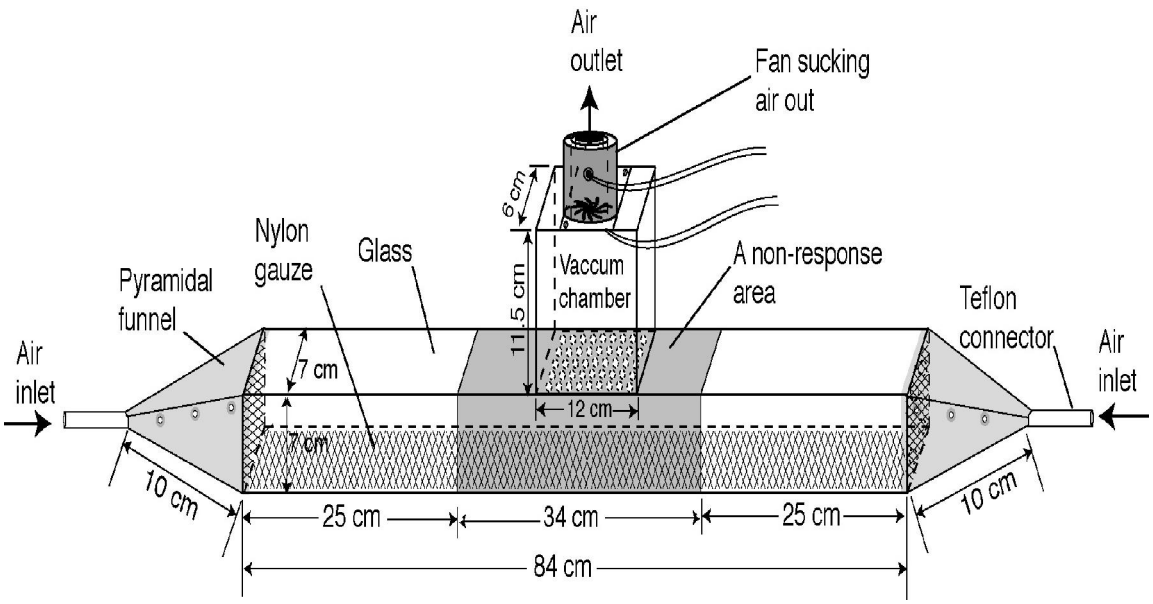

Figure 2.2 Diagram of the two-choice rectangular wind tunnel rectangular tubing with air inlets in both ends for testing anemotactic responses of the locusts (adopted from Rono *et al.*, 2008).

2.3 Sources of volatiles for bioassays and chemical analyses

2.3.1 Live locusts and their faecal pellets

Ten live gregarious male and female adult locusts, separate male and female locusts or their respective freshly collected faecal droppings were used as sources of volatiles. With regard to sources of body volatile sources, two groups of live adult locusts comprising the immature adults (1 - 14 days) and the mature adults (>14 days old) were used. Another batch of adult locusts of the same age to those used as volatile sources were tested and used as the recipients in the olfactometric arena. With regard to faecal volatiles, 4 – 8 g of faecal pellets produced overnight prior to the tests by 20 gregarious adult locusts were used as the volatile sources. The faecal pellets were held in a tightly sealed quick-fit tube (dimension: 20 x 2.5 cm diameter) (ARS, Gainesville, USA) and were aerated with charcoal filtered compressed air (BOC gases, Nairobi, Kenya). For the control, a clean empty quick-fit was used. The test locusts were from the same group from which the faecal pellets had been collected.

2.3.2 Experimental dispensers for synthetic compounds

Two types of dispensers were used to release the synthetic odours. Polyethylene centrifuge tubes with flat bottom (KART730, VWR International) (dimension: 2.5 x 0.5 cm diameter) were used to dispense identified active components of the body and faecal volatiles of *L. m. capito* (Plate 2.3) to determine their role in aggregation. The effects of phenylacetonitrile on aggregation and anemotaxis of *L. m. capito* was assessed using 3.7 ml glass vial drilled with 1.5mm hole in the caps (Plate 2.4) as

volatile dispenser. The vial was suspended in a 2 litre round-bottomed flask which was sealed at the top. The top seal had a Teflon® connector tubing that was connected to the olfactometer and the flask (Torto *et al.,* 1994).

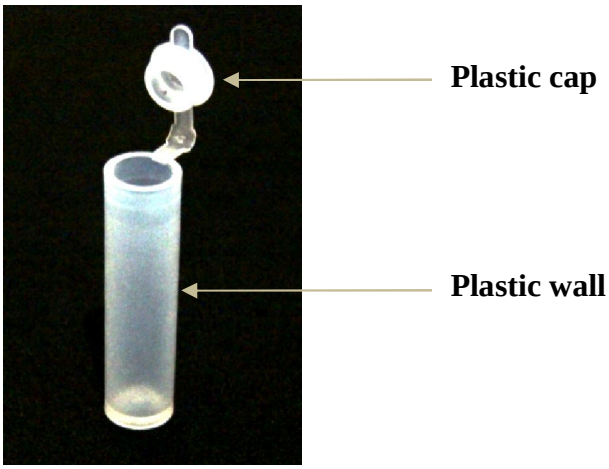

Plate 2.3 Polyethylene centrifuge tubes used for releasing volatiles.

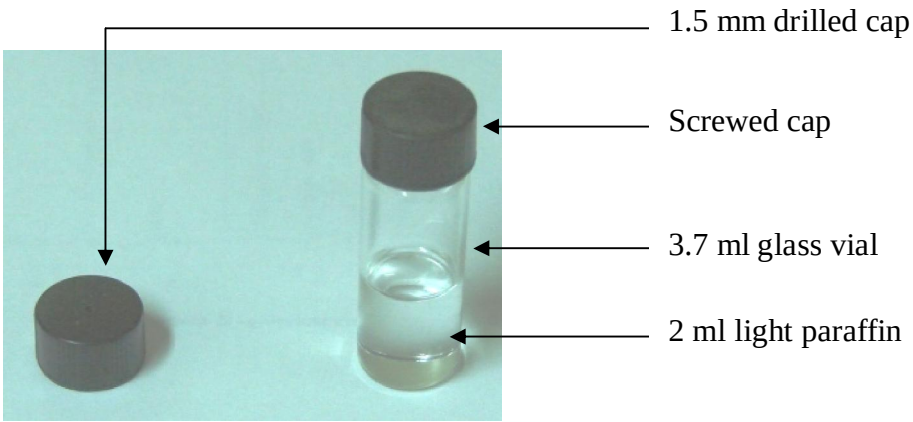

Plate 2.4 Glass vial (3.7 ml) holding 2 ml light paraffin oil and synthetic PAN with a screwed cap having 1.5 mm diam. Hole for releasing the volatiles.

2.4 Bioassay tests

2.4.1 Aggregation tests

Responses of adult *L. m. capito* to their body and faecal volatiles were bioassayed in the two choice single-chamber olfactometer, into which gregarious adult locusts were released either singly or in groups of ten. Locusts were introduced into the chamber and left for 15 minutes to settle. In tests in which groups of locusts were tested, batch of ten locusts was introduced and used as recipient throughout ten readings whereas in tests using single insect, tested locust was discarded after five readings and a new one introduced into the chamber. Each experiment was replicated ten times and each replicate comprised of ten readings taken at intervals of 10 minutes for 100 minutes. Readings consisted of the records of the preferred position of locusts within the arena. After every test, the chamber and the flasks were flushed with clean air under the operating exhaust hood for 30 to 45 minutes to prevent any contamination by the volatiles. Control and test flasks were also regularly switched alternately to avoid any positional bias through possible learning by the test locusts.

2.4.2 Anemotactic tests

Anemotactic tests to PAN vapour of different concentration were carried out using similar protocol as described in the above section (section 2.4.1). Thirty replicates were performed for each experiment using separate adult male and female locusts and fifth instar nymphs as recipients. One locust was introduced each time into the chamber and left for 15 minutes to settle. Thereafter, its position was recorded after five minutes then the locust was discarded and a new one introduced into the

chamber. After every ten replicates, the olfactometer and the flasks were flushed with clean air under the operating exhaust hood for 15 minutes to prevent any contamination by volatiles. Control and test flasks were also regularly switched alternately to avoid any positional bias through learning.

2.5 Aggregation (A.I.) and Attraction Indices (A.I.)

In the two bioassay tests (section 2.4.1 and section 2.4.2), the number of locusts choosing either side of the chamber was taken as responders, while those observed in the separation area in the middle were considered as non-responders. The aggregation and attraction indices (A.I. and At. I.) were calculated using the following formula:

$$\text{A. I. or At. I. (\%)} = 100 \times (T-C)/N$$

N is the total number of locusts tested; T, number of locusts responding to the volatiles; and C those in control area of the chamber.

2.6 Collection and trapping of volatiles

Volatile samples for gas chromatographic (GC) analyses were collected from separated sexes of gregarious adult live locusts. The sources of volatiles were: immature locusts (7 – 12 days old), the sexually mature locusts (14 days old and older ones) or 4 - 8 g of their respective freshly collected faecal droppings. Twenty live adult locusts were put either into trapping glass tubes (ARS, Gainesville, USA) (dimension: 45 x 5 cm diam.) or in a 2-liter three-necked round-bottomed flask and

then aerated with clean charcoal-filtered and humidified air from a gas cylinder (BOC gases, Kenya). At its outlet tip, clean Super-Q polymer adsorbent (30 - 50 mg; 80 - 100mesh; Alltech, Nicholasville, KY) was connected to a vacuum pump (Air cadet, Cole Parmer Instruments, Mass., USA) for suction. Air circulation was regulated at 120 mm/minute by flowmeter (Aalborg, Orangeburg, NY, USA) and the standard duration for trapping duration was one hour.

The same procedure as described above for the live locusts, was used for the freshly collected faecal pellets, however, smaller quickfit glass tubes (ARS, Gainesville, USA) (dimension: 20 cm x 2.5 cm diam.) were used as containers (Plate 2.5). The equivalent synthetics of the identified compounds from the body as well as the faecal pellets were collected using appropriate dispensers as described in section 2.3.2 and then later quantified and used for bioassays.

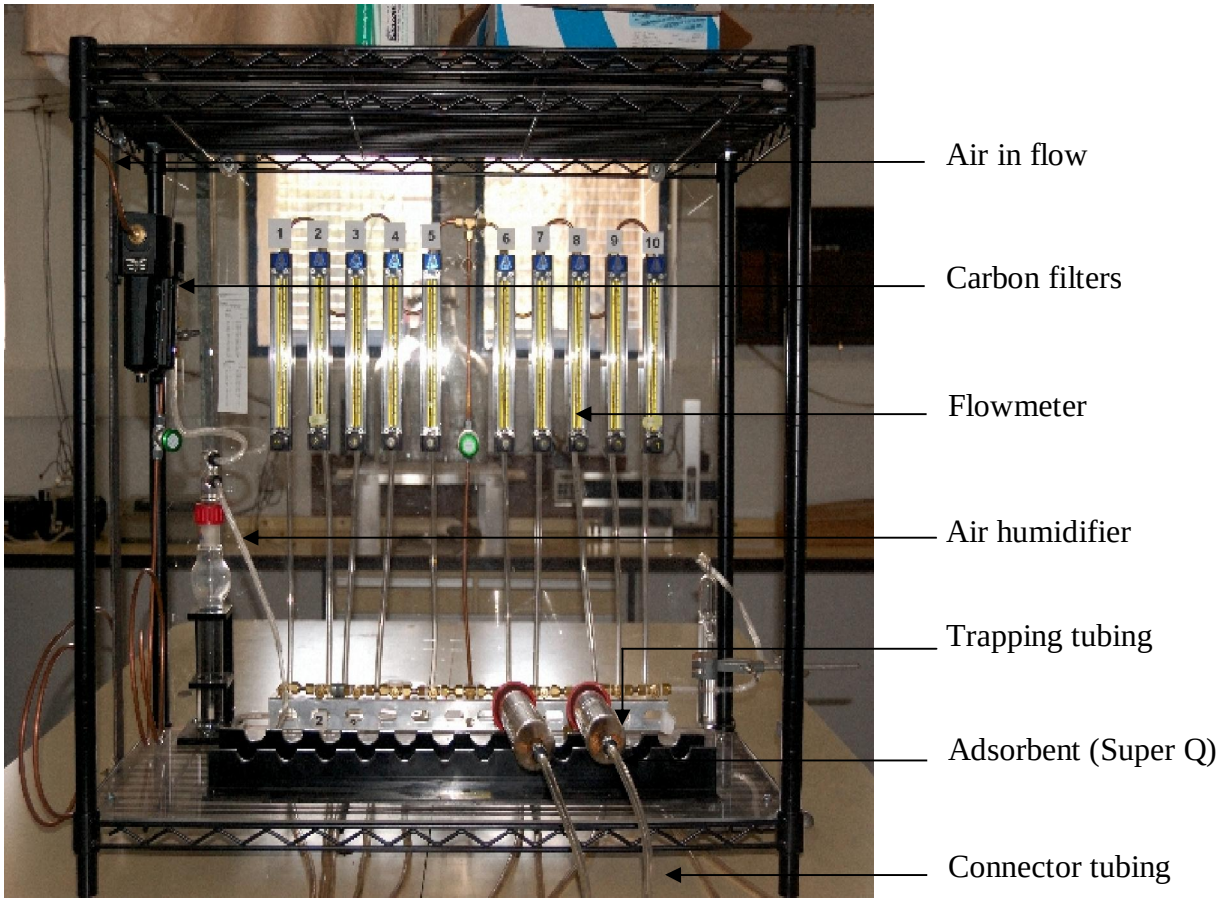

Plate 2.5 Air entrainment system for collection of locust body and faecal volatiles

(Photography by Razafindranaivo)

2.7 Elution and preparation of trapped volatiles

Volatile traps were eluted from the adsorbent in 200 µl of dichloromethane (HPLC grade, Fluka 99.9%) on ice under a stream of nitrogen into 2 ml vials (Sigma Aldrich, USA). Before injection, 1µl of an internal standard equivalent to 4.59 ng of methyl salicylate (Sigma Aldrich, USA) was added to 40 µl of the eluate and immediately analyzed. Used Super-Q adsorbent was cleaned by flushing with 300 µl of high grade dichloromethane and flushed with nitrogen and the traps were then sealed with clean Teflon® tape and stored until re-use.

2.8 GC analysis and quantification of identified compounds

Analyses and quantifications of volatiles were carried out using HP-5890 Series II gas chromatograph equipped with a Flame Ionization Detector (FID) and an autosampler injector HP 7673 (Plate 2.6 and Plate 2.7). The GC was interfaced to a HP computer monitor (Dell Optiplex X 520) via a 3365 MSD ChemStation software (G1701EA E.02.00.493) on the screen on which the chromatograms of each analysed blend were displayed. The GC oven was fitted with a nonpolar capillary column HP1 ultra I methyl silicate gum, 30 m x 0.25 mm ID x 0.25 µm (Agilent, Palo Alto, California, USA) and using nitrogen (BOC gases, Kenya) as carrier gas at a flow rate of 0.35 ml/min. The oven temperature was programmed at an initial temperature of 60ºC for three minutes then increased to 280°C at a gradual increase temperature rate of 10°C/min and held there for 10 minutes. The FID had a mixture of clean medical air and hydrogen gas (from a generator, Dorminick, USA) at a flow of 31 ml/min and 405 ml/min, respectively (Mburu, 2009). Aliquots of 1µl of the samples were

injected into the GC. Five injections were done for each volatile sample of both the locusts and their faeces while only three injections were done for the synthetic standards. For the quantifications, GC chromatographic relative peak areas were compared to the internal standard.

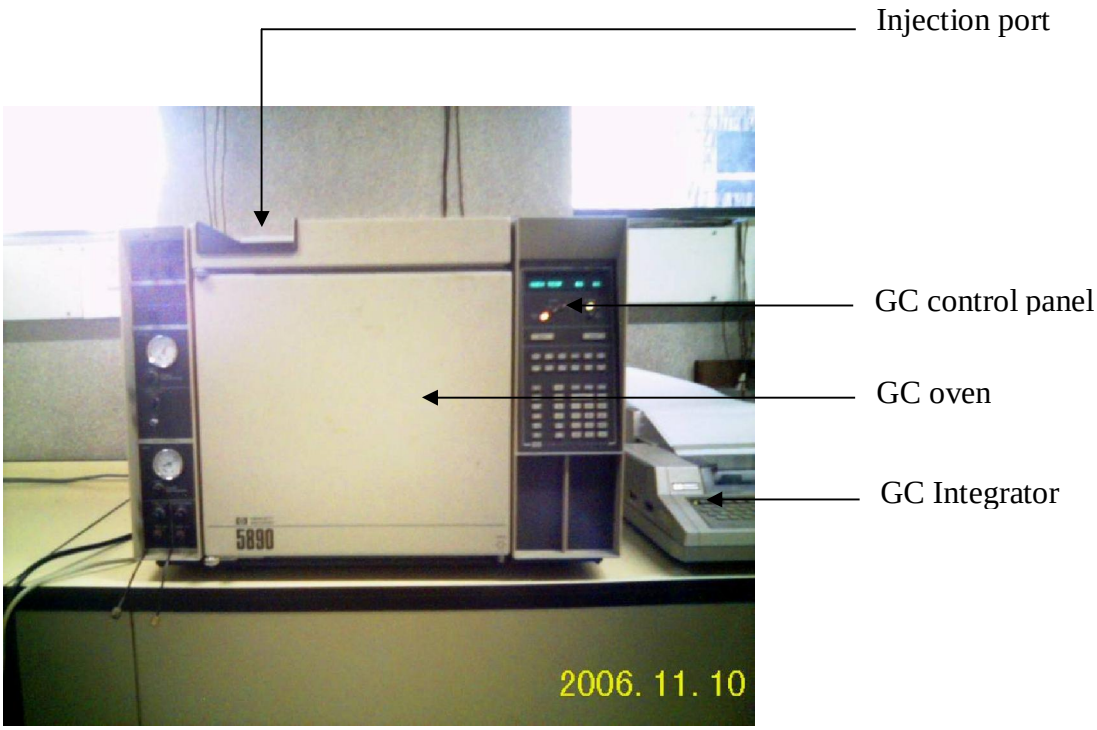

Plate 2.6 GC-5890 series machine for analyses and separation of volatiles.

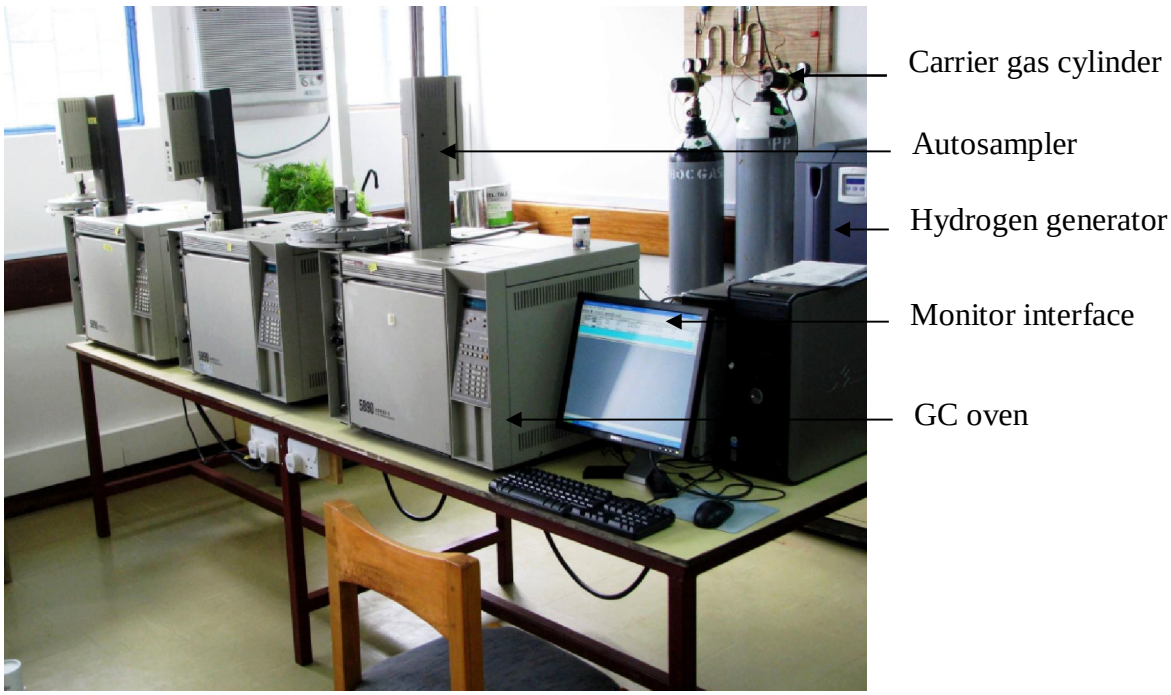

Plate 2.7 Gas chromatographs attached with autosamplers on the injector port and connected to a HP (Dell) monitor and PC (Photography by F. Kimbokota).

2.9 Coupled Gas Chromatography Electroantennographic Detector (GC-EAD) analysis

Detection and identification of active components of body and faecal volatiles of *L. m. capito* using their antennae as biological sensor were performed on HP-5890 Series II gas chromatograph equipped with FID and coupled to an EAD system (Plate 2.8).

Volatile eluates were concentrated under a stream of nitrogen at 0ºC before injection and aliquots of 5 µl were analyzed. The GC oven was programmed with shortened ramp with the oven temperature conditions starting from 60ºC and held for three minutes, then increased at 10ºC/min up to 130ºC, then at 35°C/min to 230°C and a further increase to 280°C at 10°C/min and held there for 10 min; giving a total of 1705 seconds for the run. Nonpolar capillary column HP1 ultra I Methyl silicate gum, 25 m x 0.25 mm ID x 0.25 µm was used and carrier gas was high purity nitrogen at flow rate of 0.35 ml/min. The FID and EAD signals were monitored synchronously using a program on a GC-EAD interface card (Syntech, the Netherlands) installed in a Personal Computer (Harvard Professional Computer, American Megatrends Inc.).

2.9.1. Antennal preparation

Locust antennae were prepared following the protocol detailed by Shi and Njagi (2004) and briefly described: antennae were prepared from adults of laboratory-reared *L. m. capito* that had been removed from the rearing cage and kept separately in individual small cages for at least 12 hours. After starving for about two hours, an

antenna was removed from each locust using fine forceps. One antenna per locust was then cut with a scalpel blade from both ends (at the base of the third broad segment and the tip end). Each end was inserted into a separate glass micropipette containing locust saline (ingredients: NaCl, KCl, $CaCl_2$, $NaHCO_3$, glucose and water) (Rustum, 1939; Duranton *et al.*, 1982; Shi and Njagi, 2004). One micropipette holding one tip of the antenna was sheathed over a silver wire recording electrode on one arm of a micromanipulator (Syntech, AMS, Netherlands). The other micropipette was sheathed over another silver wire electrode held on the other arm of the micromanipulator that served as an indifferent electrode and was grounded through a wire to complete the circuit. The recording electrode was connected via a probe to a Universal AC/DC UN 05 amplifier (Syntech, AMS, Netherlands) and to a computer (PC) with an EAD card. The micromanipulator was positioned such that the antennal preparation was directly in the path of the effluent from the opening of the stimulus delivery tube connected to the GC. The computer recorded the responses automatically (Shi and Njagi, 2004). Three replicates were carried out for each source of volatiles using the antennae of both sexes.

Plate 2.8 Gas chromatograph in tandem with electro-antennographic apparatus for the detection of active components and recording of locust antennal activity to components of the volatiles.

2.10 Coupled Gas Chromatography - Mass spectrometric (GC-MS) analyses

Preliminary identification of body and faecal volatiles of *L. m. capito* were carried out on HP 5790 gas chromatograph coupled to VG Masslab 12-250 mass spectrometer operating in the electron impact (E.I) mode at 70eV (Plate 2.9). The oven was temperature-programmed at 60ºC for five minutes, followed by an increasing of 10ºC/min up to 130ºC and held for five minutes then increasing of 5°C/min up to 280°C and held for 10 minutes.

Further identification and confirmation were carried out on coupled GC-MS using a Hewlett Packard (HP) 6890 series at the Pheromone Group Laboratory in Lund, Sweden (Plate 2.10) then on coupled GC-MS using a Hewlett Packard (HP) 7890 A series GC (Agilent technologies, Wilmington, DE, USA) in tandem with a 5975 C series mass spectrometer fitted with a 7683 B autosampler and a triple axes detector at *icipe,* Kenya. Both GC machines were equipped with a nonpolar column HP5 MS 5% phenyl methyl siloxane measuring 30 m x 0.25 mm ID x 0.25 µm (Agilent, Palo Alto, California, USA). Nitrogen was used as carrier gas blown at the flow rate of 0.35 ml/min. The oven program was temperature-programmed at 60ºC for three minutes, followed by an increasing of 10ºC/min up to 280ºC and held for 10 min. Identification of constituent compounds was based on the interpretation of the mass spectral fragmentation patterns obtained from MS libraries (NIST05 and WILEY275) followed by comparisons with spectral data of synthetic standards (Sigma Aldrich, USA).

Plate 2.9 VG-MassLab12-250 coupled GC-MS.

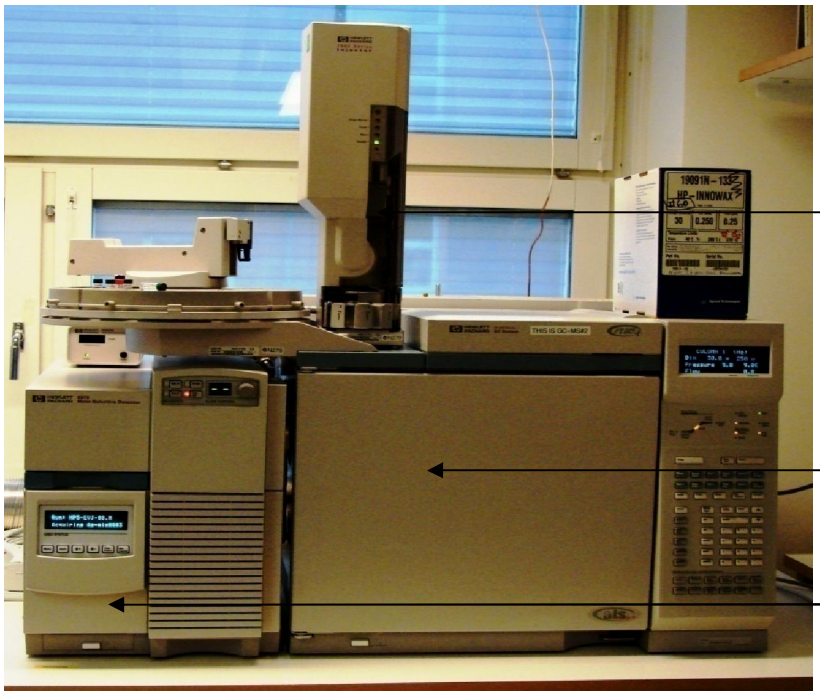

Plate 2.10 HP 6890 series GC-MS with an attached autosampler used for chemical identification of volatile components (Pheromone group – University of Lund, Sweden).

2.11 Statistical analysis

Data on aggregation responses of locusts to their body and faecal volatiles and to synthetic standards and PAN were analysed using Chi-square goodness of fit test (χ^2; $\alpha = 0.05$) (SAS, Institute, 2003, version 9.1). Aggregation Indices (A. I.) of different sexes of nymphs, immature and mature adults to various volatile sources were subjected to ANOVA ($\alpha = 0.05$) (SAS, Institute, 2003, version 9.1). When ANOVA results were significant, means were separated using Student – Newman - Keuls test (SNK, $\alpha = 0.05$).

CHAPTER THREE

3.0 RESPONSES OF ADULT *LOCUSTA MIGRATORIA CAPITO,* (SAUSSURE, 1884) TO THEIR BODY AND FAECAL VOLATILES

3.1 Introduction

Since biblical times, migratory locusts have been considered as a serious plague across the ancient world. Restricted to Madagascar, hopper bands and swarming gregarious adults of *L. m. capito* are known to be the most devastating agricultural pests. It has ability to fly in dense groups for long distances and to devastate extensive areas under cultivation. It is a voracious feeder when gregarious, justifying its status as the first ranked pest of crops and pastures in Madagascar (Zehrer, 1999).

Aggregation and gregariousness in locusts are fundamentally induced adaptation reactions in response to gradual increase of density and crowding (Roffey and Popov, 1968). Shift from the *solitariform* to the *gregariform* and vice versa, passing through *transiens* that characterise locusts is the striking determinant feature of *L. m. capito,* similar to well known locust pests (Boughdad, 1991). Although physiological changes may occur earlier, it is the behavioural characteristics that manifest first after only few hours of crowding (Roessingh and Simpson, 1994).

The Malagasy migratory locust, *L. m. capito* was isolated on Madagascar Island since early times. However, this locust species has retained the characteristic reversible propensity to gregarize, similar to other polymorphic acridids but with some specific attributes. The phase change from shy and the cryptic individuals in their solitarious phase, *L. m. capito* adopts progressive, highly interactive and

cohesive population formations of gregarious locusts when a threshold number of 2000 to 2500 individuals per hectare is reached (Lecoq, 1975; Andrianasolo, 1979). Moreover, processes that maintain cohesiveness in the swarm formations even under unsuitable environmental conditions and the recurrent and strong intensity of its outbreaks remain unknown. These phenomena may imply the use of specific cues, such as pheromones associated to their specific phase status (Chapuis, 2008; Pener and Simpson, 2009). These facts raise questions about their chemical ecology, in particular, whether they utilise volatile emissions from the body and faeces to aggregate and whether these may have other effects on the phase shifts of this locust.

It is hypothesised that similar to species of other polymorphic acridids that have been studied, gregarious adults of *L. m. capito* use body as well as faecal volatile emissions in their aggregating behaviour and these olfactory cues may play a primer role in their phase shift process. The objective of this study was to determine the role of airborne body and faecal volatiles released by gregarious adult *L. m. capito* on their aggregation behaviour.

3.2 Materials and methods

3.2.1 Locust strain and rearing conditions

Gregarious adults of *L. m. capito* that were used for experiments were from a colony reared and maintained in the Biocontainment Unit (BU, quarantine) at *icipe* (Nairobi, Kenya) following the rearing methods and under the conditions outlined in detail in section 2.1.

3.2.2 Olfactometer chamber

The single chamber dual choice olfactometer that was used to evaluate aggregation responses of adult *L. m. capito* to their body and faecal volatiles is described in section 2.2.1. Gregarious adult locusts were released into the dual choice chamber either singly or in groups of ten and left for 15 minutes to settle. Thereafter, volatiles were released into one side of the chamber, the position of the locust was recorded at regular intervals of ten minutes for 100 minutes. Each experiment was replicated ten times (Section 2.4.1).

After every test, the chamber and the flasks holding the volatile source were flushed with clean air under the activated exhaust hood for 30 to 45 minutes to prevent any contamination by the volatiles. Control and test flasks were also regularly switched alternately to avoid any positional bias of locusts through learning.

3.2.3 Bioassay tests

The aggregation bioassays were carried out using the single-chamber olfactometer (Obeng'Ofori *et al.*, 1993) as described in section 2.2.1 and the bioassay procedure as outlined in detail in section 2.4.1 was followed. Responses of single locusts singly or in groups of ten to body odours of their conspecifics and to their faecal volatiles were initially carried out. In a second experiment, the responses of immature locusts and separate male and female locusts to the volatiles from mature adult male and female locusts respectively, were evaluated. For the latter, locusts were only tested singly in the arena. Recipient locusts were given a choice between an airstream

permeated with volatiles from conspecifics and another of clean air. Each experiment was replicated ten times (Section 2.4.1).

3.2.3.1 Responses of adult locusts in groups

Responses of groups of adults of *L. m. capito* to body volatiles from live conspecifics and to faecal volatiles were carried out in the olfactometer arena as outlined in section 2.4. For each test, ten gregarious adult locusts were released within the dual choice chamber and their position was recorded. Tests were replicated as outlined in section 2.4.1.

3.2.3.2 Response of single adult locust

Using similar volatile sources as described above (section 3.2.3.1), locusts were released and tested singly within the olfactometer chamber in order to eliminate possible visual and tactile stimulus effects between test locusts. Each locust was presented with test volatiles from conspecifics through one side of the arena and clean air through the other side being the control. Bioassay tests were carried out following the general protocol outlined in section 2.4.1 of this thesis.

3.2.3.3 Cross sex aggregation assays

Volatiles from batches of ten mature male locusts and ten females were separately tested against immature locusts, mature male and female locusts to assess their aggregation responses. One locust at a time was introduced into the arena. Prior to any recordings of its position preference between the two provided volatile sources (volatiles *vs.* clean air), the locust was allowed 15 minutes to settle. Five recordings

of its position, within the arena, were carried out every 10 minutes for a period of 50 minutes after which the locust was removed and another one was similarly tested to complete the ten readings forming a replicate. The number of replicates was set as outlined in section 2.4.1.

3.2.4 Aggregation Index (A. I.)

The aggregation index (A. I.) was calculated using the formula A. I. (%) = 100 x (T-C)/N, where N is the total number of insects tested; T, number of insects responding to the volatiles; and C those in control area of the chamber.

3.2.5 Analysis of data

Count data were analysed and subjected to Chi square (χ^2, $\alpha = 0.05$) test and to ANOVA (SAS, 2003) as described in section 2.11.

3.3 Results

3.3.1 Aggregation responses of adult *Locusta migratoria capito* to body volatiles from conspecifics

3.3.1.1 Response of adults tested in groups

When tested in group, aggregation bioassays showed that locusts preferred the side of the chamber where their own volatiles were provided. There were no significant differences ($F_{df\ (5,\ 54),} = 1.34$, N = 60, P = 0.26, SNK, test) between the aggregation responses of adult male and female of the two age ranges (Figure 3.1). For the mature locusts, females responded with a relative slightly higher percentage to their own volatiles (A. I. = 22 ± 5%) whereas mature male locusts and mixed sexes of test

locusts responded equally to their own volatiles with a low aggregation index (A. I = 13 ± 3%).

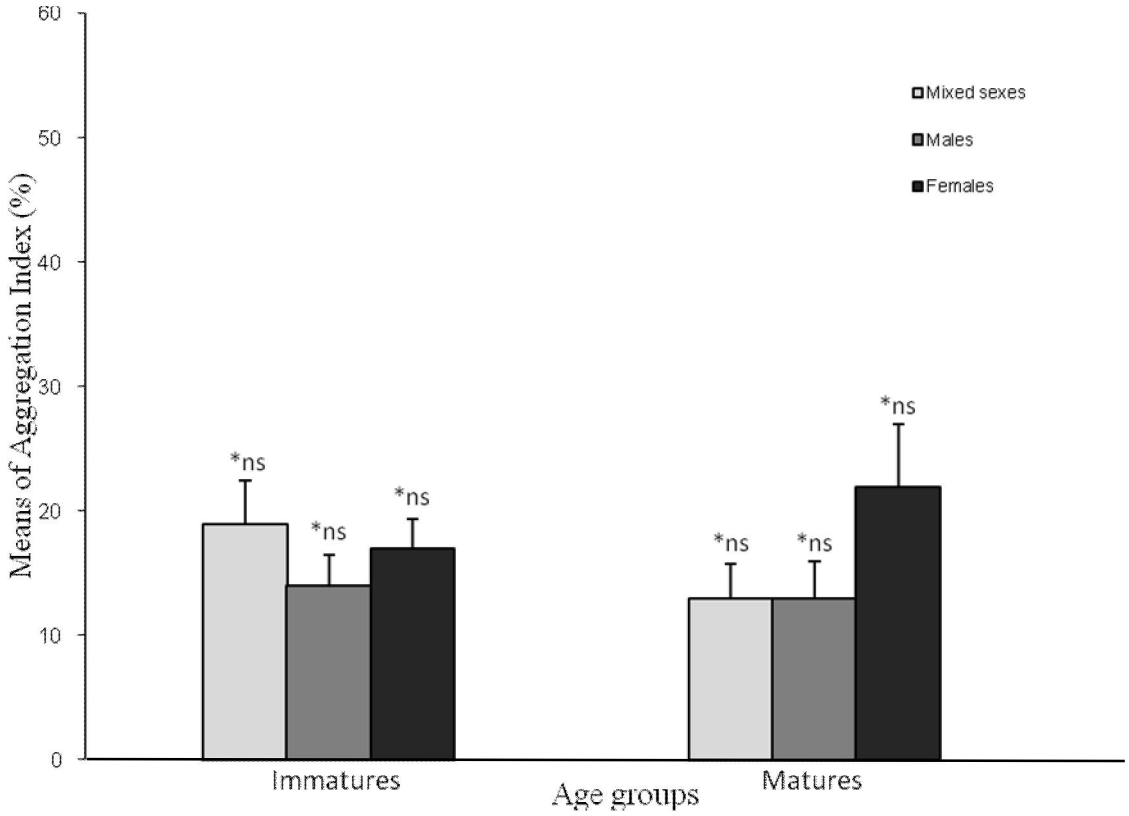

Figure 3.1 Aggregation responses (mean ± SE) of immature and mature locusts, *Locusta migratoria capito*, tested in groups of ten either in separate sexes or males and females together against body volatiles of conspecific adult locusts. * indicates differences were significant (Chi-square tests at P = 0.05). ns indicates no significant differences between means (P = 0.05, ANOVA tests)

3.3.1.2 Response of locusts tested singly

For the locusts tested singly, mature males responded strongly (A. I. = 48 ± 9%) to their own volatiles whereas females had consistently moderate responses to their own volatile emissions (A. I. = 29 ± 8%) (Figure 3.2). Responses of locusts in groups mixed sexes when used as recipients, were almost at a level similar to those of male locusts alone but lower (A. I. = 35 ± 9%). Tests showed no significant difference ($F_{df\ (5, 54)} = 1.45$, N = 60, P = 0.22, SNK, test) between males and females for the two age groups (immature and mature) of adult locusts, though the responses were higher when locusts were tested singly than when tested in groups (Mixed sexes: A. I. = 29 ± 7% and 35 ± 9%; Mature males: A. I. = 22 ± 5% and 48 ± 9% and Mature females A. I. = 29 ± 8% and 25 ± 8%). The aggregation responses indicate that both sexes of adult *L. m. capito* release almost identical chemical signals for aggregating their adult conspecifics.

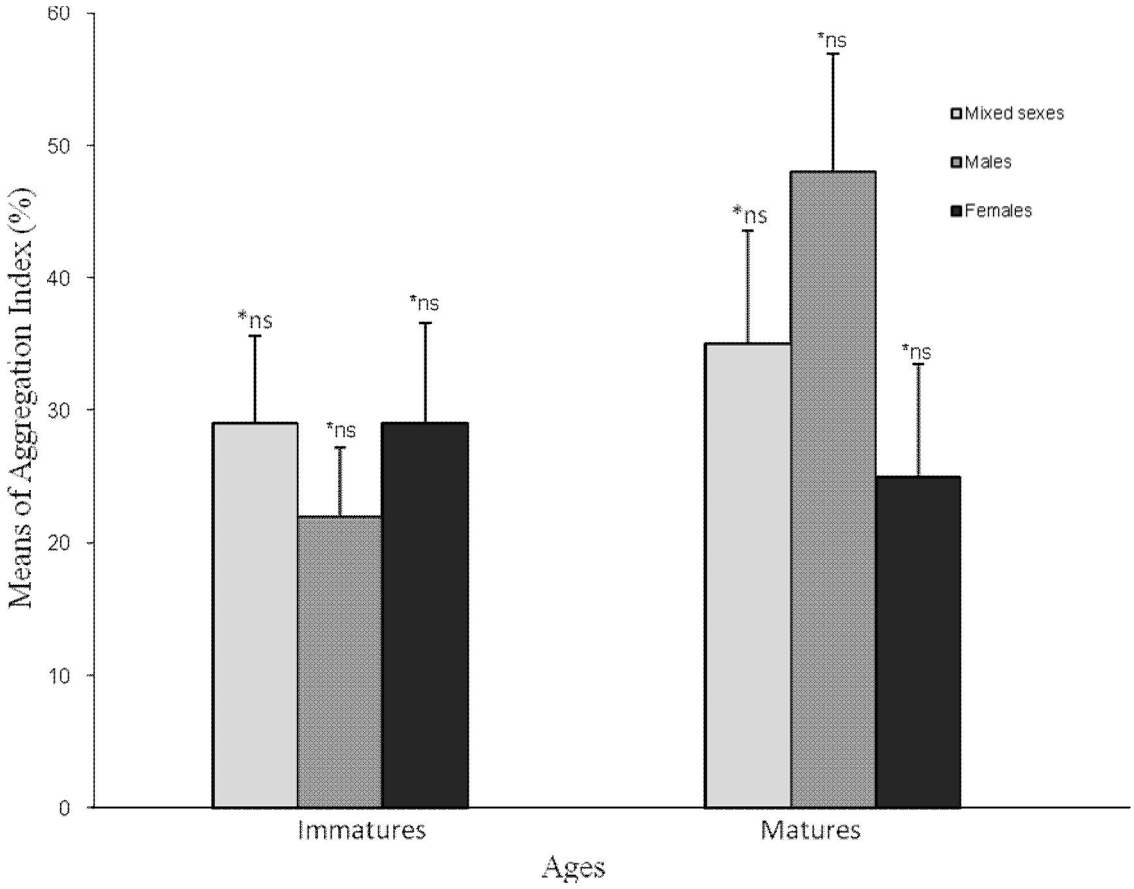

Figure 3.2 Aggregation responses (mean±SE) of immature and mature locusts respectively for the three groups: the mixed sexes, separate males and females of *Locusta migratoria capito* tested singly to the body volatiles of conspecifics adult locusts. * indicates differences were significant between test and control (Chi-square tests at P = 0.05). ns indicates no significant differences between means of the three groups (P = 0.05, ANOVA tests)

3.3.1.3 Cross sex aggregation assays of adults of *Locusta migratoria capito*

In cross sex bioassays and taking as reference the levels of responses of the mature males and the females that were tested singly to their own volatiles (section 3.3.1.2), only mature males showed significant responses to their own body volatiles ($F_{df\,(5, 54)} = 3.18$, N = 60, P = 0.0139, SNK, test). Locusts from the mixed sexes group of immature locusts and mature females responded weakly to volatiles of mature males. Both sexes of mature and immature adults of *L. m. capito* displayed relatively moderate responses (respectively A. I. = 29 ± 7% and A. I. = 20 ± 6%). However, there were no significant differences in aggregation responses to volatiles from the mature females (Figure 3.3).

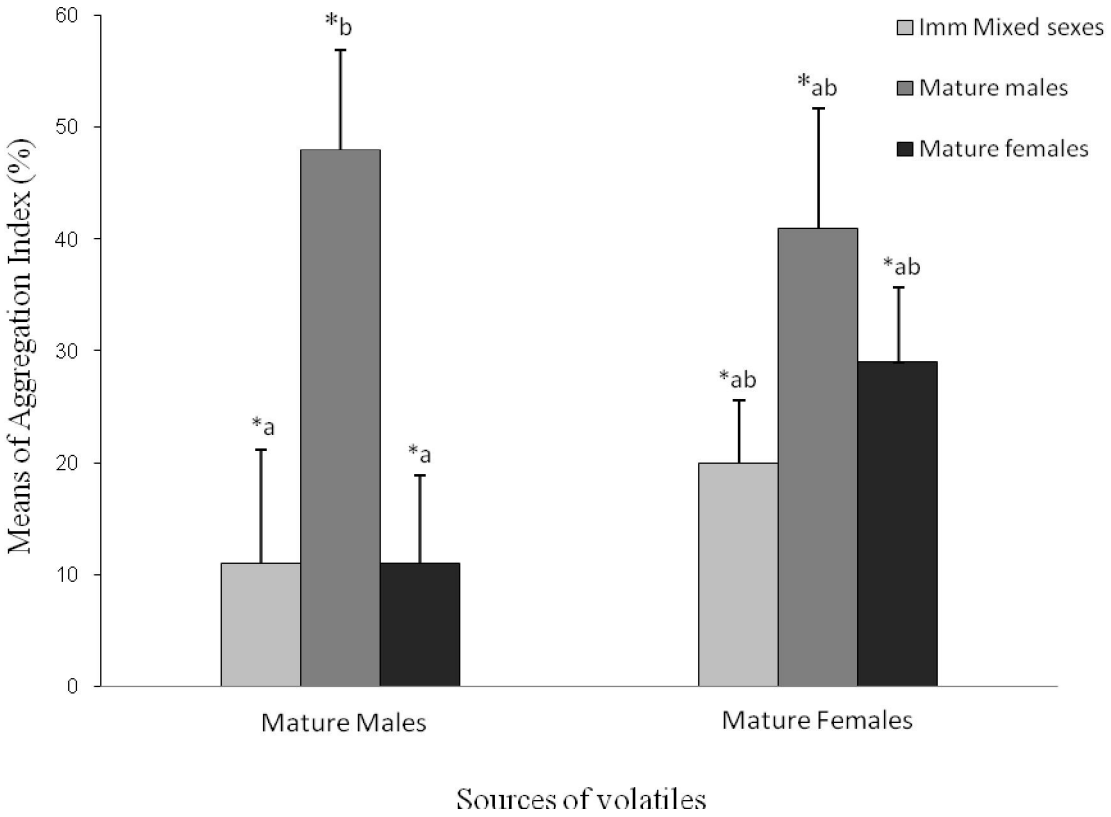

Figure 3.3 Aggregation responses (mean ± SE) of immature adult, mature males and females of *Locusta migratoria capito*, respectively to body volatiles of mature male and mature female conspecifics. * indicates differences were significant between test and control (Chi-square tests significant at P = 0.05). Different letters indicate significant differences in the means (P = 0.05, ANOVA tests).

3.3.2 Aggregation responses of adult *Locusta migratoria capito* to faecal volatiles from adult conspecifics

The general pattern of responses of adults of *L. m. capito* to their faecal volatiles showed different responses depending on the maturation status of the locusts. Only the immature adult locusts responded to their faecal volatiles. Immature stages of *L. m. capito* prefer the side of the two-choice arena permeated with their faecal volatiles, either as groups of males and females or when tested separately. The level of their aggregation responses were relatively low (maximum A. I. = 13 ± 5% for the immature females). On the contrary, sexually mature locusts either tested in groups of the same sex or mixed sexes showed a tendency of behavioural avoidance to faecal volatiles from conspecifics. This was more evident in mature male locusts ($F_{df\ (5, 54)} = 15.94$, N = 60, P = 0.0001 , SNK test) (Figure 3.4).

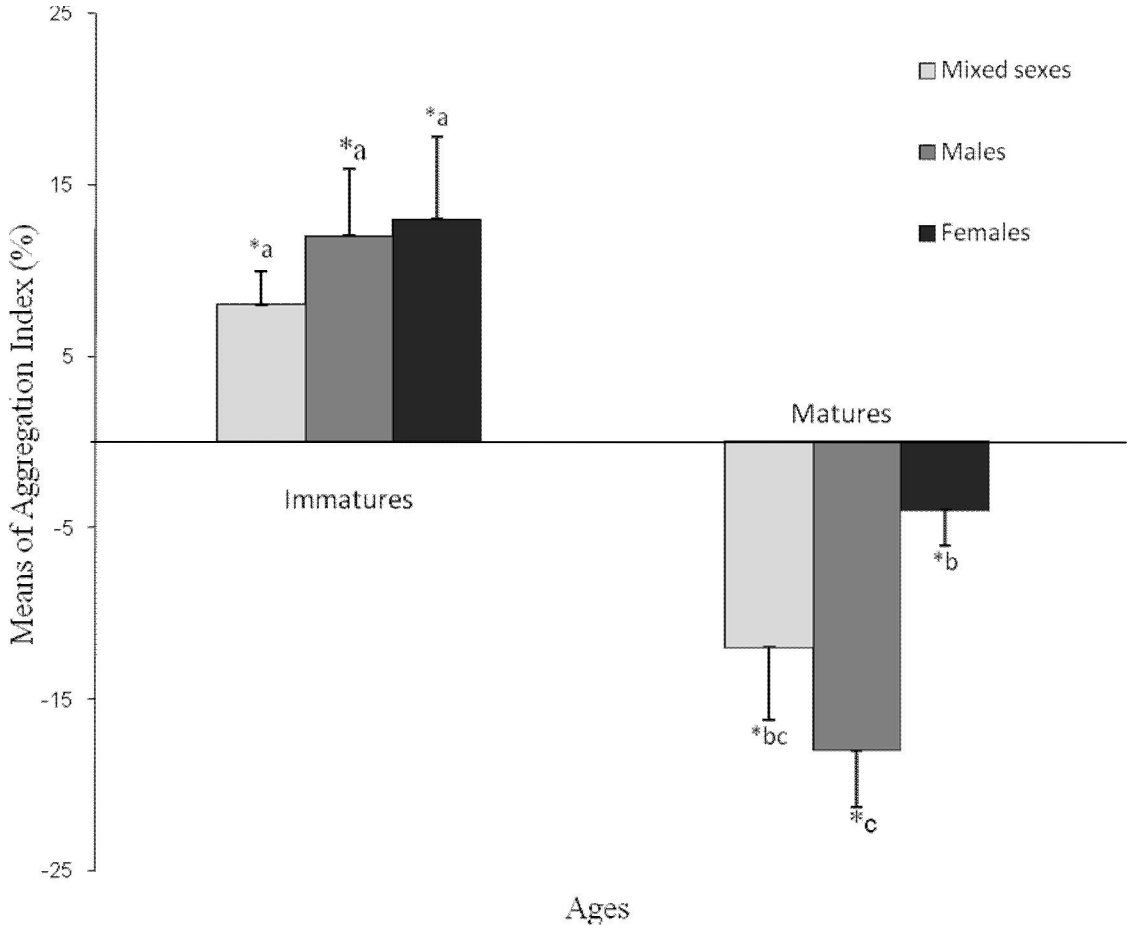

Figure 3.4 Aggregation responses (mean ± SE) of immature adult, males and females of *Locusta migratoria capito* tested in groups to volatiles from conspecifics. * indicates differences were significant (Chi-square tests at P = 0.05) Different letters indicate significant differences in the means (P = 0.05, ANOVA tests).

3.4 Discussion

Both males and females of *Locusta m. capito* produced body and faecal volatiles to which they responded variably. Thus, as in other locust species whose chemical ecology has been studied, various components in these volatiles may play a role in mediating aggregation, with the faecal volatiles being involved more with the grouping of the immature adults of this locust (Obeng'Ofori *et al.*, 1993, 1994; Torto *et al*,. 1994; Niassy *et al.*, 1998).

Although the levels of the aggregation responses of the adults of *L. m. capito* were almost the same when locusts were tested singly or in groups, the responses were always higher when locusts were tested individually. This may be an indication that the distribution of the olfactory stimulus throughout the arena due to presence of other stimuli-emitting conspecifics decreased their choice of one side of the arena, in addition to the visual cue provided by their presence. This led to low responsiveness toward the actual volatile source provided when locusts were tested in groups, in contrast to when locusts were tested singly.

With regard to responses of the locusts to volatiles from male and female conspecifics, sexually mature males responded strongly (A. I. = 48 ± 9%) to their own volatiles and to those of mature females. It is possible that, the male-produced volatiles have components that act as a male-male recognition signal to which male conspecifics are more sensitive compared to the immature and mature female recipient locusts tested. In contrast, locusts of different sexes tested either singly or in groups responded to the volatiles produced by mature female locusts. Thus,

volatiles from female locusts may play a more universal role in the aggregation behaviour of this species. Overall, unlike in the adult desert locust, *S. gregaria,* for which the aggregation pheromone is produced solely by the males (Torto *et al.,* 1994), components in body volatiles of sexually mature female *L. m. capito* seem to play a major role in the grouping behaviour of this species, perhaps augmented to an extent by similar components in volatiles from male locusts. This is almost similar to the aggregation system that was proposed for the African migratory locust, *L. m. migratorioides* for which adult males and females were found to respond to each other volatiles (Niassy *et al.,* 1998). In this system, population build up would be around nuclei of groups of aggregating female locusts into which other conspecific locusts of both sexes are recruited by responding to the olfactory cues and other stimuli emanating from these groups and from the environment.

The responses of *L. m. capito* to their volatiles were characterized by low aggregation indices (maximum 48 ± 9%) when compared to those reported for the desert locust, *S. gregaria* (67 ± 7%; Torto *et al.,* 1994) and *L. m. migratorioides* (on average 55%; Niassy *et. al.,* 1998). These were indicative of the chemical cues eliciting loose cohesiveness, suggesting that in addition to these cues, *L. m. capito* may have adapted to using other cues to aggregate. In the breeding and outbreak habitats of this locust in Madagascar, their occurrence in large numbers and the recurrent outbreaks within short periods (Lecoq, 2004) suggest unavoidable increased interaction between the locusts using visual and tactile cues and probably, acoustics. Thus, over an extended period of time this may lead to a reduction in the reliance on odour cues in the interactions between individuals.

The faecal volatiles elicited different behavioural responses in immature and mature adult locusts, with only the former showing weak aggregation responses to the volatiles. This reflects on the transitional nature of the immature stage of locusts, which after fledging, remain in the company of late fifth instars as the fledglings develop their flight muscles. During this period, faecal volatiles may partly provide the cue for cohesion, while on attaining sexual maturity, the mature locusts respond to the more relevant stimuli released in the body volatiles of their conspecifics. In the desert locust, S. *gregaria* the immature adults were found to respond only to their faecal volatiles and to those of nymphs (Obeng'Ofori *et al.*, 1994).

Results of the aggregation bioassays in this study revealed that olfactory cues in body and faecal volatiles of gregarious adults of *L. m. capito* mediate cohesion and aggregation behaviour. The results of this study validates the hypothesis that gregarious adult *L. m. capito* utilise semiochemicals derived from body and faecal volatiles for aggregation. The identification of the chemical components in these olfactory cues using chromatographic and mass spectrometric techniques is reported in the next chapter.

CHAPTER FOUR

4.0 IDENTIFICATION OF COMPONENTS IN VOLATILES OF ADULT MALAGASY MIGRATORY LOCUST, *LOCUSTA MIGRATORIA CAPITO*

4.1 Introduction

Species of acridids classified as locusts have close similarity with regard to their aggregation and phase shift pathways and design (Hassanali *et al*., 2005). The factors and signals responsible for these behavioural and morphological changes and adaptations are also likely to be similar. Amongst these signals, pheromones have been demonstrated to play a significant role in the dynamics of phase change and are amongst the important factors that maintain gregariousness in gregarious populations of locusts with regards to their intra-specific communication (Hassanali *et al.*, 2005). Results presented in the previous chapter confirmed that olfactory cues in the body volatiles of adult *L. m. capito* arrested and retained conspecific adults to aggregate. This strengthens the evidence of the role of the pheromonal emissions in the cohesion and aggregation process of the adults of Malagasy migratory locust.

Pheromone receptors in each species are specific and detect distinct specific compounds that generate the necessary biological effects, hence, the appropriate behavioural responses (Visser, 1979, 1983, 1986). Detection and recognition of the specific odour cues from a range of odours prevalent in the ambient environment requires a suitable adapted and highly sensitive olfactory system. These specific adaptations enable the locust to recognize and detect specifically the pheromone

emitted by conspecifics. In two different species of locusts namely the desert locust *S. gregaria* and the African migratory locusts *L. m. migratorioides,* different pheromone systems are involved in their respective aggregation process (Byers, 1991; Obeng'Ofori *et al.*, 1993, 1994; Niassy *et al*., 1998).

Variations in adaptations across different geographical subspecies of the latter group show biological, physiological and behavioural differences within *L. migratoria* species, including embryonic development (Farrow and Colless, 1980; Heifetz *et al*, 1994; Jing and Kang, 2003), degree of phase plasticity (Heifetz and Applebaum, 1994; Tanaka, 2003) and reproduction behaviour (Zhu and Tanaka, 2002; Jing and Kang, 2004). Eco-geographical distribution is the basis of the sub-species taxonomic differentiation. In terms of volatile emissions, preliminary comparative studies have shown that in three of its subspecies namely: *L. m. manilensis,* Meyen, *L. m. capito,* and *L. m. migratorioides,* production patterns of phenylacetonitrile are closely identical (Deng and Torto, unpublished.).

To-date, no reports are available on the identification of the aggregation pheromone system of *L. m. capito*. The objective of this study was to identify potential candidate compounds involved in aggregation and cohesion process by adults of *L. m. capito.*

4.2 Materials and Methods

4.2.1 Locust stocks and mass rearing of colony

Gregarious adults of *L. m. capito* from colony maintained at *icipe* (Nairobi, Kenya) were used for volatile sources. Rearing of locusts was described and outlined in

detail in section 2.1. Rearing of nymph and adult stages was conducted separately, minimizing cross-pheromonal effects throughout their developmental breeding.

4.2.2 Body volatile and faecal volatile collections

The protocol for collection and trapping of volatiles from body and faecal pellets of gregarious adults *L. m. capito* was described in sections 2.6 and 2.7, respectively of Chapter 2.

4.2.3 Gas Chromatographic analysis and quantification of pheromone components

Trapped volatiles were eluted on ice from the adsorbent in 200µl dichloromethane (HPLC grade, Fluka 99.95 %) under nitrogen stream. Before injection, 1µl of an internal standard equiv. 4.59 ng of methyl salicylate; (Sigma Aldrich, USA) was added to 40µl of the eluate and immediately analyzed. Quantification procedure and calculations as outlined in details in section 2.8 were applied.

4.2.4 GC-Electro-antennographic Detector (GC-EAD) and GC-Mass spectrometry (GC-MS) analyses of volatiles

4.2.4.1. GC-EAD analysis

Coupled GC-EAD settings as described in section 2.9 were used to identify, as well as to assess electroantennogram activity of locust antennae elicited by the chemical components in body and faecal volatiles of locusts. Analyses and preparation of antennae were performed as outlined in sections 2.9 and 2.9.1, respectively.

4.2.4.2 GC-MS analysis

The composition of body and faecal volatiles of *L. m. capito* were identified using the GC-MS methods and analysis as outlined in detail in section 2.10.

4.2.5 Standard chemicals co-injection analyses

Candidate synthetic standards namely anisole, benzyl alcohol, veratrole, guaiacol, beta-ionone, n-decanoic acid, butanediol, palmitic acid in powder form were purchased from Sigma Aldrich, USA and their purity ranged from 98% to 99.5%. An aliquot of 5 µl of each synthetic standard was added to 5 ml of high purity grade dichloromethane out of which 5µl of the prepared solution were injected into GC. Synthetic standards having identical retention times to those obtained and identified previously in the volatile samples by GC-MS were subsequently used for GC-EAD for final the confirmation of their chemical identity using antennae of *L. m. capito* as a biological detector. Similar procedure for GC-EAD as described in section 2.9 was followed.

4.3 Results

4.3.1 Analysis of body and faecal volatiles released by adults of *Locusta migratoria capito*

4.3.1.1 Body volatile emissions

GC analysis of volatile emissions of adults *L. m. capito* at different sexual maturity stages for both sexes showed similar chromatogram profiles with regard to their head-space volatile productions. However, male and female chromatogram profiles

displayed quantitative differences in the amounts of the different components throughout the maturation process. These quantitative differences were dependent on the age status of the locusts and were more evident when locusts were fully mature, especially during the mating period (Figure 4.1). The highest variation was observed in the amounts of phenylacetonitrile (PAN) (R.T: 11.87 minutes) that was released in substantial amount when mature males were mounting receptive females ready to mate followed by an increase in the release of veratrole (R.T: 11.92 minutes).

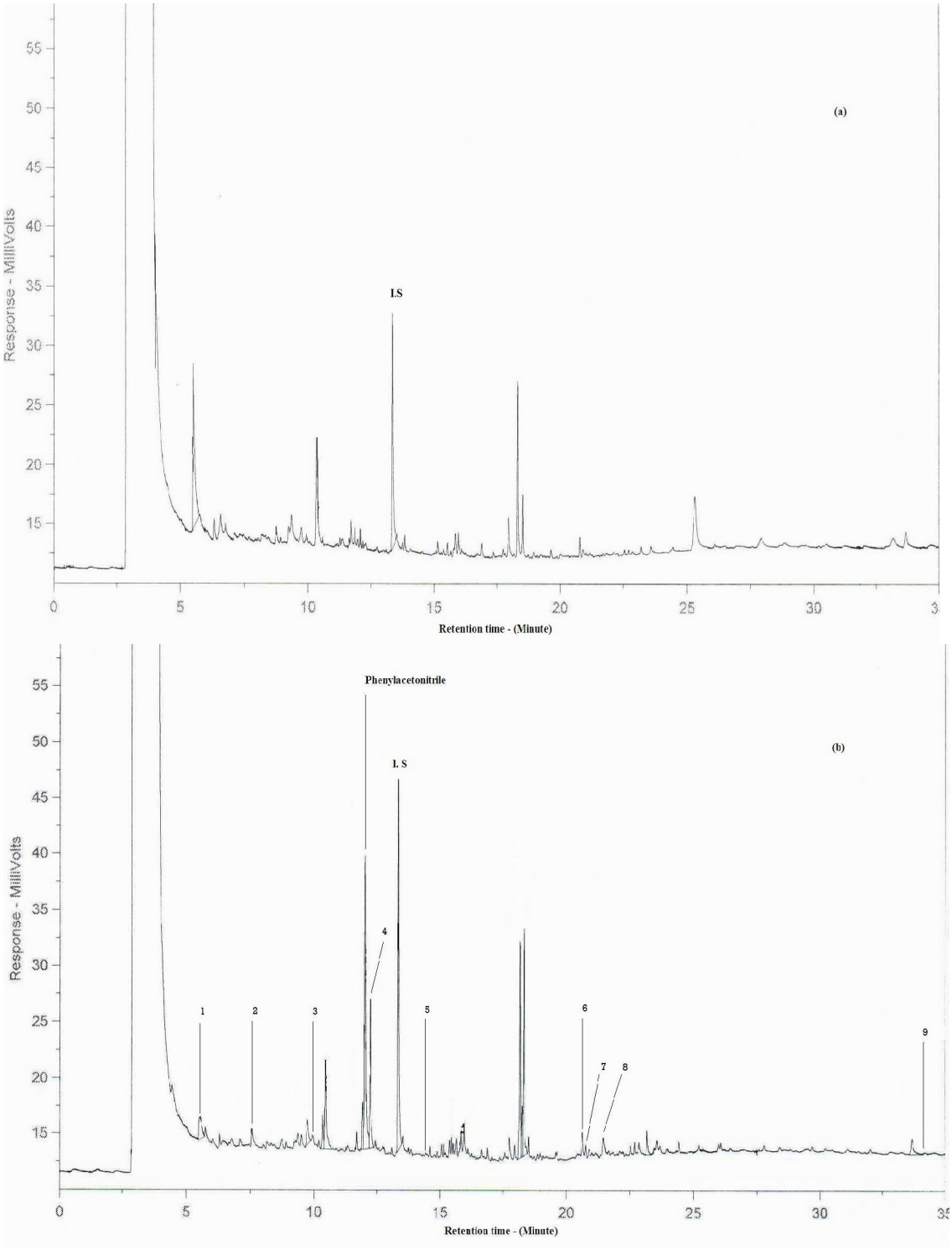

Figure 4.1 Comparison of representative gas chromatograms of volatiles collected from 18 day-old mature females (a) and males (b) *Locusta migratoria capito* injected onto HP1 ultra I methyl silicate gum capillary column (25m long) and with methyl salicylate as an internal standard (I.S. : 13.42 minutes).

4.3.1.2 Faecal volatiles odours

For both male and female adults of *L. m. capito* irrespective of their age, faecal volatiles had chromatographic profiles that were quantitatively and qualitatively similar, with the major constituents, compound detected corresponding to guaiacol (R.T : 10.70 minutes) (Figure 4.2).

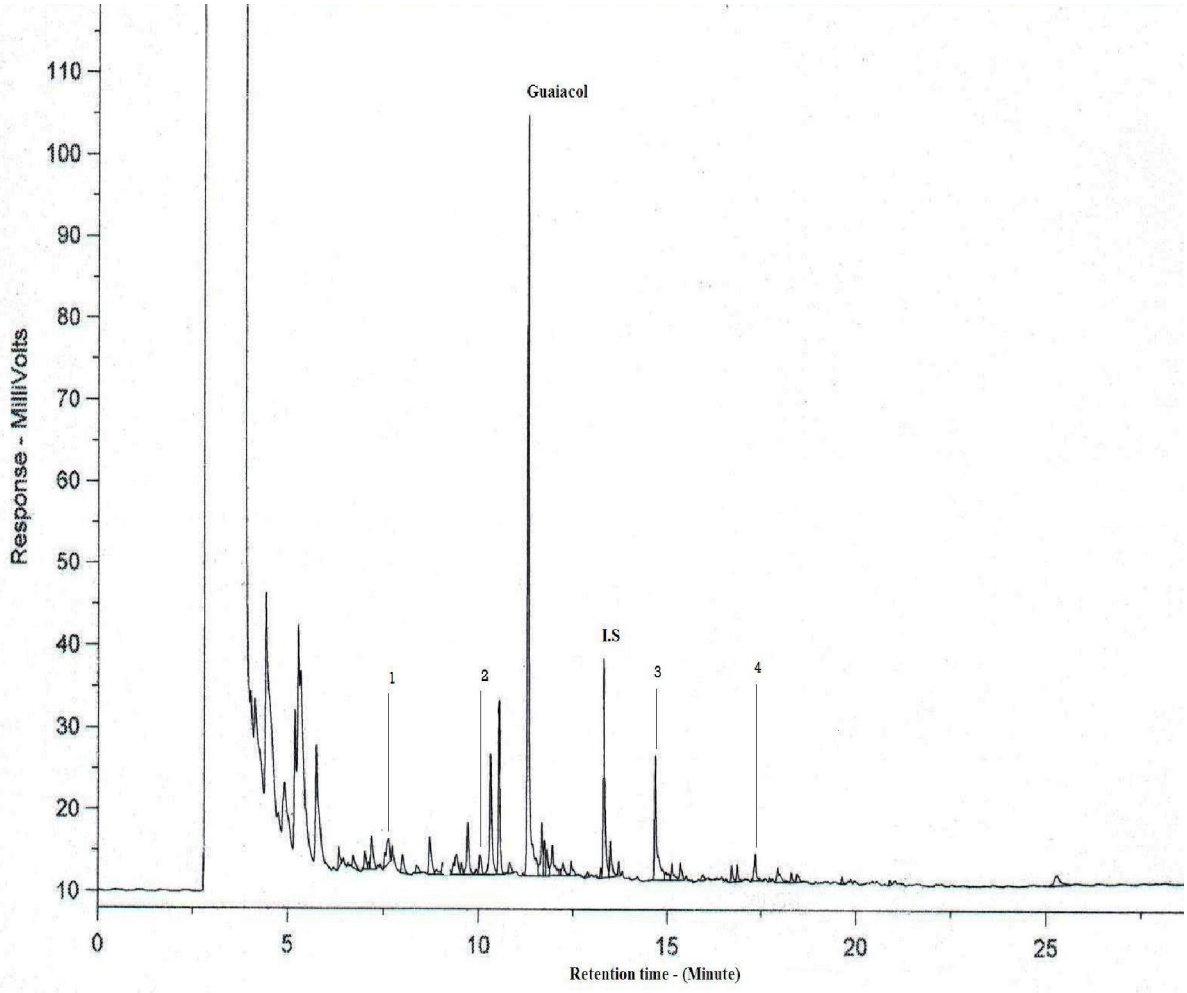

Figure 4.2 Representative gas chromatogram of volatiles collected from fresh faecal pellets of adult *Locusta migratoria capito* injected onto HP1 ultra I methyl silicate gum capillary column (25m long) and with methyl salicylate as an internal standard (I.S) (RT: 13.42 minutes).

4.3.2 EAD responses of the adults' antennae

4.3.2.1 EAD responses to locust body volatiles

The antennal electroantennogram (EAG) responses were recorded using crude body odours of *L. m. capito* at natural concentrations as effluents. Antennae of male and female locusts showed EAG responses to nine compounds. These compounds correspond respectively to retention times at 176s, 272s, 390s, 496s, 694s, 860s, 912s, 960s and 1456s (Figure 4.3).

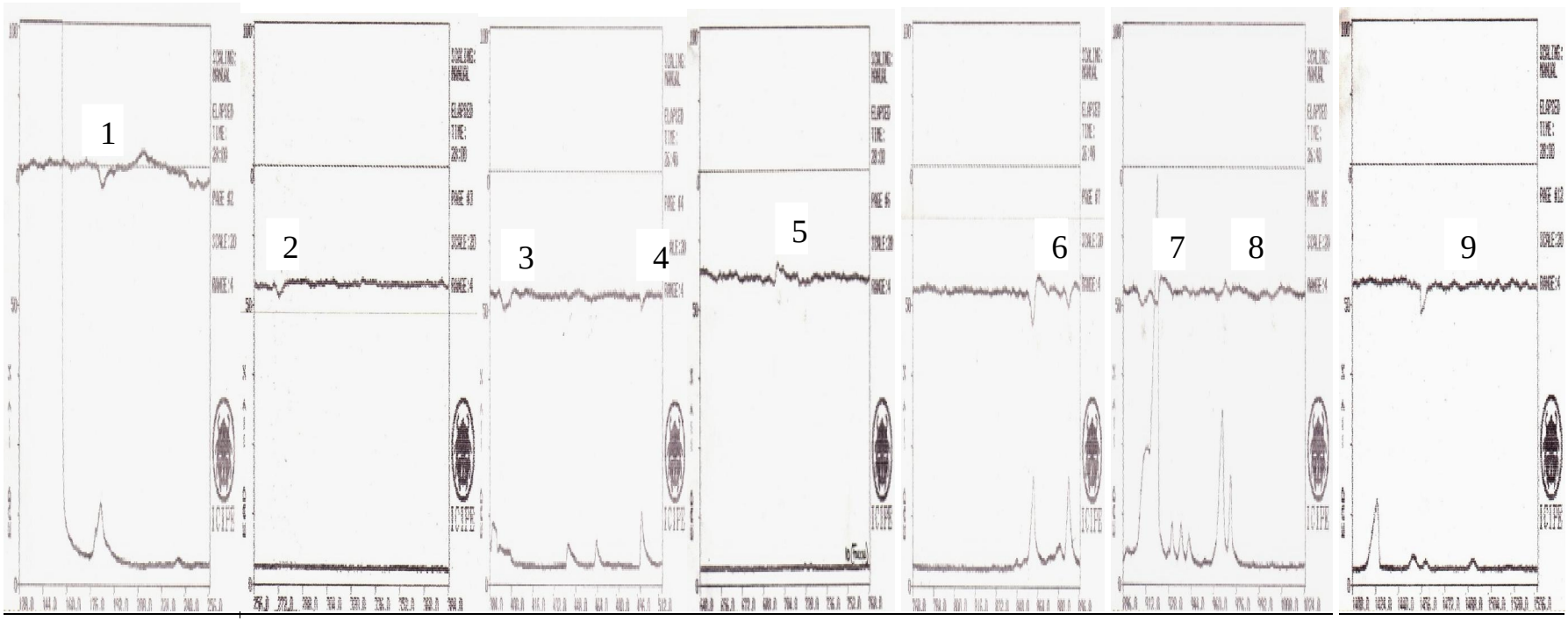

Figure 4.3 Representative EAD recordings from antennae of adults of *Locusta migratoria capito* responding to components in their crude body volatiles (EAG responses generated by compound peaks labelled 1, 2, 3, 4, 5, 6, 7, 8 and 9).

4.3.2.2. EAD responses to faecal volatiles

With regard to *L. m. capito* faecal volatiles, five compounds with retention times at 272s; 390s; 440s; 656s and 752s at natural concentrations evoked EAG responses in antennae of both sexes. The compound occurring at 440s corresponding to the major component of the faeces and identified to be guaiacol in GC-MS analysis as previously mentioned evoked strong EAG responses (Figure 4.4).

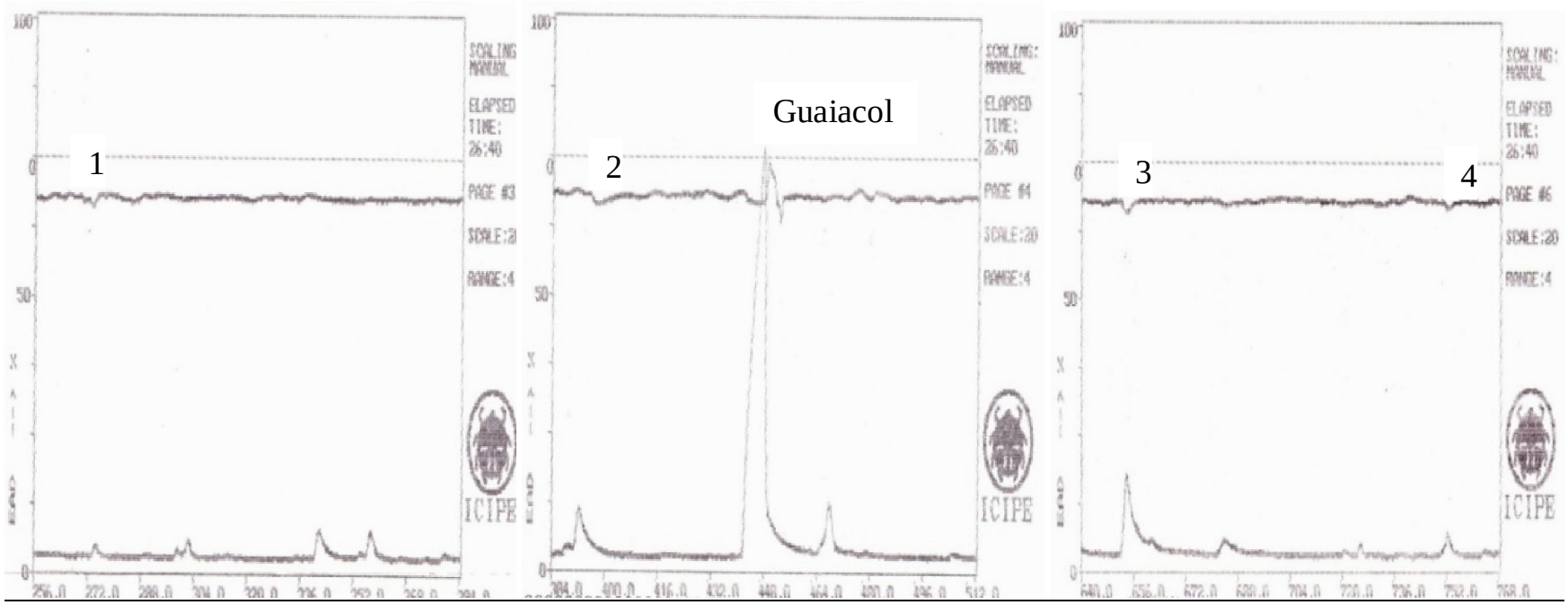

Figure 4.4 Representative EAD recordings from antennae of adults of *Locusta migratoria capito* responding to components in their crude faecal volatiles (EAD responses generated by compounds denoted by peaks labelled 1, 2, 3, 4, and guaiacol).

4.3.3 Mass Spectrometric identification and quantification of EAG-active the body and faecal components

Coupled GC-Mass spectrometric analyses identified a total of 12 of the EAG-active compounds, of which nine compounds were from body emissions and five from faecal volatiles. Two compounds namely anisole and benzyl alcohol were shared by the two different sources. Four compounds from the body volatiles have been confirmed among the nine recorded with EAD techniques in the crude volatiles namely, anisole, benzyl alcohol, veratrole, and n-hexadecanoic acid. The retention time of n-decanoic acid standard closely corresponded to body volatile active compounds at 696s, and therefore, antennal electrophysiological responses could not be obtained even with enhanced concentrations. Three of the compounds that were not fully identified not fully identified and confirmed were probably 3-penten-1-ol (176), (C_{15}) (Z) 6-pentadecen-1-ol (860s) and (C_{18}) (Z) 9-octadecanoic acid, methyl ester (960s). One unidentified compound occurred at 1490s. Faecal emissions had five EAG-active compounds, four of which were identified to be anisole, benzyl alcohol, guaiacol, and β-ionone. The additional compound at 656s remained unidentified (Table 4.1).

Table 4.1 Fully identified electro-physiologically-active potential candidate compounds with their relative amounts of the body and faecal volatiles of *Locusta migratoria capito* analysed on the Masslab 12-250 GC-MS and HP 7890 GC-MS.

N°	Rt (s)	Body volatiles (M/z) *- Standard Retention time*	Average amount (ng/h/locust)	Faecal volatiles (M/z) *Standard Retention time*	Average Ratio (ng/h/g)
1	176	***3-Penten-1-ol***	MM.0.47±0.06 *Av=0.85±0.2* MF.1.23±0.25	-	-
2	272	ü **Anisole*** **(M/z : 108,54,26,82,80)** ***- 269'***	MM.0.12±0.02 *Av=0.23±0.05* MF.0.34±0.06	ü **Anisole*** **(M/z : 108, 54, 26, 82,80)** ***- 269'***	*0.60±0.24*
3	390	ü **Benzyl Alcohol*** **(M/z : 39,51,79,91,108)** ***- 386'***	MM.0.04±0.006 *Av=0.07±0.02* MF.0.11±0.03	ü **Benzyl Alcohol*** **(M/z : 39,51,79,91,108)** ***- 386'***	*0.52±0.25*
4	440	-	-	ü **Guaiacol*** **(M/z : 81, 109, 124)** ***- 440'***	*1.49±0.55*
5	496	ü **Veratrole*** **(M/z: 77,95,123,138)** ***- 494'***	MM.0.05±0.003 *Av=0.04±0.003* MF.0.04±0.003	-	-
6	656	-	-	***Unidentified***	*0.27±0.09*
7	694	***n-Decanoic acid*** **– 696'**	>0.01	-	-
8	752	-	-	ü **β-ionone*** **(M/z=177,43,91,135,178)** ***- 750'***	*0.45±0.14*
9	860	***(Z) 6-pentadecen-1-ol***	-	-	-
10	912	ü **n-Hexadecanoic acid*** **(M/z : 43,60,78,83,97,115,129, 213,256)** ***- 915'***	MM.0.26±0.003 *Av=0.22±0.04* MF.0.17±0.09	-	-
11	960	***(Z) 9-Octadecanoic acid methyl ester***	MM.0.11±0.008 *Av=0.07±0.02* MF.0.04±0.005	-	-
12	1456	**Unidentified (C27-C28)**	>0.01	-	-

*Confirmed with co-injected standards and EAG activity. (MM=Mature males; *Av=Average* amount; MF=Mature females).

4.4 Discussion

Aggregation bioassays using live *L. m. capito* adults as recipients of their own body and faecal volatile emissions have previously shown evidence implicating their mediation in the aggregation process. No significant differences were noticed during aggregation assays with regard to response by both sexes to their own body volatiles. The identical chromatogram profiles obtained during chemical analyses of body volatiles produced by males and females of *L. m. capito* support and are in accordance with results recorded during the previous aggregation bioassays conducted using these same sources of volatiles (Chapter 3). Relative differences shown by the behavioural assays previously obtained possibly are due to the quantitative differences in addition to the relative low sensitivity of male locust antennae compared to females to the various odour components (Pers observations).

Results of the chemical identification showed *L. m. capito* produces some components that are present in volatiles of the African migratory locust, *L. m. migratorioides* while other components overlap with those of adult desert locust, *S. gregaria* (Torto *et al.*, 1994; Niassy *et al.*, 1998; Schmidt, 1999). Phenyl derivatives such as anisole and veratrole were previously reported in volatiles of the desert locust in addition to phenylacetonitrile which was also detected and identified in volatiles of *L. m. capito* as identified earlier by Fuzeau-Braesch *et al.* (1988) and Torto *et al.* (1994). Alcohols and aliphatic chain compounds most likely similar to those identified in volatiles of *L. m. migratorioides* were also detected in *L. m. capito* though not fully characterised (Obeng'Ofori *et al.,* 1993; Torto *et al.,* 1994, 1996;

Niassy *et al.,* 1998; Schmidt, 1999). Their identity in the volatiles of the Malagasy migratory locust was not confirmed due to the unavailability of some of the synthetic standards for further confirmation.

Identified components in body volatiles of *L. m. capito* fell into two distinct groups on the basis of their chemical structures. On one hand, high volatility molecules comprising phenyl derivatives formed the first set and assumed to constitute the volatile pheromone, probably responsible for the aggregation behaviour. These compounds are assumed to act at long and middle-range distance because of their volatile physico-chemical property to activate the aggregation process in this locust species. Thus, they may be considered to be the releaser pheromone for aggregation (Schmidt, 1997). On the other hand, heavy compounds that include the long aliphatic chain hydrocarbons and their derivative acids and alcohols possibly constitute the cuticular contact pheromone and/or secreted metabolites due to induced effects of the gregarious status (Heifetz *et al.,* 1996; 1998). These compounds probably form either the short range and contact pheromone involved in conspecific and mate recognition, courtship and sexual behaviour, or phase change (Whitman, 1990a; Heifetz *et al., 1996*; Hassanali *et al.,* 2005). Indeed, during volatile collection, it was noted that mature locusts produced white waxes that coated the glass tubings. These probably correspond to the heavy hydrocarbon compounds that were only observed in mature locusts and also when locust populations were reaching a certain level of gregariousness. This suggests their possible role in the maintaining of gregarious characteristics and is hypothesised to constitute secretions of a primer gregarising pheromone in this species as proposed previously in the nymphs of the desert locust

that may also influence their sexual behaviour (Heifetz *et al*., 1997; Schmidt, 1997; Heifetz *et al.,* 1998).

Sexually maturing female *L. m. capito* release relatively higher amounts of all volatile components compared to their male conspecifics, with the exception of phenylacetonitrile. Conversely, release of heavy compounds follow the pattern of phenylacetonitrile emission, with relative high amounts released by mature males compared to females (Table 4.1). The foregoing supports the hypothesis advanced earlier that, aggregation and gregarisation processes are triggered by releaser and primer pheromones respectively that are produced by female and male locusts recruited into nuclear groups of locusts.

GC analysis on faecal volatile emissions showed identical profiles regardless of sex and age of the adult locusts and showed predominance of guaiacol, as reported previously in different species of gregarious acridids (Obeng'Ofori *et al*., 1994). Anisole, Benzyl alcohol and beta-ionone were also identified among faecal volatile active component in the Malagasy migratory locust species similar to faecal volatile composition of the *L. m. manilensis*, as reported earlier by Yu *et al.,* (2007).

Locusta m. capito is assumed to originate from and is biologically related to the mainland species *L. m. migratorioides*, arguments that probably support to some extent the chemical ecology similarities to the original ancestors (Wintrebert, 1970). On the other hand, identical ecological environment leading to similar breeding adaptations to the semi-arid regions prevalent in the south-western part of Madagascar possibly point to the convergent chemical ecology adaptations of *L. m.*

capito with regard to the resemblance with the desert locust. These adaptations may have enabled *L. m. capito* to evolve and display flexible adaptability to climate and environmental changes within the outbreak area. It remains to elucidate whether both the origin of the species and adaptation to a new environment have an effect on the chemical ecology of the species.

The effects of the identified physiologically-active compounds on the Malagasy migratory locust and their roles in the aggregation process have not been investigated. Results of their roles in the aggregation processes are presented and discussed in the following chapter.

CHAPTER FIVE

5.0 ROLE OF BODY AND FAECAL VOLATILES OF ADULTS OF *LOCUSTA MIGRATORIA CAPITO* IN AGGREGATION BEHAVIOUR AND SOLITARISATION EFFECTS ON NYMPHAL CONSPECIFICS

5.1 Introduction

Olfaction has been demonstrated to take a preponderant and major role in modulating behaviour in many insects and also play a role in aggregation and cohesion behaviour in *L. m. capito*. The development of new control strategies using pheromone has gradually opened up a promise of offering efficient and environment friendly method in locust control since the isolation and identification of "locustol". This compound, a faecal volatile component, was assumed to be the olfactory factor involved in mediating aggregation in the migratory locust (Nolte *et al.,* 1973). However, locustol as a component of faecal volatiles was shown to be inexistent in subsequent studies (Fuzeau–Braesch *et al.,* 1988; Whitmann, 1990; Torto *et al.,* 1994; Schmidt, 1997; Ferenz and Seidelmann, 2003; Schmidt and Albutz, 2004).

The identification of two different pheromone systems that control aggregation behaviour of the desert locust *S. gregaria* in all stages, namely, the nymphal pheromone blend (NPB) for the nymphs and the adult pheromone blend (APB) strengthen the current findings and indicate the possible use and integration of pheromones as environmental friendly tactic in locust management strategies (Obeng'Ofori *et al.,* 1993; Torto *et al.,* 1994; 1996). These two pheromonal systems showed antagonistic roles when applied to either of the stages, leading to disruption

of the grouping behaviour of the nymphs when treated with adult pheromone (Bashir *et al.,* 2000b; Kane, 2004).

In gravid females, acetophenone and veratrole produced by the egg pod froth, have been demonstrated to trigger communal and synchronous oviposition, hence its designation as "oviposition pheromone" (Saini *et al*., 1995; Rai *et al.,* 1997). These, together with compounds deposited in the sand, viz (Z)-6-octen-2-one, (E,E)-3,5-octadien-2-one and its geometric isomer (E,Z)-3,5-octadien-2-one, stimulate the grouping of hatching nymphs in the early stages and maintain their gregarious characters (Assad *et al*, 1997; Niassy *et al*., 1998; Torto *et al.,* 1999; Hamouda *et al.,* 2009).

Aggregation and gregarisation processes are postulated as following the same pattern in gregarisable locusts (Hassanali *et al.,* 2005). Four of the nine antennographic active compounds in body volatiles of *L. m. capito* were identified as anisole, benzyl alcohol, veratrole, n-hexadecanoic acid. The other five compounds were not fully identified but are potential candidates in the composition of the pheromone bouquet in *L. m. capito*. However benzyl alcohol was of faecal origin due to their large amount in the faecal volatiles. Blend comprising anisole and benzyl alcohol with guaiacol and beta-ionone in addition to an unidentified compound constitute the faecal bouquet. The role of these compounds, singly or in blends, in the aggregation behaviour are crucial as well as pivotal in the population dynamics and control of each stage in the phase shift process in this species (Ferenz *et al*., 1994; Klause de Pupka, 1997; Cork *et al*., 2003).

There is need to determine the role of these compounds in the aggregation behaviour of adult locusts and their effects on nymphal grouping behaviour and the phase shift process. The objective of this study was to characterize the aggregation pheromone system of gregarious adults of *L. m. capito* followed by laboratory assessment on whether the constituent active compounds also play a role in the grouping behaviour as well as their solitarisation potential to nymphal stages.

5.2 Material and Methods

5.2.1 Rearing of locusts

Gregarious adults and fifth instar nymphs *L. m. capito* from a colony reared and maintained at the insect rearing and quarantine facilities at *icipe* (Nairobi, Kenya) were used. Rearing conditions are outlined in section 2.1. Adult and nymphal stages were kept separately, minimizing cross-pheromonal effects, throughout their development.

5.2.2 Synthetics of the identified compounds used in tests

Anisole, benzyl alcohol, veratrole, guaiacol, beta-ionone, n-decanoic acid, butanediol, methyl salicilate and palmitic acid in powder form were purchased from Sigma Aldrich, USA and used. Purity ranged from 98% to 99.5%.

5.2.3 Calibration of dispenser

5.2.3.1 Preparation of dispensers and collection of volatiles

For calibrations of dispensers, three different increasing concentrations namely one, two and four (µl) in 500 µl light paraffin oil (Merck) were prepared for each identified synthetic compound and kept in polyethylene centrifuge tubes (section 2.3.1). Prior to their use, dispensers were kept in ambient temperature in a glass Petri dish (dimension: 10 mm x 10 cm diameter) for three to five days, thereby allowing release rates to stabilize, and then used as source of volatiles. One dispenser served as source of volatiles for collection at a time. Volatile collection procedure was outlined in sections 2.6 and 2.7, respectively. Volatile collections were repeated three times for each concentration of synthetic standard prepared and also for the two other concentrations of the same compound.

5.2.3.2 GC analyses and calibration of the dispensers

Collected volatiles were analysed and their corresponding release rates served as the basis in the calculations and the plot of the release calibration line of the corresponding compound. Quantification of all collected volatiles were carried out with chemical analysis routine using HP-5890 Series II gas chromatograph equipped with FID detector as described in section 2.8. GC chromatographic relative peak areas were used for calculations of the amounts of volatile release relative to the internal standard.

Amounts of standards to be added to 500 ml light paraffin oil to provide the desired and expected quantity of volatiles to be released for bioassays were calculated using equations obtained from the previous calibrations with known concentrations. The procedure as described in section 5.2.3.1 was followed. Volatile sources for bioassays were prepared according to these calculations.

5.2.4 Sources of volatiles for bioassays

Volatile sources comprising identified synthetic chemicals as listed in section 5.2.2 were prepared in dispensers. The amount of synthetic standards obtained after calculations of the release rates of the dispenser was prepared in order to allow approximate volatile releases of 25 to 40 locust equivalents. For each synthetic compound, the rounded ratios of their volumes in 500 µl paraffin oil used were: anisole (0.5 µl); benzyl alcohol (0.15 µl); veratrole (0.10 µl); guaiacol (0.15 µl), beta-ionone (1 µl); Faecal blend with anisole, benzyl alcohol, guaiacol and beta-ionone (0.5:0.15:0.15:1 µl); body volatile blend comprising anisole, benzyl alcohol and veratrole (0.5:0.15:0.10 µl); faecal blend without benzyl alcohol (0.5:0.15:1 µl) and body blend out of benzyl alcohol (0.5:0.10 µl). Confirmation of the estimated release rates of the dispenser was carried out following the same procedure as for the quantification previously outlined in section 5.2.3.

5.2.5 Aggregation bioassays

Aggregation responses to single or blends of previously identified active compounds were assessed using olfactometric assays described in section 2.2.1 and following identical protocol outlined in section 2.4.1. Single synthetic compounds or their

blends as previously prepared in dispensers were used as sources of volatile (section 5.2.4).

5.2.6 Solitarisation tests

Fourth instar nymphs (N_4) that synchronously moulted to the fifth instar (N_5) were exposed continuously to the identified single compounds or blends of electro-antennographically-active body and faecal volatile components until their final moult. Nymphs were kept individually in banks of separate cages for isolation. Same dispensers as previously used were utilised to release odours (section 5.2.5) within each solitary cage except for the powdery palmitic acid which was prepared by addition of 0.12 grams of palmitic acid (Aldrich Sigma, USA) in 300µl light paraffin oil (Merck) and spread on filter paper (Manchery-Nagel, MN615, Germany) (125 mm diameter) then presented to nymphs. An additional control treatment consisting of exposure of a batch of nymphs to filter paper impregnated with only paraffin oil was included in parallel. Batches of thirty two fifth instar nymphs formed the whole treatment for each volatile source tested. Normal solitarisation free of volatile exposure comprising of a batch of thirty two nymphal locusts served as control. The experimental locusts were kept under the same rearing conditions to avoid effects that may arise from changes and proper ventilation was provided for the experiment.

Measurements of the lengths of hind femora (F) and Elytra (E) and the width of the head (C) of the new fledglings after two days of cuticle hardening were performed using an electronic calliper (Mitutoyo, Absolute precision calliper, UK). Gregarious and solitarised newly fledged locusts were determined by morphometric ratio

measurements established by Launois-Luong (1980) such that individuals from the two distinct populations display morphometrics ratio indicated in table 5.1 below.

Table 5.1 Morphometric ratios of the two different phase populations in *Locusta migratoria* kin by Launois-Luong (1980).

Ratios	Solitary	Gregarious
E/F	< 2.00	> 2.00
F/C	< 3.30	> 3.30

Numbers and percentages of solitarised individuals within the whole batch of treatments were counted and compared against the control treatment.

5.2.7 Aggregation Index (A. I.) and data analysis

The aggregation index (A. I.) was calculated using the formula A. I. = 100 x (T-C)/N %, where N is the total number of insects tested; T, number of insects responding to the volatiles; and C those in control area of the chamber.

Data were analysed as described Chapter two, section 2.11. With regard to the solitarisation tests, count data were analyzed with Chi-square test at $\alpha = 0.05$) (SAS, 2003).

5.3 Results

5.3.1. Calibrations of release rates of dispenser

Synthetic standards used, namely phenyl derivatives including anisole, benzyl alcohol, veratrole, guaiacol and beta-ionone, which form the pheromone components, were released in the proportion of increasing amounts following the gradual increase of their corresponding concentrations. Calibration trends were identical in linear model in amounts released as defined by linear equations anisole ($Y_{anisole} = 13.965X$ with $R^2 = 0.9799$); benzyl alcohol ($Y_{benzyl\ alcohol} = 14.459X$ with $R^2 = 0.9413$); veratrole ($Y_{veratrole} = 14.029X$ with $R^2 = 0.8686$) and guaiacol ($Y_{Guaiacol} = 13.583X$ with $R^2 = 1$) whereas beta-ionone was comparably low, but followed similar linear trend defined by the equation $Y_{beta\text{-}ionone} = 0.0608X$ with $R^2 = 0.5258$) (Figure 5.1). Release rate of phenyl derivatives gave approximate release rates of 25 to 37 locust equivalents and equivalent faecal weight ranging between 1g to 10g (Table 5.1).

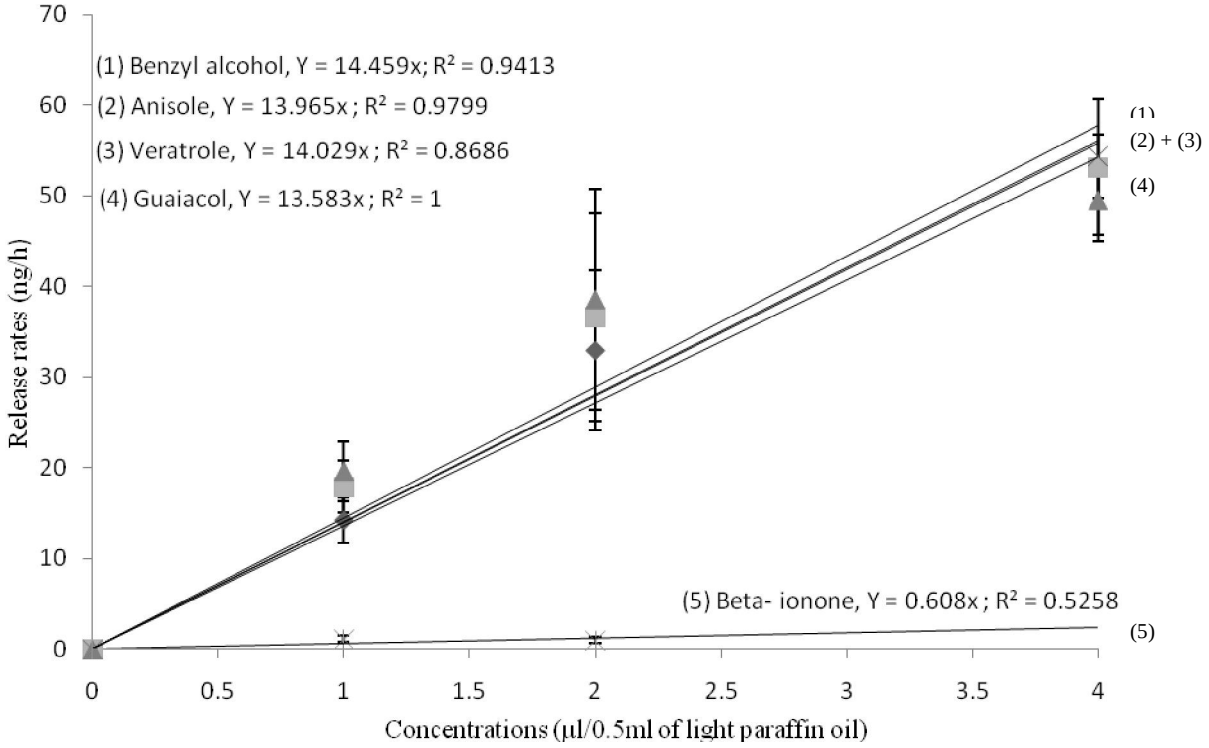

Figure 5.1 Release rate calibration plots for different synthetic standards at different concentrations (1, 2 and 4 µl) in 500 µl paraffin oil using 1 ml polyethylene centrifuge tubes as dispenser.

Table 5.2 Release of synthetic standards using 1 ml polyethylene centrifuge tube as dispenser and their equivalent locust body and faecal (grams) sources.

Sources of volatiles Standard/Paraffin oil	Average release amount of standard (ng/h)	Locust and Faeces Releases (ng/h)	Number of replicates	Average locust equivalent (Meq)	Average Faeces Equivalent(g/h)
Anisole	5.75±1.52	0.23±0.05	3	25	10
Benzyl alcohol	1.75±0.13	0.07±0.02	3	25	3.5
Guaiacol	1.39±0.07	1.49±0.55	3	-	1
Veratrole	1.51±0.43	0.04±0.003	3	37	-
Beta-ionone	0.36±0.14	0.45±0.14	3	-	1

5.3.2. Aggregation responses to identified EAG-active body and faecal volatile components

To single volatile component, both sexes responded positively to anisole, guaiacol and beta-ionone whereas benzyl alcohol elicited reverse responses especially to mature males (Figure 5.3). Anisole and beta-ionone presented the highest responses to females (A. I. = 24 ± 7% and 26 ± 13%), whereas guaiacol induced low responses (A. I. = 4 ± 15%). Conversely, these three volatile sources induced similar level of responses by mature males (A. I. = 18 ± 9%; 17 ± 8% and 14 ± 10%). With regard to veratrole, only mature females responded positively to this compound. Mature males responded inversely by weak repellence (A. I. = -9 ± 9%) (Figure 5.2).

A blend of anisole, benzyl alcohol and veratrole, three components of the body volatile amongst the five physiologically active compounds, elicited weak positive responses to mature females but not to the mature males. A blend without benzyl alcohol enhanced aggregation responses to both sexes giving the highest index (A. I. = 30 ± 9%). Results show that the blend of anisole and veratrole likely act as the aggregation pheromone used by gregarious adult locust *L. m. capito* in their cohesion and aggregation behaviour. This blend evoked aggregation response close to that by crude body volatiles previously tested (Figure 5.2).

Blend of four active compounds amongst the five physiologically-active ones produced by faeces elicited repellence to both sexes of mature adults *L. m. capito* (A. I. = -15 ± 12% and -13 ± 13%) (Figure 5.2). Random distribution across the arena was observed when benzyl alcohol was not included in the blend.

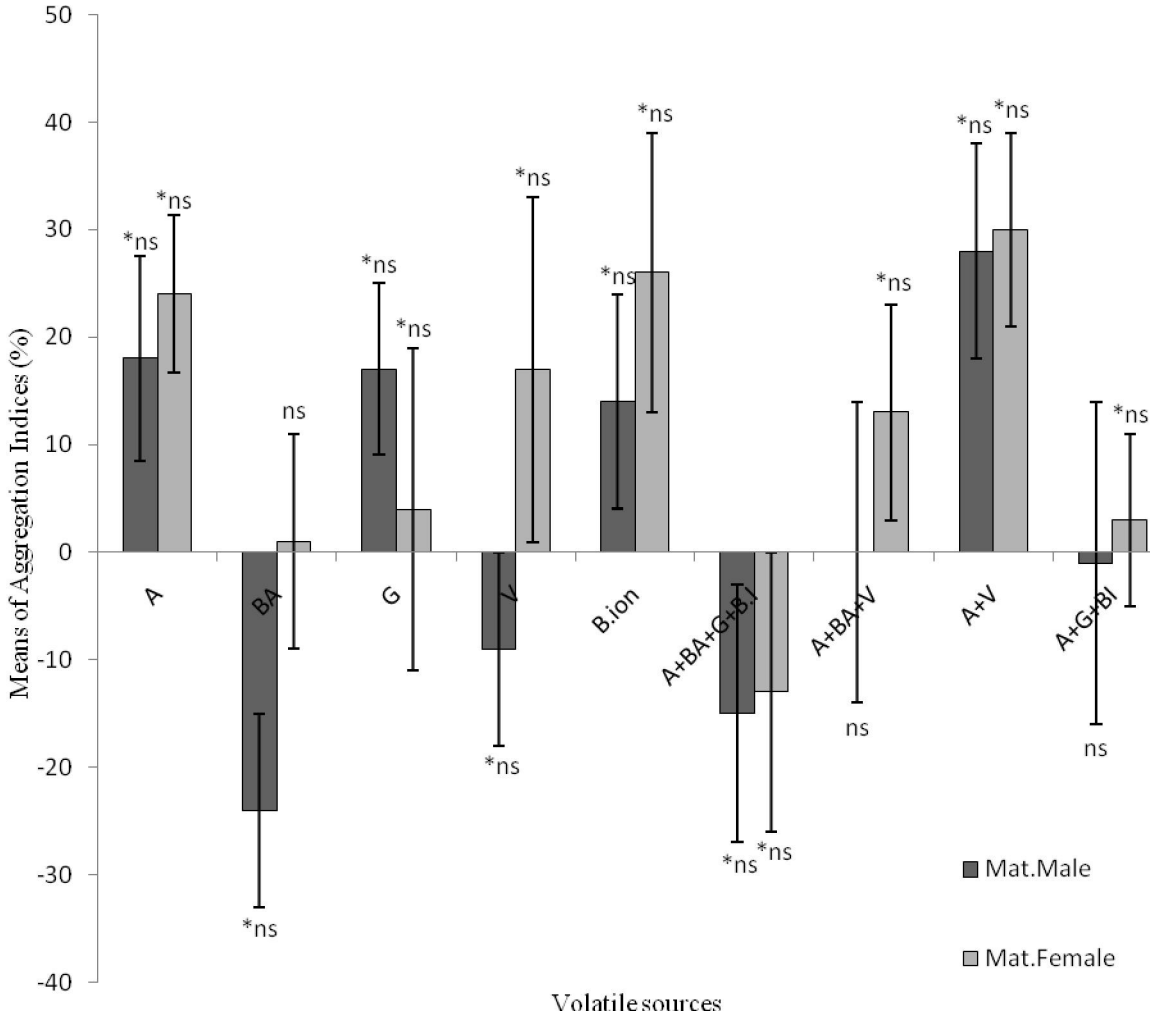

Figure 5.2 Aggregation responses (mean ± SE) of mature male and female locusts *Locusta migratoria capito* individually tested to single compounds and blends of the electro-antennographically-active compounds. (Locust tested: Mat. Males= Mature Males and Mat. Female = Mature Females). (Source of volatile: A = Anisole; BA = Benzyl alcohol; G = Guaiacol; V = Veratrole; BI = Beta-ionone). * indicates differences were significant (Chi-square tests at P = 0.05). ns indicates no significant differences between means (P = 0.05, ANOVA tests)

5.3.3 Solitarisation effects of identified EAG-active body and faecal components on fifth instar nymphs (N_5)

Solitarisation experiments exposing nymphal stage of *L. m. capito* to single compounds of the highly volatile active electro-antennographically-active components of body and faecal odour namely anisole, benzyl alcohol, veratrole guaiacol and beta-ionone, showed that all compounds presented to nymphs gave an identical pattern and induced apparition of solitary characters in the newly emerging fledglings with the exception benzyl alcohol. Benzyl alcohol elicited no significant differences between the test and control nymph with low rate of solitarisation in adult fledglings ($\chi^2 = 0.157$, D.F = 1, N = 64, P = 0.69).

Blend of compounds comprising the three active components, namely anisole, benzyl alcohol and veratrole, amongst the five volatile compounds identified from the body volatile as well as the blend of four active components identified from faecal volatiles comprising anisole, benzyl alcohol, guaiacol and beta-ionone, showed no significant differences in solitarisation rates compared to the control ($\chi^2 = 0$, D.F = 1, N = 64, P = 1) ($F_{df\,(7,\,248)} = 1.92$, N = 270, P = 0.06, SNK, test) (Figure 5.3).

The only high molecular weight component available, namely n-hexadecanoic acid induced low rate of solitarisation that was significantly different when compared to the control locusts that were exposed to light paraffin oil but was not significantly different when compared to the untreated control ($\chi^2 = 2.12$, D.F = 2, N = 96, P = 0.634) ($F_{df\,(2,\,93)} = 1.06$, N = 96, P = 0.35, SNK, test) (Figure 5.4).

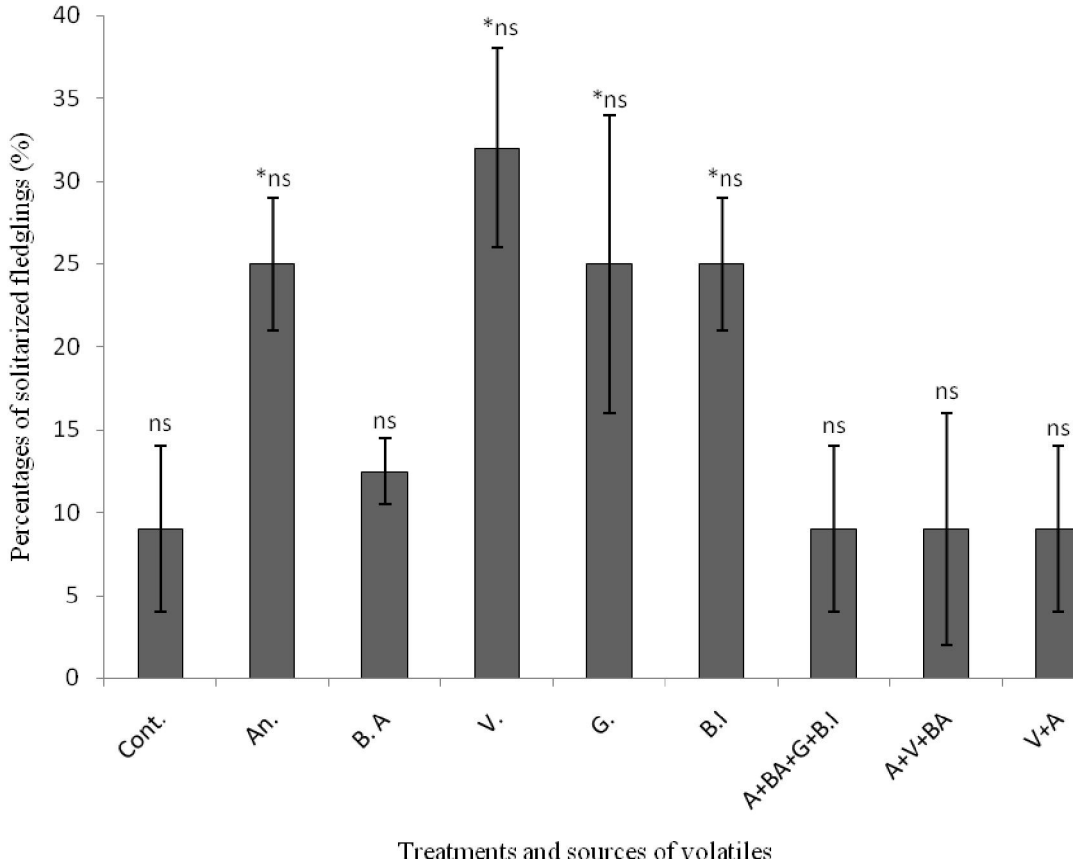

Figure 5.3 Percentages of solitarised fledglings (mean ± SE) *Locusta migratoria capito* after exposure to single compounds and blends of the electro-antennographically-active compounds. (Source of volatiles: Cont = control; A = Anisole; BA = Benzyl alcohol; G = Guaiacol; PAN = Phenylacetonitrile; V = Veratrole; B. I = Beta-ionone, A+BA+G+B.I, A+V+BA and V+A = Blends of the respective compounds). * indicates differences were significant (Chi-square tests at P = 0.05). ns indicates no significant differences between means (P = 0.05, ANOVA tests)

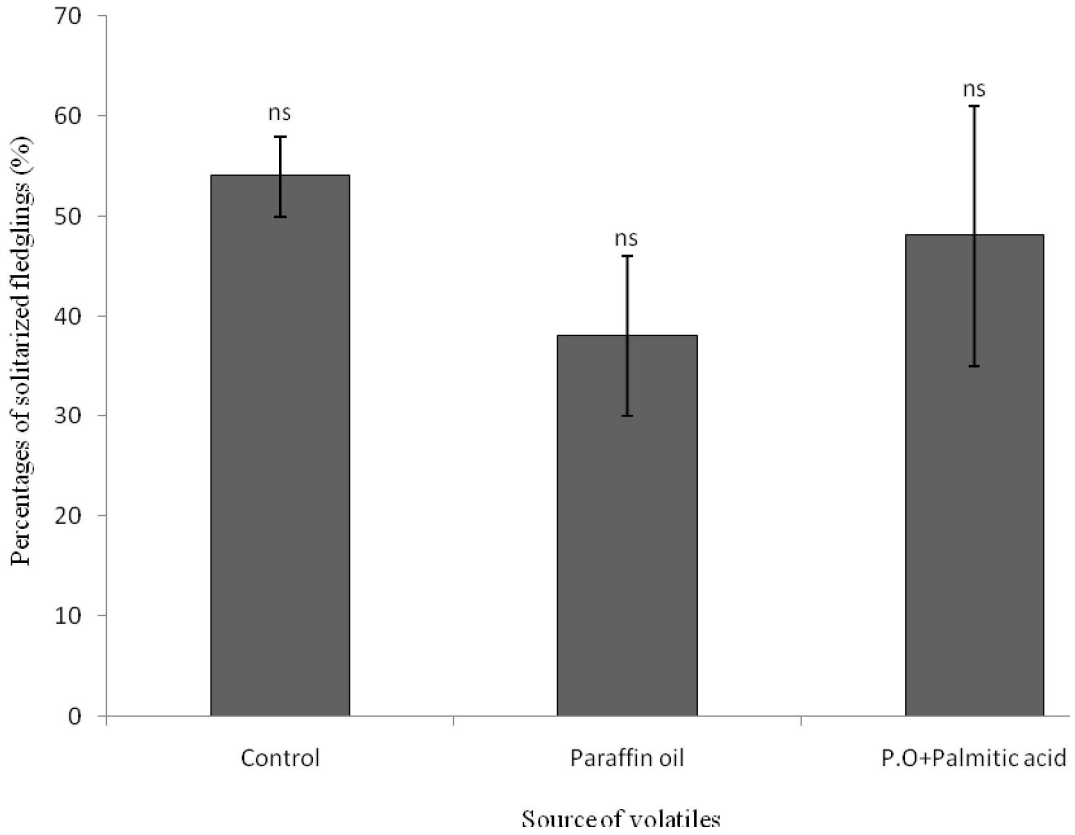

Figure 5.4 Percentages (mean ± SE) of solitarised fledglings of *Locusta migratoria capito* after exposure to n-hexadecanoic acid. Thirty two locusts were tested singly for each test. (P.O = Paraffin oil). ns, means not statistically significant, SNK test.

5.4 Discussion

In the previous chapter, analyses on the volatiles of adult of *L. m. capito* led to the identification of veratrole and anisole in the body volatiles while guaiacol, anisole, benzyl alcohol and beta-ionone were present in faecal volatiles. Nolte *et al.* (1973) identified guaiacol in volatiles of *L. migratoria* which was also found by Fuzeau-Braesch *et al.* (1988) together with veratrole and phenol in volatiles surrounding cages of these locusts. These three components were also identified to be part of the blend of the aggregation pheromone of adult desert locust, *S. gregaria* (Torto *et al*., 1994). However, in the present work, phenol was not among the physiologically active components in the faecal odours of *L. m. capito*. Subsequent aggregation bioassays performed using these compounds tested singly or in blends, showed similar trend of aggregation responses to those obtained with the crude volatiles (Chapter 3). The presence of these compounds confirms the earlier findings by other workers on the characterisation of the aggregation pheromones in *Locusta* spp (Fuzeau-Braesch *et al*., 1988, Heifetz *et al*., 1996).

With regard to components identified in body volatiles, both males and females responded to anisole while only females responded to veratrole. Further, both sexes of adult locust responded strongly to the blend of these two compounds. This suggests that the two-compound blend is part of the aggregation pheromone used by gregarious adult locust *L. m. capito* in their cohesion and aggregation behaviour. They gave almost the same level of responsiveness as obtained previously for the crude body volatiles. The lower responses of the locusts to the synthetic as compared

to those from the body could be due to the fact that the two unidentified compounds were lacking from the blend and these could have a synergistic or additive effect on aggregation of locusts if included in the synthetic blend.

It is worthwhile to note that anisole elicited aggregation responses in males and females of *L. m. capito* contrary to observations on the desert locust in which, although this compound is present, it was found to be behaviourally inactive (Torto *et al.,* 1994).

On the other hand, while veratrole is produced by both sexes, only the adult female locusts showed aggregation responses to it. Differences in detection in the two sexes were prevalent and it may be an indication of the potential role of veratrole in modulating the recruitment, aggregation and retention of different sexes in nature. During the trapping of volatiles, it was observed that, female and male *L. m. capito* produce veratrole after mating and during probing of the oviposition substrate (Pers. observations). This suggests that, mature ovipositing female locusts may also utilize this compound to congregate during oviposition, similar to gravid females of the desert locust, *S. gregaria* (Rai *et al.,* 1997).

With regard to the identified synthetic of faecal volatiles, male and female locusts responded positively but variably to anisole, guaiacol and beta-ionone. However, benzyl alcohol was strongly repellent to males. Also the blend was rendered repellent to males and inactive to females when benzyl alcohol included the blend. This is in agreement with the results obtained with the crude faecal volatiles (Chapter 3) and identifies benzyl alcohol as the factor responsible for the repellency of faecal

volatiles to mature adult locusts. In nature, the mature adult locusts hardly come into contact with faecal volatiles even when roosting. Thus, it is not surprising that the faecal volatile components do not play an important role in the aggregation of mature adult locusts.

With the exception of benzyl alcohol and palmitic acid, solitarisation treatments exposing the gregarious fifth instar nymphs *L. m. capito*, to each single compound of the body and faecal volatiles of the conspecific adults led to triggering of acquisition of some solitarious locust characteristics. On the contrary, exposure to the blends that mimic the body and faecal volatiles did not affect the locusts with regard to solitarisation effects. Under natural conditions, gregarious locusts either in hopper bands or in swarms of adults are each within an atmosphere that is predominantly permeated by a blend of their own odour but not to single compounds. The property of the blends that may be involved in maintaining the gregarious characteristics in locusts is hypothesized to include some of these compounds, some of which are common to volatiles of adults and nymphal locusts (Razafindranaivo, pers comm.).

The level of detection of the olfactory cues and their behavioural effects depend on its qualitative and the exact proportions of its different components (Visser, 1986; Schlyter *et al.*, 1989; Cardé and Millar, 2004). These factors, together, determine the induced behavioural effects leading to either aggregation or repellence of locusts. Aggregation is a density-driven process in gregarising locusts. The importance of the threshold number of the first nucleus of locust formed determines the amount and strength of the olfactory signals released in the surroundings. The variations and

fluctuations of the olfactory cue concentration may lead to completely different behaviour. This may explain the instability of the behavioural aggregation responses of laboratory-reared locust due to fluctuations in the population density during rearing. In addition, the adaptation undergone by locusts to the confined rearing conditions may have influenced their level of behavioural responsiveness to some extent. Under natural conditions, the fragmentation of huge swarms into many smaller swarms may result from the dilution of the olfactory cues arising from dispersion of individuals during flights and changes in ambient climatic conditions.

Findings in this chapter support previous findings of characterization of the aggregation pheromone in the closely related mainland African migratory locust species with regard to the use of these same compounds (guaiacol and veratrole) in their aggregation and cohesion behaviour. This partly contributes in resolving the controversy regarding the phylogenetic relation of the two subspecies at least from a chemical ecological perspective. However, two additional compounds were identified as part of the aggregation pheromone composition of *L. m. capito,* including anisole and beta-ionone. This could have taken place during speciation and the separation of the two subspecies. Alternatively, it could be as a result of the technique used for separation and identification of the pheromonal components which did fail to identify these two compounds in the volatiles of the African migratory locust.

Despite the isolation and evolution in different ecosystems which have led to different geographical adaptations in adult locusts, they moderately, aggregate in

response to their body and faecal volatiles, though at a lower level compared to the closely related African mainland species, *L. m. migratorioides*. The new adaptations in *L. m. capito* are expressed in the reduction on the reliance of the use of pheromone in the aggregation and cohesion without losing fully the original traits. As phenylacetonitrile has been identified in the body volatiles of *L. m. capito* and also revealed to be a promising behaviour control agent in the desert locust, its presence raises questions with regard to its possible role in the behavioural of *L. m. capito*.

CHAPTER SIX

6.0 EFFECTS OF PHENYLACETONITRILE ON AGGREGATION AND ANEMOTAXIS IN NYMPHS AND ADULTS OF *LOCUSTA MIGRATORIA CAPITO*

6.1 Introduction

Since the identification of phenylacetonitrile (PAN), which forms the major component of the desert locust body volatile emissions, this compound offers a promise in locust control (Obeng'Ofori *et al.*, 1993). After its aggregative property was demonstrated, many controversial statements related to its implications have also been advanced (Torto *et al.*, 1994, Seidelmann *et al.*, 2000). At first, PAN has been identified to be specific as the major and key component of the aggregation pheromone in the adult stage of males of the desert locust and not produced by any ages of the nymphal stage (Obeng'Ofori *et al.*, 1993; Torto *et al.*, 1994 and 1996; Ferenz and Seidelmann, 2003). Besides, PAN also has been demonstrated to induce repellence in mature males and this seemed to contradict the aggregative role as claimed previously (Seidelmann, 2002). These studies attributed sexual roles to PAN, namely courtship inhibition in mating locusts for female guarding to prevent sperm competitions from competing males, and preventing homosexual encounters between males (Seidelmann *et al.*, 2002; 2003). All these findings serve to highlights the precise roles played by PAN in the biology and behaviour of the desert locust. However, the findings are still disputable and further indepth supporting investigations.

Phenylacetonitrile (PAN) properties, namely its behavioural modulating potential, in addition to its developmental effects have brought into attention its very promising potential as tool for preventive control of build up of hopper band in the field. Moreover, PAN displays high application flexibility and compatibility either on its own in combination with conventional pesticides or locust specific biopesticides namely the fungi, *Metharizium anisopliae* strains (Kane, 2004). In addition, PAN treatment was able to bring gregarious hoppers gradually to solitarisation by inducing dispersion of individuals, thereby enhancing the likelihood of being attacked by predators. Its use reduces drastically to a very low amount the quantity of conventional pesticides required as well as biopesticides when treating hopper bands (Kane, 2004). Moreover, applications of entomopathogenic fungi (Green Muscle®) at a quarter of the recommended dose in combination with minute doses of PAN offers control of the treated hopper bands in 10 days (Hassanali and Bashir, 2007).

In the process of seeking for an environment friendly control method to monitor the onset of outbreaks of the Malagasy migratory locusts, PAN has been advocated to be the best candidate based on to the desert locust model. However, prior to its integration as biotechnological method to control the Malagasy locust, there is a need to determine its precise role in the attraction and aggregation behaviour of locust.

The objective of this study was to determine the possible role played by phenylacetonitrile in the aggregation behaviour and anemotactic attraction of nymphs and adults of the Malagasy migratory locust *L. m. capito*. The amounts of PAN

produced by both sexes of *L. m. capito* were quantified. In addition, bioassays to assess the aggregation behaviour and the anemotactic effects of PAN.

6.2 Materials and Methods

6.2.1 Locusts and rearing conditions

The fourteenth generation of gregarious nymphal stages and adults of *L. m. capito* from the colony reared and maintained at the facilities at *icipe* (Nairobi, Kenya) under laboratory conditions as described in section 2.1 were used. Nymphal and adult stages were kept separately thereby minimizing cross-pheromonal effects throughout their development.

6.2.2 Quantification of release of PAN

6.2.2.1 Body volatile collections

Volatiles for quantifications were collected from separate batches of mature male and female gregarious adult locusts on clean Super-Q polymer (30 - 50 mg, 80-100mesh, Nicholasville, KY) adsorbent as outlined in section 2.3.1.

6.2.2.2 Synthetic chemicals

Phenylacetonitrile (purity, 98% to 99.5%) was purchased from Sigma Aldrich, USA..

6.2.2.3 Experimental chemicals

Sources of volatiles were prepared in 2 ml of light paraffin oil (Merck) into which 4, 8 and 32 µl of phenylacetonitrile (Sigma Aldrich, USA) were added and held in 3.7

ml glass vials drilled stoppered with a screw caps that had a 1.5 mm for release of the volatiles. Vials were suspended inside 2 litre round-bottomed flasks and then sealed at the top (Torto *et al.,* 1994) and connected to the olfactometer as described in Chapter two, section 2.3.2.

6.2.3 Release of volatiles in bioassays

The trapping method and preparation of samples were as outlined in sections 2.6 and 2.7, respectively. Three different concentrations of PAN were prepared and released from the dispenser into the olfactometer as outlined in section 6.2.2.3.

6.2.4 GC analysis and quantification of PAN release from the dispenser

Quantification analyses of volatiles were carried out using HP-5890 Series II gas chromatograph equipped with FID detector. Analyses and quantifications were performed following the protocols outlined in sections 2.8 and 5.2.3, respectively.

6.2.5 Behavioural effects of PAN on nymphal and adult locusts

6.2.5.1 Aggregation bioassays

A set of ten replicates of aggregation bioassay using two locusts per replicate was performed. For each replicate, a single locust was introduced into the olfactometer arena and its preferred position was recorded every ten minutes for 50 minutes and the test was terminated and the locust removed. Another locust was introduced to complete the set of ten readings. Male and female fifth instar nymph and adult locusts were used as recipient (test) locusts against different concentrations of PAN in a vertical two-choice olfactometer chamber as described earlier in chapter two,

sections 2.2.1 and 2.4. Choice was given to locust in the arena between the two sides, one permeated with PAN vapour and the other with clean air as control.

6.2.5.2 Anemotactic responses of locusts to PAN

A similar protocol and device outlined in Chapter two, sections 2.2.2 and 2.4, respectively and with the same number of replicates as described above, section 6.2.5.1, was carried out.

6.2.6 Aggregation and Attraction Indices and data analysis

The aggregation index (A. I.) and attraction Index (At. I.) were calculated using the formula A. I. or At. I. (%) = 100 x (T-C)/N, where N is the total number of locusts tested; T, number of locusts responding to the volatiles; and C those in control area of the chamber. Data were analyzed as described in Chapter two, section 2.11.

6.3 Results

6.3.1 Quantification of PAN release

The three concentrations of PAN (4, 8 and 32 µl/ 2 ml paraffin oil) from the dispenser provided the equivalent of PAN produced by 11, 29 and 42 mature males respectively (Table 6.1). Although both male and female *L. m. capito* release PAN in their volatiles(phenylacetonitrile), female locusts produced amounts of PAN that were ten times lower than those emitted by males. However, no statistical significant differences separated the two release amounts in males and females ($F_{df\ (4,\ 14)}$ = 22.63, N = 19, P = 0.001, SNK, test). Quantification of PAN released by *L. m. capito*

measured on an hourly release basis averaged 0.38 ± 0.04 ng/male *vs.* 0.04 ± 0.003 ng/female (Table 6.1).

Table 6.1 Phenylacetonitrile release rates by individual mature male and female *L. m. capito* and from the 3.7 ml glass vial dispenser with different concentrations of phenylacetonitrile in paraffin oil (P.O).

Sources of PAN Locust	Average release amount of PAN (ng/h)	No. of replicates	Average mature males equivalent (Meq)
Mature male	0.38 ± 0.12a	5	1
Mature female	0.04 ± 0.003a	5	-
Glass vial dispenser PAN/2ml P.O			
4µl/2ml	4.45 ± 1.83a	3	11
8µl/2ml	11.30 ± 2.40b	3	29
32µ/2ml	16.00 ± 3.05c	3	42

Different letters indicate significant means (P = 0.05, ANOVA tests)

6.3.2 Aggregation responses of mature adults and fifth instar nymphs (N_5) of *Locusta migratoria capito* to PAN

Aggregation responses of the three groups of locusts tested against the three different concentrations of PAN corresponding to the population equivalents of locusts showed no significant difference across the different batches of locust ($F_{df\ (8,\ 81)}$ = 1.53, N = 90, P = 0.15, SNK, test). The locusts were not responsive to PAN and were repelled by it with gradual increase in it concentration. However, only mature locust males aggregated to PAN at 29Meq (A.I = 23 ± 9%) (χ^2 = 8.73, D.F = 1, N = 20, P = 0.025) (Figure 6.1).

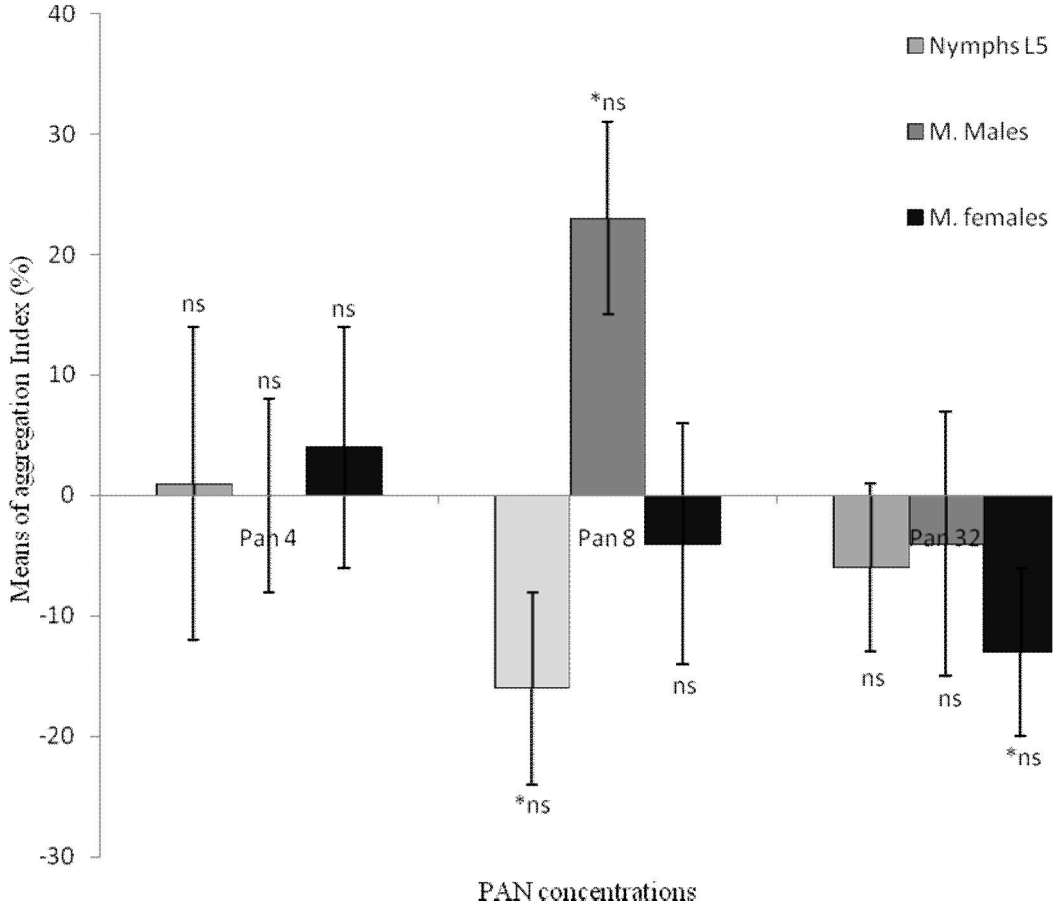

Figure 6.1 Aggregation responses (mean ± SE) of fifth instar nymphs (L_5), mature male and females *Locusta migratoria capito* to different concentrations of PAN (4 µl, 8 µl and 32 µl) in 2ml light paraffin oil. (Test locusts: M. Males = mature Males and M. Females = mature Females). * indicates significant differences (Chi-square tests at P = 0.05). ns differences between means not significant (ANOVA tests, P = 0.05)

6.3.3 Anemotaxis responses to PAN by males and females mature and nymphal stage (N_5) *Locusta migratoria capito*

With regards to the general pattern of responses, PAN elicited no significant attraction effects to all the batches of locusts tested at the three concentrations used ($F_{df\,(8,\,261)} = 1.06$, N = 270, P = 0.39, SNK, test). However, the fifth instar nymphs had a relatively high attraction response to PAN at 11Meq (A.I = 24 ± 14%) (χ^2 = 4.99, D.F = 1, N = 60, P = 0.0031). Mature males and females were all repelled at all the concentrations of PAN tested (Table 6.2).

Table 6.2 Anemotactic responses (mean ± SE) of fifth instar nymphs, mature male and mature female *Locusta migratoria capito* to different concentrations of PAN (4 µl, 8 µl and 32 µl) in 2 ml light paraffin oil.

Sources of volatiles	Sexes and stages of locust	Number of locusts tested	Anemotactic Attraction Indices (At. I.) (%)
PAN 4	Nymph (N_5)	30	27±14*a
	M. Males	30	-10±6*a
	M. females	30	-16±12*a
PAN 8	Nymph (N_5)	30	-13±14*a
	M. Males	30	0±6a
	M. females	30	-24±3*a
PAN 32	Nymph (N_5)	30	0±9a
	M. Males	30	-6±18a
	M. females	30	0±6a
Control	Mixed sexes	30	2±8a

* indicates differences were significant (Chi-square tests significant at P = 0.05). a indicates no significant differences in the means (ANOVA tests, P = 0.05)

6.4 Discussion

The foregoing results confirm that phenylacetonitrile (PAN) is released by mature gregarious male locusts *L. m. capito* that are ready to mate with their female counterparts (Torto and Deng, unpublished). The mature female locusts also release PAN but in lower amounts. While this indicates similar synthetic metabolic pathways, differences in the production of PAN by the two sexes may depend on the availability of the precursors and the intermediate metabolic products, or the necessary enzymes during the sexual maturation process in the locusts.

Although no statistical significance was obtained in the overall levels of various behaviours, aggregation and anemotactic attraction tests to PAN by both stages of *L. m. capito* show that mature male and late fifth instar nymph (N_5) were responsive to PAN at given locust population equivalents (29 and 11 Meq) (Figure 6.1 and Table 6.1). Attraction responses were recorded in the nymphal stage when an average number of 11 mature male equivalents (A. I. = 24 ± 9%) while aggregation responses of gregarious adult males were observed when an average number of 29 mature male equivalents (A. I. = 23 ± 14%). These population equivalents locusts possibly correspond and reflect the range of critical amounts of PAN equivalent to the threshold number of congregating locusts that is required to elicit physiological and behavioural reactions in the two different stages. These findings are in agreement with the appearance of the gregarious traits and aggregation behaviour involving marching and synchrony of movements observed only at a very precise number of individual locusts in a given area (Buhl *et al.*, 2006).

The mature male locusts had dose-dependent aggregation responses to PAN. At above threshold concentration, mature males had positive aggregation responses while at a four times higher dose, there were indications of it being repellent to these locusts. This suggests that, like in the desert locust, *S. gregaria,* it may play a role as an aggregant at low physiological doses, while at relatively higher doses it is a sexual recognition signal to their male conspecifics (Seidelmann, 2002). When the mature male L. m. capito mount on their conspecifics to mate, they release phenylacetonitrile (PAN) (Pers observation.) This suggests that, these locusts may also use it for mate guarding from other competing males. PAN may be used as an aphrodisiac that affects the physiology of female locust to ensure sperm transfer during and after mating, a period during which physical attacks and assaults by other male locusts can also occur (Parker *et al*., 1972; Seidelmann *et al.,* 2002; Tanaka and Zhu, 2003; Rono *et al.,* 2008). Furthermore, male conspecifics may use phenylacetonitrile emission during copulation as an indirect olfactory cue possibly to indicate the presence of female locusts and their sexual receptiveness status in a narrow range of concentrations (Rono *et al.,* 2008). Increasing concentration of PAN indicates overcrowding of mature males and females copulating, similar to the release and use of ethyl acetophenone by ovipositing females, which arrest mature males for mating (Schmidt, 1999, Seidelmann *et al.,* 2005), hence reduced likelihood to mate competition. Conversely, low or sub-threshold amounts of PAN are either not capable of being detected to trigger behavioural responses or it could also be an indication of sexual immaturity of conspecifics.

Fifth instar nymphs of *L. m. capito* release low amounts of PAN (Razafindranaivo, pers comm.). In this study, nymphs were found to respond to PAN at a low concentration. This suggests that, it may serve as an attractant cue that enables the nymphal stages to locate groupings of their conspecific stages, which in turn ensures synchrony in their behaviour and development. In addition, production of PAN by the nymphs may provide them protection against predators because cyanide compounds are known to be used for defence (Seidelmann *et al.,* 2000; Lechtenberg, 2001).

Use of PAN as behavioural cue may probably be secondary and opportunistic in the aggregation of the gregarious adult stage *L. m. capito*. Because of the positive aggregation and attraction responses of fifth instar nymphs (N_5) and mature male locusts to it, PAN cannot therefore, be excluded to play role in these behaviour aspects though its use in these behavioural aspects is minor compared to *S. gregaria*, which use PAN as the key compound in the aggregation behaviour (Torto *et al.,* 1994).

Other studies done on the desert locust hoppers and adults with regard to the toxicity of PAN have revealed the levels of acridicide potency. Hoppers and adult stages exposed to a range of very low doses of PAN vapour showed sensitivity to its insecticidal effects and knock down effects of PAN indicating the possibility of its application at fractional doses as insecticide (Torto *et al.,* 2008). Repellent effects of PAN may probably precede the primary effects of PAN vapour on its knock down effects as demonstrated by Torto *et al.* (2008) in the absorption/release metabolism in

the desert locust. At this stage of the laboratory study and the assessment on the possible integration of PAN as a behaviour modulating agent for gregarious adults *L. m. capito*, the foregoing results show that, to some extent, phenylacetonitrile elicits aggregation and anemotactic responses in *L. m. capito* adults and hoppers, although at lower levels compared to the desert locust.

Further tests using other bioassay techniques in the laboratory and under field conditions are necessary to determine whether PAN can be integrated into the management strategy for *L. m. capito as* a behaviour modulating agent. Other aspects that need to be studied include the metabolism and release pathways, its role in biological and physiological aspects such as its involvement in the body detoxification metabolism during development and maturation processes. In addition, requiring further investigation is the use of PAN as mediating olfactory signal since cyanide compounds are assumed to be toxic to insect metabolism (Prestwich, 1985; Seidelmann *et al.*, 2000).

The possible role of PAN in the behavioural ecology of solitarious locusts of *L. m. capito* this species also remains unknown. Detailed studies and further investigations are necessary on this aspect as well as its toxicological effects as an acridicide and/or biological pesticides adjuvant prior to its possible large scale use on *L. m. capito*. Assessment on its effects on non-target insects, human health and environment should be conducted before application in the field and wide scale use.

CHAPTER SEVEN

7.0 GENERAL DISCUSSION, CONCLUSIONS AND RECOMENDATIONS

7.1 General discussion

In the breeding habitats and outbreak areas of the Malagasy migratory locust, *L. m. capito,* hopper bands and swarm formations occur cyclically depending on the abiotic factors involving the ecosystem and the biotic factors involving the locusts themselves. These factors influence the dynamics of hopper band and swarm population build up. Food foraging, reproduction (mate location and selection of suitable oviposition sites) and predator avoidance are key behavioural activities that influence their adaptation to the environment and a wide range of semiochemicals and, possibly, other cues that may play important roles.

The Malagasy migratory locust has been assumed to have been derived from the African migratory locust as part of the population that was isolated after the drift of the island from mainland Africa to its current geographical location (Wintrebert, 1970; Farrow and Colless, 1980). The drift probably created a different variant of the original ecosystem forcing the locust to adapt to the new environment compared to the original one. Although the evolving species may not have altered significantly, the general features of its aggregation behaviour and its continued reliance on pheromonal signals produced from the body and faecal odours being retained over time.

The low aggregation and attraction responses to the identified blend of pheromonal compounds obtained in bioassays clearly demonstrate that, other cues are involved in the gregarisation process and aggregation. The blend of identified compounds was solely not sufficient to explain fully the complex mechanism of the gregarious population build up and the remarkable cohesion of the hopper bands and adult swarms.

Modulation of the release of these different components throughout the life cycle of the locusts in tandem with their adaptation to the eco-climatic changes that occur within the breeding and outbreak areas may underlie the aggregation/solitarisation processes of this species. In addition, a combination of different cues including visual, tactile and acoustics may also be involved in hopper band and adult swarm build up, as means of transfer of contact semiochemicals and general communication.

7.2 General conclusion

Body and faecal volatiles in together with suitable environmental factors lead to mediation of the aggregation processes. However, these are subset of the all different cues that need to be taken into account in monitoring of gregarious populations of the Malagasy migratory locust.

Several chemical components similar to those identified in the desert locust and the African migratory locust were detected in the headspace of the volatiles from the adult Malagasy migratory locust. The blend of anisole and veratrole, likely acts as the aggregation pheromone of *L. m. capito* eliciting the highest levels of aggregation

in male and female adult locusts. During the present study only some of components, mainly the more volatile ones, were identified whereas the chemical identities of most of the heavy components could not be fully confirmed. These components may also play a significant role in the maintaining gregarious traits in gregarizing and gregarious populations of *L. m. capito*.

Detection of combinations of several components in specific ratios by antennal olfactory receptors may generate varying levels of aggregation responses in locusts of the two sexes. Each compound in the body volatiles, with the exception to benzyl alcohol, was also capable of inducing visible solitary phase characteristics in fledglings when late fifth instar nymphs were exposed to them.

Since some components of faecal and body volatiles of adult Malagasy migratory locusts *L. m. capito* have demonstrated to have aggregating and solitarizing effects. Their combination may provide a basis for a future pheromone-based preventive method of locust management involving monitoring of the build up of hopper bands and swarms of adult locusts and applications of an appropriately formulated solitarising agent. Unlike in the desert locust, phenylacetonitrile did not elicit strong aggregation and anemotactic behaviour with clear pattern in nymphal and adult stages of *L. m. capito*. Hence, there is a need for further indepth studies to fully elucidate the possible primer and/or releaser roles that PAN may play in the biology and behaviour of this locust prior to recommending it for use as behaviour-modulating agent for management of this locust.

7.3 Recommendations

During the course of execution of the objectives of this study and the resultant findings reported above, other important questions arose with regard to the whole range of stimuli that may be involved in the aggregation and gregarization processes of the Madagascar migratory locust, *L. m. capito*. These could not be addressed during the time available for the research work for this thesis and are recommended below as follow-up research topics that comprise projects for postgraduate students:

1. The full identification and characterization of the unidentified EAG-active components in body volatiles of *L. m. capito* need to be pursued further in order to eludate their role in mediation of the aggregation behaviour of *L. m. capito*, either individually or in blends with the other identified compounds;
2. There is need to carry out further studies to clarify the actual role of PAN in the aggregation and attraction behaviour of *L. m. capito*, more importantly in combination with the other compounds in locust body volatiles including the ones that were not fully characterised if this is done as in (1) above;
3. From the levels of responsiveness of the locusts to components in their volatiles, it was evident that, in addition to the chemical signals, other stimuli most likely including visual and, tactile cues and acoustics are involved in mediating aggregation behaviour in *L. m. capito*. Their role, either alone or in combination with each other or with the semiochemicals needs to be elucidated;
4. Electrophysiological single-cell tests are important in identifying compounds that may be potent in evoking antagonistic and agonistic neural activity in sets of neurons

in higher centres of the olfactory sensory system including the antennal lobe and the central nervous system (CNS) of insects. Similar studies on the identified components in locust volatiles may provide further information that will help in determining those that are involved in aggregation (agonists) and those that are repellent (antagonists) to the locust behaviour;

5. Mass-reared insects in the laboratory may not exhibit the same levels of responsiveness to stimuli as their counterparts in the field. It is necessary to carry out comparative chemical ecological studies with insects from field populations to confirm the chemical composition of their volatiles and their level of responsiveness to them; and

6. In locusts, distribution (sparse or clumped) of specific host plants and emissions of plant volatile organic compounds (VOCs) and metabolites during different seasons have been shown to be involved in triggering the initial aggregation of locusts that leads to gregarisation and upsurges of their localized populations, leading to locust outbreaks (Hassanali *et al.*, 2005; Speight *et al.*, 2008). Studies on plant-locust interactions with *L. m. capito* may determine whether there are certain host plants in its breeding habitats and outbreak areas that influence their behavioural biology and phase dynamics.

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