Chemical and molecular analysis of the pheromone phenylacetonitrile in desert locust, *Schistocerca gregaria* Forskål (Orthoptera: Acrididae)

by

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Declaration

I, Peris Wanza Amwayi declare that the thesis, which I hereby submit for the degree of Doctor of Philosophy (Entomology) to the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature:……..

Date:…….

Peris Wanza Amwayi

Dedication

To my parents, Jas and Hellen Amwayi with love, my spouse Marius Gunga and children Chelsea and James Gunga for the immeasurable love, care and patience.

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Note: Chapter 2 to 5 were written as separate publications and submitted to appropriate international journals, consequently there is some overlap of information and references in this thesis.

Chapter 2: Peris W. Amwayi, Daniel K. Masiga, Prem Govender, Peter E. A. Teal, Baldwyn Torto (2012). Mass spectral determination of phenylacetonitrile (PAN) levels in body tissues of adult desert locust, *Schistocerca gregaria*. Journal of Insect Physiology 58(8) 1037 – 1041

Chapter 3: Peris W. Amwayi, Daniel K. Masiga, Prem Govender, Peter E. A. Teal, Baldwyn Torto. The influence of pheromone titers in mating response of the desert locust *Schistocerca gregaria* (Orthoptera: Acrididae) (Submitted to Chemeoecology)

Chapter 4: Peris W. Amwayi, Baldwyn Torto, Prem Govender, Peter E. A. Teal, Daniel K. Masiga. Molecular basis of aggregation behavior and pheromone release in the desert locust (*Schistocerca gregaria;* Orthoptera) (Submitted to PLoS One)

Chapter 5: Peris W. Amwayi, Baldwyn Torto, Prem Govender , Peter E. A. Teal, Daniel K. Masiga. Quantitative real time PCR analysis of genes controlling aggregation behaviour in the desert locust (*Schistocerca gregaria*) (submitted to PLoS One)

Chemical and molecular analysis of the pheromone phenylacetonitrile in the desert locust, *Schistocerca gregaria* Forskål (Orthoptera: Acrididae)

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Summary

The desert locust, *Schistocerca gregaria* (Orthoptera: Acrididae) is a destructive pest when gregarious but harmless in solitary form. Reports demonstrate the involvement of aggregation pheromones in mediating behavior of the desert locust in the gregarious phase. Phenylacetonitrile (PAN), the major component of the adult aggregation pheromone, can act as an adult aggregant for both sexes and as a male homosexual avoidance pheromone. However, the origin of PAN and its biosynthesis pathway are not well understood. In the present study, chemical and molecular approaches were employed to elucidate the site of production and biosynthesis pathway of PAN. In a previous study, wings and legs of mature gregarious adult desert locusts were shown to emit notable amount of PAN. However, the present study revealed that PAN is widely distributed in body parts of both sexes in varying amounts. This is the first study that detects noticeable amounts of PAN in the body parts of female and suggests that the pheromone is present in both sexes but that the release of this pheromone is restricted to sexually

mature adult males. Also, there were significant differences in PAN released by sexually mature gregarious males at various time intervals during photophase (day) and scotophase (night). More PAN was emitted during the photophase than scotophase and there was a positive correlation of PAN emitted with temperature. Sexual receptivity of both sexes in relation to PAN production was determined. These results suggest that both sexes of gregarious desert locusts are involved in mate finding, which may be influenced by sight, age and levels of PAN released by both sexes of adults. Subsequently, forward and reverse subtracted cDNA libraries were created using the suppression subtractive hybridization (SSH) technique. The potential genes encode energy metabolism genes and represent the up-regulated genes that could be responsible for triggering aggregation behavior. Relative expression revealed that the genes were up- and down-regulated in gregarious and solitary phenotypes, indicating that there is a molecular mechanism that switches on and off during phase change and PAN production.

Table of contents **Page**

CHAPTER ONE

The biology of the desert locust, *Schistocerca gregaria*, invasion and management: A Literature Review

Introduction

Plagues of locusts have been known since time immemorial from biblical and Quran records. The desert locust, *Schistocerca gregaria*; the African migratory locust, *Locusta migratoria migratorioides*; the red locust, *Nomadacris septemfasciata Serville*; the tree locust, *Anacridium melanorhodon* and the brown locust (*Locustana pardalina)* are the main economically important locust species in Africa (Steedman 1988). The desert locust is a member of the orthopteroid insects that present a varying level of threat because of their tendency under certain conditions to multiply and aggregate in large numbers forming huge migrating swarms in breeding and invade new areas. During outbreaks, the gregarious locust can invade an estimated area covering 29 million km^2, about 20% of the world's land surface area (Steedman 1988). They are also capable of rapidly damaging pastures and crops due to their polyphagous nature (Uvarov 1977; Steedman 1988; Magor et al. 2008).

Two extreme phase shift occur in the desert locust: a harmless solitarious (*solitaria*) and a damaging gregarious (*gregaria*) (Uvarov 1966; Pener and Yerushalmi 1998). Phase shift result from interactive causal links, including phase-specific physiological and behavioural traits, ecological factors in breeding areas and tactile stimulations at higher densities that may lead to the production of neurochemicals such as serotonin (Ainstey et al. 2009), which may be responsible for switching on traits typical of gregarious phase, including changes in hormone

levels, emission of pheromones that mediate key behavioural and physiological traits of gregarious locust such as social cohesion, synchronous maturation, communal oviposition and trans-generational transfer of phase traits.

In 1988, a group of French scientists reported the volatile composition of gregarious adult males of the desert locust. Although, veratrole, guaiacol and phenol were identified in the volatiles, the behavioural significance of these components in the ecology of the locust was unknown (Fuzeau-Braesch et al. 1988). Following this discovery, the aggregation pheromone system of gregarious phase desert locust was first described in the 1990s by scientists at the International Centre of Insect Physiology and Ecology (ICIPE). These pheromones may have releaser effects that inducing the immediate behavioral responses that result to aggregation of the gregarious locust or primer effects that initiate have long-term physiological transformation on physiology, development or behavior of the solitarious insects to the gregarious phase.

The releaser and primer function(s) of these pheromones has been a subject of controversy and confusion. Studies on exposure of the solitarious locust individuals to volatile emissions from their gregarious counterparts (Heifetz et al. 1996; Roessingh et al. 1998; Hassanali et al. 2005) showed no primer phase transformation. However, phase shift of solitarious locust to gregarious locust was the actual physical contact between the insects that experience crowding and two stimuli, a chemotactile stimulus associated with the hydrocarbon fraction of locust cuticle (Heifetz et al. 1996, 1997) and a site-specific mechanotactile stimulus associated with outer face of hind femurs (Hägele and Simpson 2000; Roessingh et al. 1998; Simpson et al. 2001) are involved. On the other hand, the releaser function of these volatiles involves cohesion responses

(Obeng-Ofori et al. 1993, 1994; Torto et al. 1994, 1996). Additionally, there was an interesting stage differentiation in the responses of nymphal and adult stages indicating two distinct cohesion pheromonal effects for the two stages with different chemical compositions, an adult pheromone system comprising a blend of benzene derivatives including veratrole, anisole, benzaldehyde, phenol, and phenylacetonitrile (Torto et al. 1994); and a nymphal pheromone system comprising a blend of short chain aldehydes and their fatty acid derivatives (Obeng-Ofori et al. 1993; 1994; Torto et al. 1996; Hassanali and Torto 1999). In mating experiments by Seidelmann and Ferenz (2002) revealed different effect of PAN as a repellent and as a courtship inhibition pheromone as opposed to cohesive responses. The repellency of PAN was further confirmed on a Y-shaped olfactometer (Seidelmann et al. 2005). However, a concentration-dependent response to PAN was established where adult male individuals were retained close to PAN source at low relative doses of PAN but avoided each other at higher relative doses.

Biology and behavior of the desert locust

The biology of the desert locust

The two phases of the desert locust exhibit different behavior, induced by chemical stimuli especially olfactory cues. Locusts in the solitary phase are secretive in behavior, and actively avoid each other. On the contrary, in the gregarious phase they aggregate into active hopper bands which develop into migrating swarms consisting of millions of individuals (Uvarov 1966). The adult gregarious locusts form day-flying swarms while the solitarious locusts normally fly at night except when they are mating (Uvarov 1977). Previous findings show that solitarious locusts are able to acquire gregarious activity within one to four hours of crowding while isolation of crowded reared insects leads to loss of the aggregation behavior (Deng et al. 1996).

Swarming or banding can arise due to favourable breeding conditions or induced switch from mutual aversion in solitarious locusts to aggregation after a few hours of forced crowing (Deng et al. 1996).

Classification

Locust belongs to the family Acrididae, which include most of the short-horned grasshoppers (Symmons and Cressman 2001). The scientific classification is as follows:

Kingdom	Animalia
Phylum	Arthropoda
Order	Orthoptera
Class	Insecta
Family	Acrididae
Genus	*Schistocerca*
Species	*gregaria*

Morphology

The morphological characteristics of the desert locust are distinctly different between the two phases, *solitaria* and *gregaria.* The body colour patterns of the solitarious nymphal stage individuals are often characterized by a green straw while the gregarious nymphal stages, on the other hand, have black patterns on a yellow background (Fig. 1.1). The adults gregarious are pinkish when immature, bright yellow as mature males or pale yellow on the head and pronotum as mature females while the adults are greyish brown (Fig. 1.2) (Norris 1952, 1954).

Fig 1.1 Two phases of the nymphal stage of the desert locust *Schistocerca gregaria*, gregarious (black with yellow background) and solitary (green).

Fig 1.2 Two phases of the adult desert locust males *Schistocerca gregaria*, gregarious (yellow) and solitary (brown).

The gregarious nymphal stage are further classified on a 0 – 5 scale based on the black emaculation on the head and other parts. Grade 0 indicates no black markings on the head, hind femora, pronotum or the abdominal tergites while grade 5 corresponds to full black markings on the body parts. The adult stage of the gregarious phase also has five grades of colour classification (grade I to V) (Norris 1954). The pink immature adults are classified as grade I, the adults with a flush of yellow on abdominal tergites, wings and thorax are classified as grade III, and grade V correspond to full bright yellow colour in full mature adults.

Morphometric measurements have also been used to differentiate solitarious and gregarious phases of *S. gregaria* (Deng et al. 1996). The morphometric E/F ratio is a ratio of the length of the elytron (E) to that of hind femur (F) while F/C ratio is a ratio of the length of the hind femur (F) to the width of the head capsule (C) (Dirsh 1953). The F/C ratio is considered more reliable than E/F ratio. However, these morphometric parameters change slowly with several generations of the desert locust. (Deng et al. 1996).

Life cycle of the desert locust

The desert locust belongs to the hemimetabolous branch of insects. This subgroup comprises insects that undergo an incomplete metamorphosis, lacking the formation of a pupal stage. This implies that the life cycle consists of three stages: the egg, hoppers (nymphs) and adults (Fig. 1.3). It begins with the hatching of the egg laid in groups of about 50 per egg pod in sand (Harjai and Sikka 1970). On average eggs hatch into nymphs/hopper within 10-25 days. This is followed by five to six nymphal stages depending on the phase, *gregaria* and *solitaria*, respectively, temperature and humidity just before fledgling into immature adults (Duranton and Lecoq 1990).

After fledgling, the immature adults take 10-15 days to become mature. The onset of maturity is signified by yellow colouration and production of the aggregation pheromone (Mahamat et al. 1993; Mahamat et al. 2000). Generally, the life span of desert locust is three to five months depending on weather and ecological conditions (Ashall and Ellis 1962; Roffey and Popov 1968; Steedman 1988) taking longer under dry weather conditions. Sexual maturity in *Schistocerca gregaria* takes two weeks to one month, while in *Locusta migratoria* it takes about a week (Norris and Richards 1964).

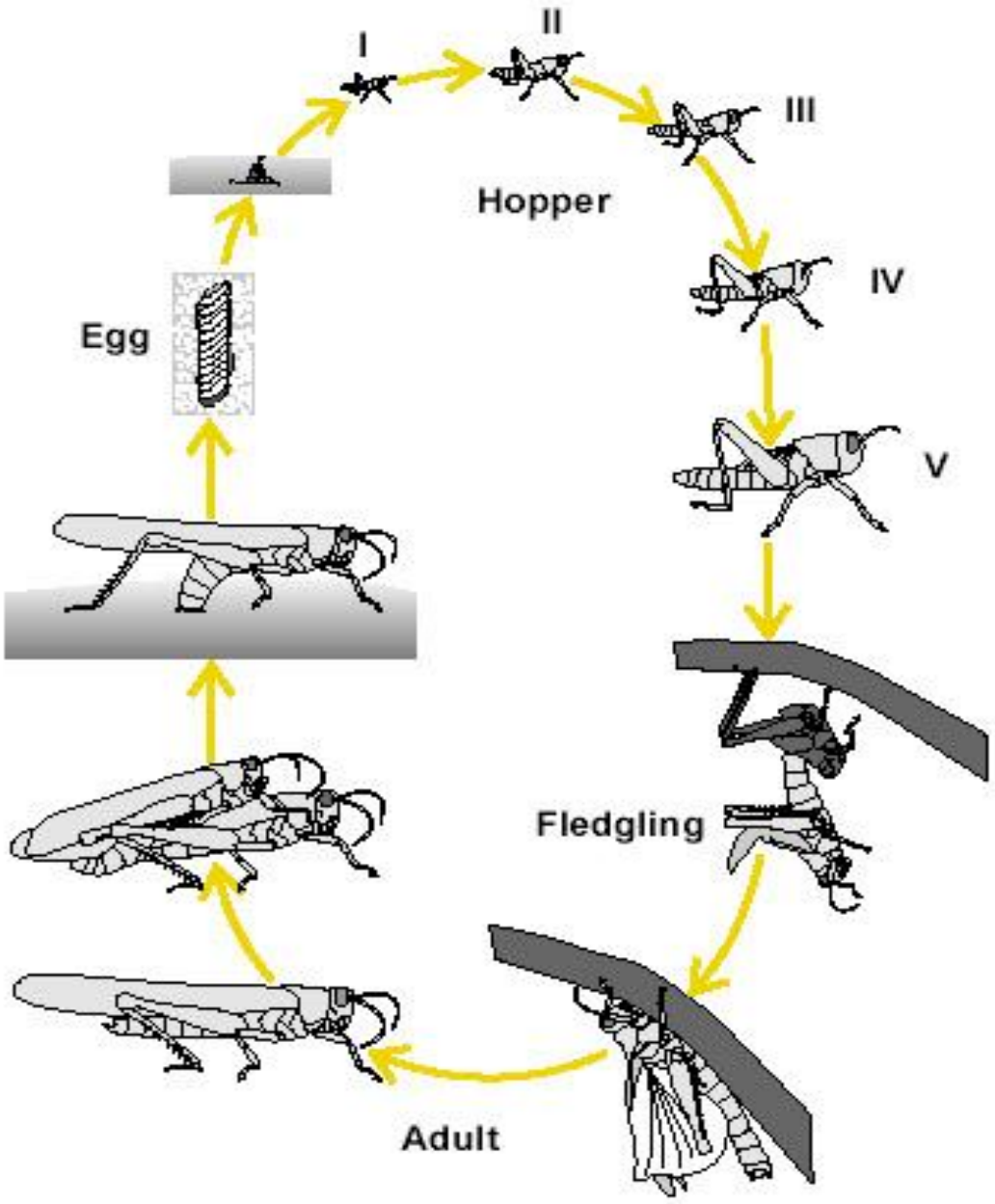

Fig 1.3 The life cycle of gregarious phase of the desert locust *Schistocerca gregaria*. (Source: Desert locust guidelines, FAO 2001).

Ecology of the desert locust

The desert locust is the most widely distributed among all species of grasshoppers and has a vast invasion area of about 29 million km^2 ranging across Africa to Western Asia (Steedman 1990) (Figure 1.4). The invasion area has a great variety of climatic conditions, soil types and vegetation. The region is characterized by seasonal rainfall averaging between 80 and 400 mm annually, varying dramatically from year to year with annual rainfall being upto 70% above or below average. The recession area is dominated by solitarious phase of the desert locust occurring as small scattered populations while swarms and hopper bands are rare.

Rainfall determines whether there is sufficient growth of vegetation to provide adequate food supply for the desert locust. Although vegetation islands can be as large as several square kilometers and the vegetation areas are not more than 10 to 30%, however, in dry years the vegetation area is well below 10% (Uvarov 1921; Roffey 1982). This means that the desert locust not only locate scarce and seasonally variable habitats, but also exploits them optimally. Adequate utilization may perhaps be explained by the phase theory, first proposed by Uvarov (1921) and further developed by Roffey (1982). According to this theory, during the dry period the desert locust occurs primarily or exclusively in the less mobile solitary phase. However, with suitable conditions involving widespread, heavy and prolonged rainfall in successive seasonal breeding areas, recession population can rapidly increase in number and size, leading to the formation of hopper bands and adult swarms. Swarms of the desert locust in the gregarious phase are highly mobile and cause serious damage over a wide range of wild and cultivated plants. This process also depends on other biotic and abiotic factors which are not clearly understood. Generally, an increasing number of locusts leads to local outbreak, regional upsurges and, in

more favourable conditions to plagues (Uvarov 1921; Roffey 1982). This hypothesis contradicts the theory of swarm continuity put forward by Rainey and Betts (1979) who argued that swarms are born from swarms. However, during some periods in the 1970s there was no confirmed evidence at all of migrating swarms. Therefore, the hypothesis was based on the assumption that large, but undetected, gregarious populations must be present even during recession periods.

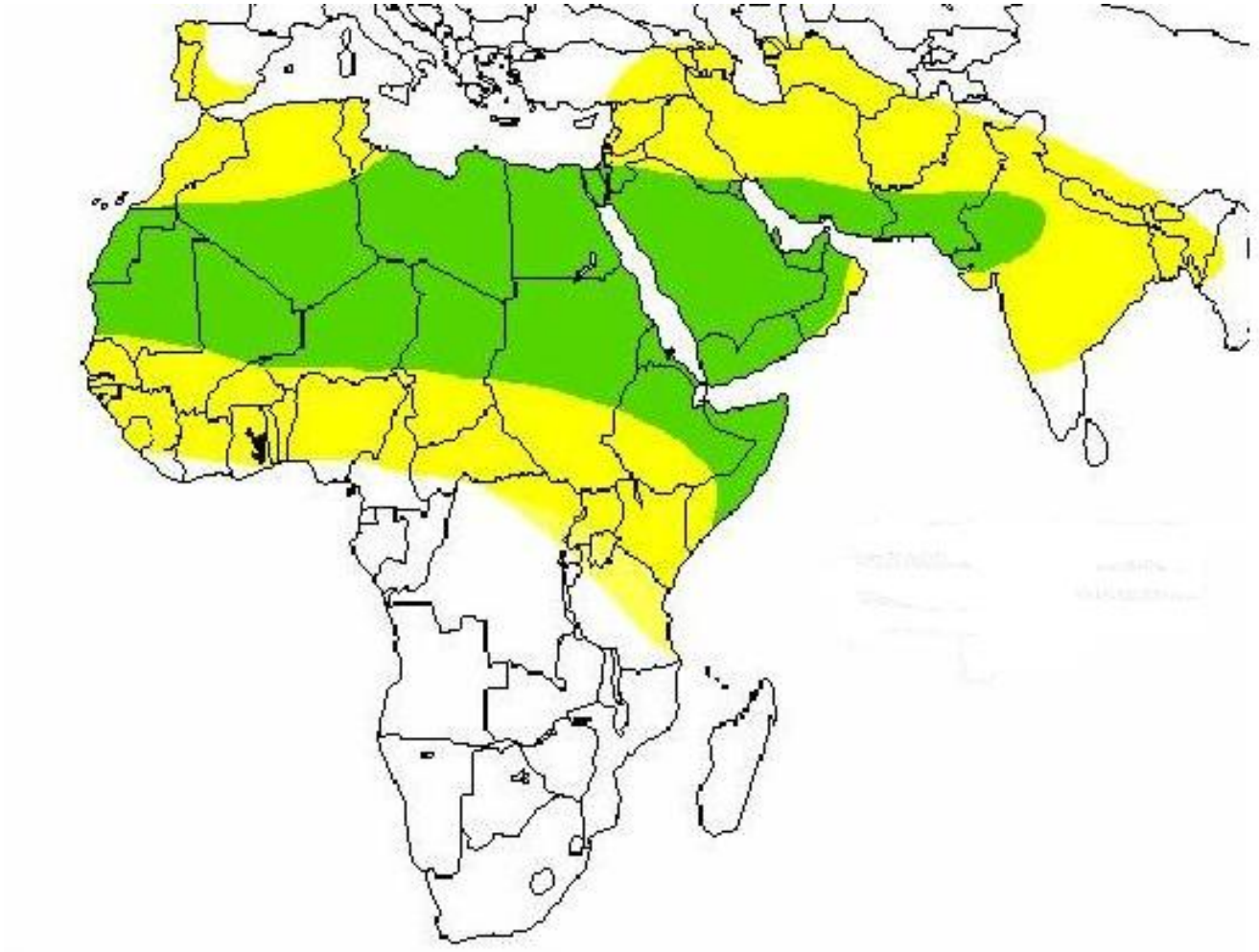

Fig 1.4 Distribution range of the desert locust *Schistocerca gregaria*. (Recession area = green; Invasion area= yellow) (Source: www.ipmworld.umn.edu).

Economic importance of the desert locust

Desert locusts feed on a wide range of plants, thus making them voracious crop pests of great economic importance in agriculture. Plagues of desert locusts have had detrimental effects to food productivity in Africa, the Middle East and Asia for centuries. In addition, they are the most destructive of all locusts. Four main factors contribute to its status as a major pest: the food intake per individual, the range of food plants and parts eaten, and the frequency of occurrence of high density populations and the mobility of the populations (White 1979). Because swarms are so mobile there is great variation in the amount of damage caused seasonally, from country to country and from region to region. A single swarm of locusts comprises several billion individuals that eat at least their own weight (2-3g) of fresh vegetation daily (possibly three times as much) and can travel great distances (Steedman 1988). During an outbreak, swarms of winged insects (up to 80 million locusts km^{-2}) can cover enormous areas of approximately 29 milliom km^2 which about 20% of the world surface (FAO 2006; Sanchez-Zapata et al. 2007). An outbreak in 2004 was the largest known since that of 1986-89 (www.fao.org, March 2005). The whole of the Sahel region was affected, from Senegal and Mauritania to the Red Sea, and from the Atlantic Ocean to the Canary and Cape Verde Islands, and to the Mediterranean sea (Sanchez-Zapata et al. 2007). The cost of control was approximately US$122 million to combat the locust swarm, and an additional $2.5 billion in harvest losses (OECD 2004).

Phase polymorphism and gregariousness

The phase polymorphism theory was proposed by Uvarov (1966), defining the ability to a biological breeding adaptation according to the level of population density. It is also referred to as phase polyphenism. The desert locust, *Schistocerca gregaria*, exhibits a population density

dependent phase polyphenism which also includes changes in behaviour, biochemistry, morphology and physiology (Uvarov 1977, Pener 1991, Pener and Yerushalmi 1998, Simpson et al. 1999). It is characterized by the ability to transform reversibly between two extreme phases *solitaria* and *gregaria* forms which pass through intermediate level of differentiation termed as *transiens.*

The acquisition of gregariousness in the desert locust is gradual and cumulative through their successive generations. The behavioral trait is first acquired after a few hours of increasing population density (Roessingh and Simpson 1994) followed by production of the aggregation pheromone and later colour change (Deng et al. 1996). After three successive generations, locusts may become fully transformed into the gregarious state. The aggregation pheromone are semiochemicals that are associated with the locust phase polyphenism (Nolte et al. 1973; Torto et al. 1994; Seidelmann et al. 2000).

Chemical communication in desert locust

Insects are the major agricultural pests destroying approximately 35% of crops annually all over the world, and 15% loss in harvest and shipping (Sword et al. 2010). Due to the high cost of broad-spectrum pesticides for locust control, two main alternatives have been exploited. The first alternative is behavior-modifying chemicals (BMCs), also referred to as semiochemicals, which occur as natural signals that affect changes in the behaviour or development of many organisms (Hardie and Minks 1999; Matthes et al. 2003). The second one involves biological control pathogens of pests such as *Metarhizium anisopliae* (Powell and Pickett 2003).

Semiochemicals fall in two major groups: chemicals used among members of the same species (intraspecific) called pheromones, and chemicals used among individuals of different species (interspecific) called allelochemicals. Pheromone types differ in the nature of the information delivered or process affected. Many insect pests communicate with each other through pheromones. There are various types of pheromones such as releaser pheromones, which trigger short-term behavioral effects and primer pheromones, which trigger long-term behavioral effects. Examples of releaser pheromones include alarm pheromones released when an individual is attacked by a predator, trail pheromone common in social insects such as ants, and aggregation pheromones, which trigger cohesive behavior. Examples of primer pheromones include maturation–accelerating pheromone in the desert locust (Mahamat et al. 1993; Mahamat et al. 2000). All pheromones consist of mixtures of several chemicals and performance of the pheromone is based on all these chemical components in the proper proportions (Shani 2000). Pheromones have been identified from hundreds of insect species, with bombykol ((E, Z)-10, 12-hexadecadienol-ol) identified as the first sex pheromone from the silk moth *Bombyx mori* (Butenandt, 1959).

Pheromones of the desert locust

A number of key pheromones controlling the behaviour of the desert locust have been identified and their roles in chemical communication elucidated. They include gregarisation, social cohesion, synchronous maturation, mating, oviposition, maternal transfer of phase traits to the progeny and courtship inhibition pheromones (Ferenz and Seidelmann 2003; Hassanali et al. 2005). For instance, gregarious and solitary locusts have been distinguished by the grouping or cohesive behaviour which is mainly attributed to aggregation pheromones (Obeng-Ofori et al.

1993; Torto et al. 1994; Torto et al. 1996). The aggregation pheromones in gregarious nymphs and adults have been shown to be different. The gregarious nymph pheromone blend is produced by both sexes and comprises C_6, C_8-C_{10} aliphatic aldehydes, their corresponding carboxylic acids, guaiacol and faecal phenols (Torto et al. 1996). The adult aggregation pheromone produced by the gregarious-phase mature males has been shown to be a blend of benzene derivatives (benzaldehyde, guaiacol, phenol and phenylacetonitrile (PAN) (Torto et al. 1994; Seidelmann and Ferenz 2002; Seidelmann et al. 2003) with PAN comprising 75-85% of the blend. There is a clear differentiation between volatiles emitted by gregarious nymphs and adults. Additionally, PAN has also been shown to have maturation-accelerating effect on immature adults (Mahamat et al. 1993; Mahamat et al. 2000). PAN seems to have a male-male avoidance function at relatively high concentrations near the source (Seidelmann and Ferenz 2002) and a cohesive effect at lower concentrations away from the source (Pener and Yerushalmi 1998; Hassanali et al. 2005; Rono et al. 2008). There was lack of courtship-inhibiting effect of PAN on female-deprived gregarious males (Seidelmann 2006). Two behaviorally active components of the oviposition aggregation pheromone of the desert locust, acetophenone and veratrole, induce gravid females to oviposit (Rai et al. 1997). However, no veratrole is found in female volatiles (Torto et al. 1994). Guaiacol and phenol are not produced by the locust themselves but by gut bacteria (Dillon et al. 2000; Dillon and Charnley 2002). On the other hand, solitary locusts use chemical signals for timed and synchronized maturation and also in locating their mates at low densities (Hassanali et al. 2005).

Strategies to combat the desert locust

The original locust control strategy assumed that plagues arose when swarms escaped from outbreak areas and bred successfully in the surrounding invasion area. There are essentially three possible strategies for desert locust control: to prevent plagues by control during the upsurge stage, to eliminate plagues by destroying nearly all the locusts and merely to protect crops and allow the plague to follow its natural course (Symmons 2009).

Plague prevention

Early surveillance of desert locust population dynamics is critical to outbreak prevention. The main areas that are known to harbour desert locust populations are surveyed using remotely sensed images that depict vegetation. Other survey options are the use of relevant global information system (GIS), meteorological and historical locust frequency information. Examples of the more remote breeding areas are western and northern Sudan and much of Mauritania. However, these areas are faced with challenges such as lack of trained manpower, scarcity of transportation to the breeding areas and sustainable scouting systems in some countries which rely to a large extent on indigenous populations, especially farmers and nomads (FAO/EMPRES 1999).

Crop protection

Another option of desert locust control is the protection of crops under immediate threat using trenches or pesticides. The swarms are targeted and destroyed before they reach agricultural perimeters especially commercially crops. Farmers of citrus crop in southern Morocco applied insecticides to destroy swarms of desert locust before they reached the. This initiative

contributed significantly to the plague decline in 1962 (Rainey et al. 1979) to the end of the 1968 upsurge (Bennett 1976) and perhaps ending the recent plague. Another attempt in 1987 and 1988 to protect the crops of subsistence farmers in the Sahelian belt by spraying locust bands near crops seemed successful. Even though damage was light, there was no adequate evidence to indicate that it was due to the applied intervention. However, the main drawback to this control option is the need to deploy resources in advance, most of which will not be needed.

Plague elimination

The plague elimination is the main control strategy that is commonly used. There are various strategies that have been used and these include physical, chemical, and biological methods. The use of chemical pesticides is by far the most efficient with organophosphates, carbamates and pyrethroids being the chemicals widely used in repetitive applications for effective control (Nasseh et al. 1993) but it has its drawbacks (Peterson and Higley 1993).

Chemical control of locusts

Dieldrin was the first insecticide to be developed and used for large scale control of locusts. It is an organochlorine insecticide known to be toxic on contact with insects. The mode of application is by spraying on the ground from a backpack, vehicle or airplane onto locust hopper bands or over the swarm in flight. Use of dieldrin allowed the treatment of barrier strips; migrating hopper bands would cross these strips and accumulate a lethal dose. In addition, the aerial application of low doses of dieldrin as a fine droplet cloud is one of the most efficient methods for control of desert locust swarms (Sayer 1959; Matthews 1992). Dieldrin was the most effective and economical control agent because of its long persistence (Bennett and Symmons 1972).

The negative environmental impact of organochlorine pesticides led to its ban. Organophosphate, carbamate, and pyrethroid pesticides replaced dieldrin for locust and grasshopper control, but these could not be tested against African locusts because populations of those insects were in recession. The substitute organophosphate pesticides, such as fenitrothion and malathion, had shorter environmental persistence and were often repeatedly applied as blanket treatments over large areas. These were less hazardous to the environment but more hazardous to human and animal health. They were followed by chemicals of the pyrethroid family such as cypermethrin and lambda-cyhalothrin, and the new phenyl pyrazole fipronil. These broad spectrums 'knock-down' chemicals are hazardous to non-target species, and requires a clearly identified locust swarm target. Ironically, such treatments may have caused greater environmental damage than the organochlorine treatments they were designed to replace (Rowley and Bennett 1993).

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

These are new and modern approaches used to control insect pest. These substances adversely affect insect growth and development Examples of IGRs include diflubenzuron and trifluormuron (Tunaz and Uygun, 2004). These products interfere with the insect development by interrupting moulting between growth stages like juvenile hormones (JHs). This implies that locusts have to be treated before they reach adulthood. Chemically and biologically, JHAs are intermediate 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 the insects. Their molecular stability leading to long persistence under field

conditions drastically reduces the number of sprays in the environment thereby making them suitable for application as barrier treatment agents for control of hopper bands. However, their toxicological effects on non-target insects and environmental impact are not known. Application is time restricted and requires early detection of the hoppers because of their effectiveness on very defined stages – the nymphal stages of the target insects.

Physical control

In the past, only mechanical methods which included herding, collecting crushing or burning the hoppers were used. They also included harrowing and tilling (Meinzengn 1993; Karrar 1974). Other methods included driving locusts away with branches, digging trenches and clearing the grass vegetation around fields (Flint and Van den Bosch 1981b; Gahukar 1988; Nwanze and Harris 1992). At times people also would eat the locusts and it has been shown to be a way of eliminating them.

The sterile insect technique (SIT) has been successful in a number of pest species (Klassen 2005). Recently, this technique was employed on grasshoppers and locusts and successful results were obtained. There was reduced number of offsprings produced by females mated with gamma-irradiated males. However, the irradiation did not affect the ability of the males to copulate (Dushimirimana et al. 2008). Also, an increased mortality rate was observed due to non-specific damage of somatic cells.

Biological control of locusts

The emphasis of chemical pesticides on locust and grasshopper control has lead to the underestimation of the potential of biocontrol approaches in the past. A mycopesticide called green muscle™ (GM) based on the spores of the entomopathogenic fungus *Metarhizium anisopliae* var. *acridum* was first developed by the LUBILOSA PROGRAMME (LUtte Biologique contre les Locustes et Sauteriaux) (Lomer et al. 2001). In a recent field study in Sudan, GM was shown to be effective control against hopper stage of the desert locust due to its specificity and virulence. The recommended dose of GM (50g/ha) gave a mortality of 73.1% and 89% of hoppers at day 6 and 9, respectively, and 100% mortality at day 15 post application. At a quarter of the recommended dose of GM (12.5 g/ha), a 51.8% and 56.7% mortality at day 6 and 9, respectively. Fungus sporulation was evident in most cadavers that were exposed to GM (Bashir et al. 2007). However, the cost of biological control has been shown to be higher than most chemical insecticides (Mahamat et al. 2000; Hajek 2004)

Pheromones as a new control tactic

Semiochemicals including pheromones are gradually being integrated as a new biotechnological control agent to insects in various domains. Because they are species specific and their mode of action mainly involves induction and/or alteration of well-defined behavior during a specific period in the lifespan of a given insect, these tools are environmentally friendly and efficient (Knights; 2010; Vacas et al. 2010; Campos and Thomas, 2010). PAN (10 ml/ha) treatment in the field resulted in fragmentation of band at day 3. The density of the hopper bands reduced from 124 to 52 at day 3 post application. A mortality rate of >95% was observed by day 15 post

application. It is expected that the high mortality rate was also attributed to other factors such as cannibalism, predation or stress-related death (Bashir et al. 2007).

Integrated pest management (IPM) of the desert locust

To effectively suppress the invasion of desert locusts, an integrated pest management (IPM) approach is required. The components of IPM strategies for desert locust include chemical insecticides, biological control, cultural methods, chemosensory based control strategies, crop protection and desert locust monitoring. An example of IPM in desert locust involve a combination of two products based on mycopesticides *Metarhizium anisopliae* var. *acridum* and a semiochemical (PAN) for preventative control or during invasion. Studies have shown that green muscle at half the recommended dose (25 g/ha) and a quarter of the recommended dose (12.5 g/ha) with PAN gave >95% mortality at day 15 post application (Bashir et al. 2007; Lecoq 2010).

Biosynthesis of PAN in the desert locust

Postulated biosynthesis pathway of PAN

The issue of pheromone origin and biosynthesis remains unclear. Previously, there were assumptions that the pheromone precursors could be traced through dietary components or synthesized *de novo*. Isotope studies on Lepidoptera indicated that most sex pheromones were synthesized *de novo* (Bjostad et al. 1987). Studies in the boll weevil, have also demonstrated that the monoterpenoid pheromone components were from both modification of dietary precursors and from *de novo* biosynthesis (Thompson and Mitlin 1979). The dietary products of normal

metabolism are modified by a few pheromone gland-specific enzymes to produce the myriad of pheromone molecules (Bloomquist and Vogt 2003).

Despite the importance of PAN in the desert locust, information on its biosynthetic pathway is not well understood. PAN comprises a benzene ring with a two carbon side chain suggesting two possible amino acids as precursors/substrate during its synthesis. In addition, biochemical studies of cyanogenesis in millipedes and in plants with PAN as an intermediate suggest that phenylalanine or tyrosine could serve as a precursor of PAN (Duffey et al. 1974; Duffey 1981; McFarlane et al. 1975; Halkier and Møller 1989; Khan et al. 1997). Recent studies by Siedelmann and coworkers have shown that when ^{14}C-phenylalanine and ^{14}C-tyrosine were incubated in body parts of the male desert locust ^{14}C-PAN was readily produced when ^{14}C-phenylalanine was the substrate. Therefore, it may be derived by removal of the carboxyl group to give phenethylamine, a reaction catalyzed by amino acid decarboxylase (AADC), also referred to as phenylalanine decarboxylases (Tieman et al. 2006). Phenylpyruvate obtained by the removal of the amine groups is another possible reaction, however, this is unlikely because PAN has a nitrogen atom on the side chain. Therefore, the metabolism of phenylacetonitrile from phenethylamine is a possible pathway. However, to fully elucidate the pathway, there is need to determine the presence and identify possible intermediates involved in the biosynthetic process (Fig. 1.5).

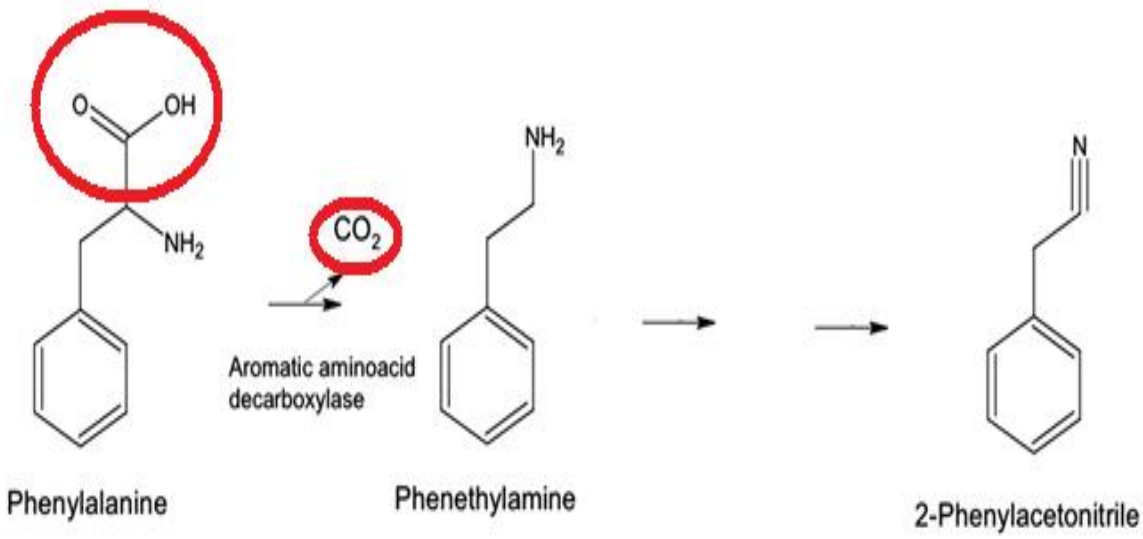

Fig. 1.5 The proposed biosynthesis pathway for phenylacetonitrile from phenylalanine as the precursor. Source (Adopted from Tieman et al. 2006).

Construction of a subtracted cDNA library

Many insects show polyphenisms, or alternative morphologies, which are based on differential gene expression rather than genetic polymorphism. The desert locust, *Schistocerca gregaria* is characterized by phenotypic plasticity involving a harmless solitarious phase and a damaging gregarious (swarming) phase. Subtractive suppression hybridization has been employed to study differentially expressed genes between closely related species, varieties and gene expressed differentially in different tissues (Hara et al. 1991). Generation of differentially expressed genes of unknown sequences was achieved by screening of genomic DNA or cDNA libraries with cDNA probes prepared from the cells in two different stages. Generally, this technique involves hybridization of cDNA from one population (tester) to excess of mRNA (cDNA) from other population (driver) and then separation of the unhybridized fraction (target) from hybridized common sequences (Diatchenko et al. 1996; Ban et al. 2007) between the two populations. Therefore, using subtractive hybridization, one is able to isolate, identify and clone mRNAs that differ in abundance in one population but not in the other. The advantage of using subtractive library is that the size of the library can be reduced amicably thus the potential genes are also reduced to ease scrutiny. By so doing, only genes that are differential between the two pools are expressed.

The literature of the desert locust shows that only gregarious males release considerable amounts of PAN when they become sexually mature (Torto et al. 1994; Seidelmann et al. 2000). A key feature of the desert locust is their ability to express morphological, physiological and behavioural differences between the two phases. Here, tissue from the sexually mature solitary population can be hybridized with gregarious male population to obtain the unhybridized

sequences which can be isolated, identified and studied further to evaluate the differences in such behaviour. Therefore, this molecular approach can be used to identify genes/proteins encoding these important aggregation pheromones. Little is known about the proximate molecular mechanisms underlying the aggregation behaviour or any other such polyphenism. Knowledge on the molecular mechanism of this process could lead to manipulation of the chemosensory systems. Similar findings by Xu et al. (2005) showed that mutation of the *Drosophila* odorant binding proteins (OBP) LUSH reduced the response to the aggregation pheromone *(Z)*-vaccenyl acetate.

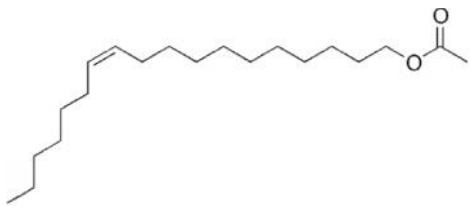

Fig 1.6 The chemical structure of (*Z*)-vaccenyl acetate.

Furthermore, identification of targets and pathways through which PAN acts might lead to the development of new rational tactics. In the current study, gas chromatography coupled to mass spectrometry (GC-MS) technology was exploited to determine the source of PAN production. The biosynthesis pathway of PAN was elucidated using suppression subtractive hybridization (SSH) technique to deduce the potential genes/enzymes that could be involved in the aggregation behaviour of the desert locust.

Justification of the study

Limitations on available locust control strategies emphasize the need for development of new strategies. Application of locust pheromone to disrupt gregarisation at critical stages of population build-up and phase transformation is viewed as a possible alternative control strategy (Byers 1991; Hassanali and Torto 1999) and was successfully tested using PAN alone and PAN combined with the biopesticide 'Green Muscle' in Sudan between 2005 and 2007 (Bashir and Hassanali, FAO 2007 report). Molecular approaches can be used to identify genes/proteins encoding the expression of PAN and other important pheromones associated with the desert locust. This requires knowledge on the molecular mechanisms of aggregation, which could lead to manipulation of the chemosensory systems. Furthermore, identification of targets and pathways through which PAN acts might lead to the development of new management tactics for the desert locust.

Objectives of the study

Overall objective

To assess the level of phenylacetonitrile at different life stages in the adult desert locust, *Schistocerca gregaria*, the biosynthesis/gene pathway and potential candidate genes associated with it.

Specific objectives

i. To determine PAN levels in different tissue and body parts of both adult males and females of the desert locust.
ii. To assess the sexual receptivity of both male and female desert locusts in relation to PAN production.
iii. To identify the differentially expressed gene that could be responsible for aggregation behaviour in desert locust.
iv. To quantify the potential gene levels that could be responsible for aggregation behaviour in both adult solitarious and gregarious desert locust.

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CHAPTER TWO

Mass spectral determination of phenylacetonitrile (PAN) levels in body tissues of adult desert locust, *Schistocerca gregaria*

Abstract

Wings and legs of the gregarious desert locust, *Schistocerca gregaria* have been shown to be release sites of phenylacetonitrile (PAN), the major adult male-produced pheromone. The pheromone is produced prior to sexual maturation of the adults. Previously it has been shown to play other roles, including social cohesion and synchronous maturation. However, there is limited information on the distribution of PAN within the locust. Here we show, using gas chromatography-mass spectrometry (GC-MS), that PAN occurs in nearly all body parts of both adult males and females of the locust in varying amounts. PAN was 20-fold more concentrated in males than in females. In females, PAN was concentrated more in the tarsal segments. The greatest amounts of PAN were in 2- and 3-week old female and male body parts, respectively. No trace of PAN was found in similar ages and sexes of the solitarious phase desert locust. The wide distribution of PAN in the bodies of both sexes of gregarious phase locusts suggests that no specific tissue is responsible for biosynthesis of the pheromone. Our results support the role of PAN as a pheromone in sexually mature gregarious males, and they provide the first insights as to its distribution in the body matrix of both sexes and phases of the desert locust.

Keywords: *Schistocerca gregaria*, PAN levels, pheromone sites, mass spectrometry

Introduction

Phenylacetonitrile (PAN) was identified as the principal component of the adult aggregation pheromone of the desert locust *Schistocerca gregaria* (Orthoptera: Acrididae) in the 1990s (Obeng-Ofori et al. 1993; Torto et al. 1994). Subsequent studies have demonstrated the multiple behavioural and reproductive functions for the pheromone in this notorious agricultural pest (Ferenz 1990; Loher 1990; Byer 1991; Seidelmann et al. 2000; 2003; Rono et al. 2008; Bashir and Hassanali 2010). The major pheromone component of the gregarious adult males has been shown to be involved in cohesive behaviour of the desert locusts at lower relative concentrations (Torto et al. 1994) and as male–male homosexual repellent at higher relative concentrations (Seidelmann and Ferenz 2002; Rono et al. 2008).

Based on histological studies, abdominal epidermal cells have been suggested as the possible site of production of the pheromone (Loher 1961; 1990). These epidermal cells are large and vacuolated in the gregarious phase of male locusts when they became sexually mature, but are undeveloped in immature males and females and solitarious locusts. The occurence of enlarged vacuolated epidermal cells has also been reported in other body parts of the locust including tarsal segments of the legs) (Kendall 1972). Subsequent studies suggested that phenylacetonitrile and benzaldehyde, the biosynthetic products of phenylalanine produced in the epidermal pheromone producing cells of the wings and legs (Seidelmann et al. 2003), where distinct body parts of the gregarious male desert locust were analyzed using solid-phase-microextraction (SPME). Therefore, we undertook a study to determine if anatomical tissues other than the wings and legs contain PAN. The following report results of studies in which we analyzed extracts of different tissues by GC-MS to determine (i) the presence of PAN in various body parts and (ii)

age related variations in the amounts of the pheromone in both males and females of two phases of the desert locust.

Materials and methods

Insects

The gregarious locusts were reared at the Insect and Animal Breeding Unit (IABU) of *icipe*, Nairobi. The colony originated from the centre's Port Sudan field station in Sudan. About 300–400 of these insects were reared under crowded conditions in aluminium cages (50 x 50 x 50 cm) in a special room (4.5 x 4.5 m) each well aerated with a duct system to maintain a negative pressure. The solitarious colony originated from Dr. Steve Roger, Department of Zoology, University of Cambridge. Solitary reared adult insects were kept individually in standard aluminium cages (10 x 10 x 24 cm) in visual, olfactory and tactile isolation from other insects. In both cases, the rooms were maintained at temperature of 30 ± 4 ºC, 40 – 50% relative humidity (RH) and a photoperiod of 12:12 L/D (Ochieng-Odero et al. 1994). The insects were fed on fresh wheat seedlings and bran daily.

Extracts

Both males and females of crowd-reared and solitary-reared adult stages of the desert locust were used in this study. Relative concentration of PAN from extracts of different body parts according to age (five ages after fledgling namely 0, 1, 2, 3 and 4 week old) males and females were analyzed by GC-MS. The following body tissues were used: fat body, and accessory gland (males and females), testis, ovaries and phallic complex (from males), ovaries and spermatheca (from females). The insects were chilled and dissected under physiological saline. The body

parts were transferred into vials containing 1ml hexane solution (Sigma Aldrich). External body parts were obtained from whole wing, femur, tibia and tarsus from both males and females, then cut into smaller pieces per individual insect and transferred to 1ml of hexane. The weights of all the body parts were recorded. All the body parts were soaked in 1 ml hexane for 10 min followed by homogenization using a vortex for 5 – 10 min. The extract was then obtained by decanting the hexane. An aliquot (1 µl) of methyl salicylate (~150 ng) was added to 40 µl of each extracted sample as the internal standard to determine the quantity of PAN per mg of body part. Three replicates of each body part were used in this study.

Chemical analysis

Gas chromatography-mass spectrometric (GC-MS) analysis and identification of compounds within each sample was conducted using an Agilent technologies 7890A® series GC coupled to a 5975C Triple Axis MSD®. The mass spectrometer has an overall mass scan range of *m/z* 1–1050 and was calibrated using heptacosa (Perfluorotributylamine) $[CF_3(CF_2)_3]_3N$ (Apollo Scientific Ltd, UK) and set to acquire data over the range of m/z 38–550, respectively. The electron ionization (EI) mode was used with electron energy set at 70 eV and emission current of 34.6 µA. The temperature of the ion source was held at 230 ºC, the Quadrupole at 150 ºC with the multiplier voltage at 1106 V. The pressure of the ion source was held steady at 8.4 x 10^{-6} mBar. A data acquisition scan cycle of 3 scans per 2 sec was used.

Samples were injected in the splitless mode and the injector was purged at 0.8 min at an injector temperature of 270 °C. Helium was used as a carrier gas at a linear flow velocity of 39.621 cm/sec. GC oven was held at an initial temperature of 35 ºC for 5 min, and then the temperature

was increased at a rate of 10 ºC/min up to 280 ºC for 10 min then 50 °C to 285 ºC for 9.9 min, the total run time was 50 min. HP5-MS low bleed GC-MS capillary column, with dimensions 30 m x 0.25 mm (i.d) x 0.25 µm (film thickness) supplied by J & W Scientific was used for separation of compounds in the extracts.

The GC-MS was linked to a computer with MS library and PAN was identified by comparing their retention times and spectra with those of authentic standards, and their fragmentation patterns.

Fig 2.1 Agilent GC-MS: Agilent technologies 7890A® series GC coupled to a 5975C Triple Axis MSD® at *icipe's* Behavioural and chemical ecology (BCED) laboratory.

Quantification

GC-MS in selected ion monitoring mode (SIM) was used to quantify PAN in the extracts. Two ions monitored in PAN were *m/z* 90 and 117 while the selected ions for internal standard, methyl salicylate were *m/z* 92, 120 and 152. Serially diluted solutions of synthetic PAN (1–10 ng/µl) were analyzed by GC-MS in SIM mode to obtain linear calibration curves (Mass area vs. concentration) which served as the basis for the external quantification.

Data Analysis

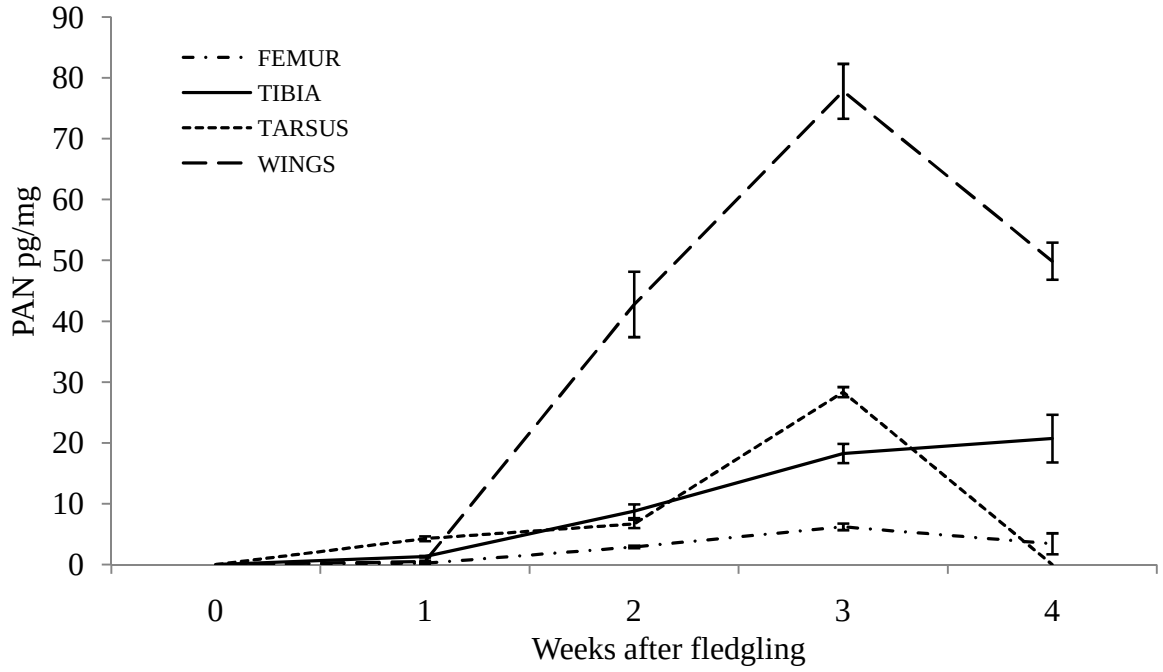
The data on the amount of PAN in the internal and external tissues of each sex were analyzed separately. Analysis of amounts of PAN in each sex was performed week by week using multi-way ANOVA in the R 2.13.2 statistical package. Means were separated by Student-Newman-Keul (SNK) test at 5% level of significance.

Results

The results of the GC-MS analysis of the amounts of PAN in both males and females with age are shown in Fig 2.2 and 2.3, respectively. There were quantitative differences in amounts of PAN associated with increasing age. No significant difference in the amount of PAN in different body parts was evident during the first week after fledgling. However, the amount of PAN increased significantly the following week (two weeks after fledgling) with the onset of sexual maturation. Extracts from both external and internal body parts contained more PAN in the second week showing age related differentiation in both sexes.

The body parts with the highest levels of PAN were detected in 3-week-old males and 2-week-old females respectively. The body parts of the females contained PAN but in lower concentrations compared to their male counterparts. There was a significant difference in amounts of PAN in extracts from external and internal tissues with external body parts of both sexes containing the highest amounts of PAN. The mean PAN amount (mean ± SE) in extracts from 3-week-old males followed the order in decreasing amounts; wings (77.82 ± 4.51 pg/mg), tarsi (28.35 ± 0.84 pg/mg), tibia (18.27 ± 1.58 pg/mg) and femurs (6.21 ± 0.54 pg/mg) ($p < 0.01$, $F = 44.48$, $DF = 3$). In females, tarsi had the highest amount of PAN at 2 weeks (3.54 ± 0.36 pg/mg), followed by tibia (1.43 ± 0.09 pg/mg), wings (0.80 ± 0.01 pg/mg) and femurs (0.28 ± 0.03 pg/mg) ($p < 0.01$, $F = 57.25$, $DF = 3$). PAN in the internal body parts of both sexes was found to be in relatively small amounts (Fig 2.2B and 2.3B). Fat bodies were amongst the highest PAN containing internal tissue in both sexes with (4.02 ± 0.44 pg/mg) in males and (0.91 ± 0.22 pg/mg) in females. Representative GC-MS chromatographs (Fig 2.4) indicating the highest and lowest PAN in wings and fat body respectively in males. No PAN was detected in any body part of solitary reared locust.

A.



B.

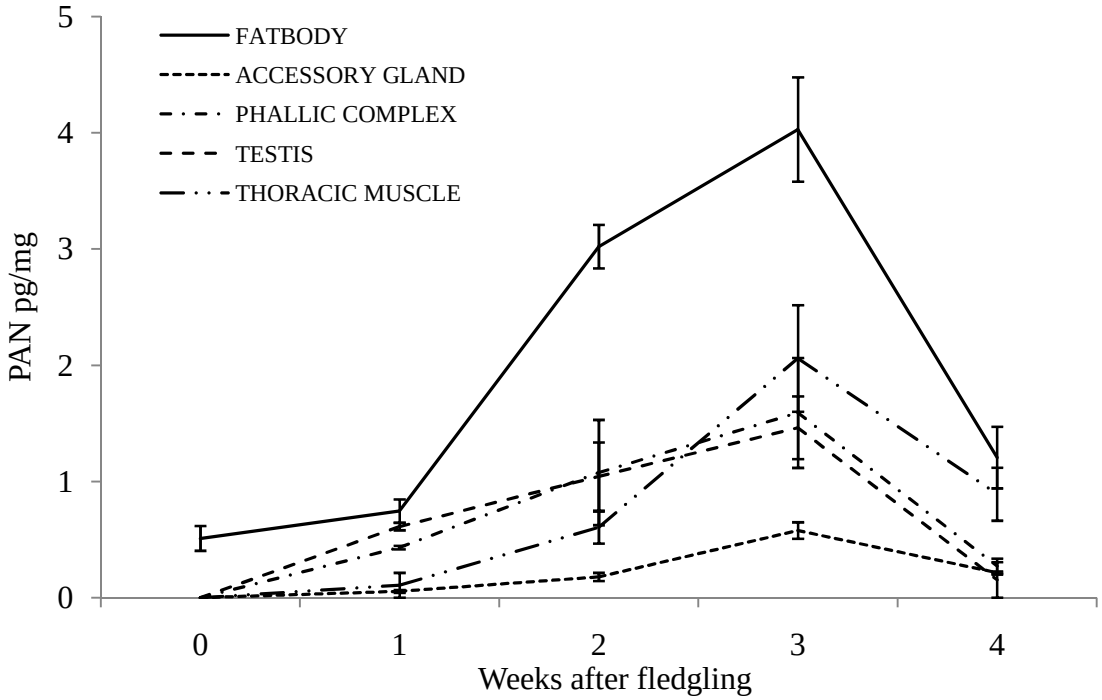
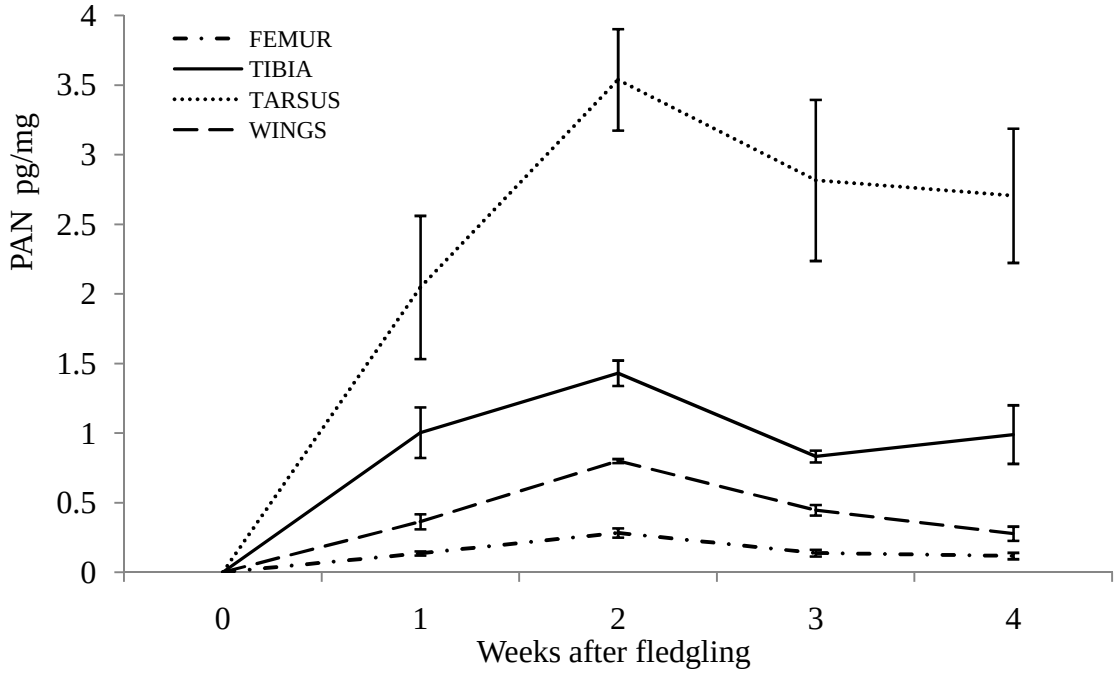


Fig 2.2 Mean phenylacetonitrile (PAN ± SE) amounts determined from differing ages of the male *Schistocerca gregaria.* (A) external and (B) internal body parts.

A.



B.

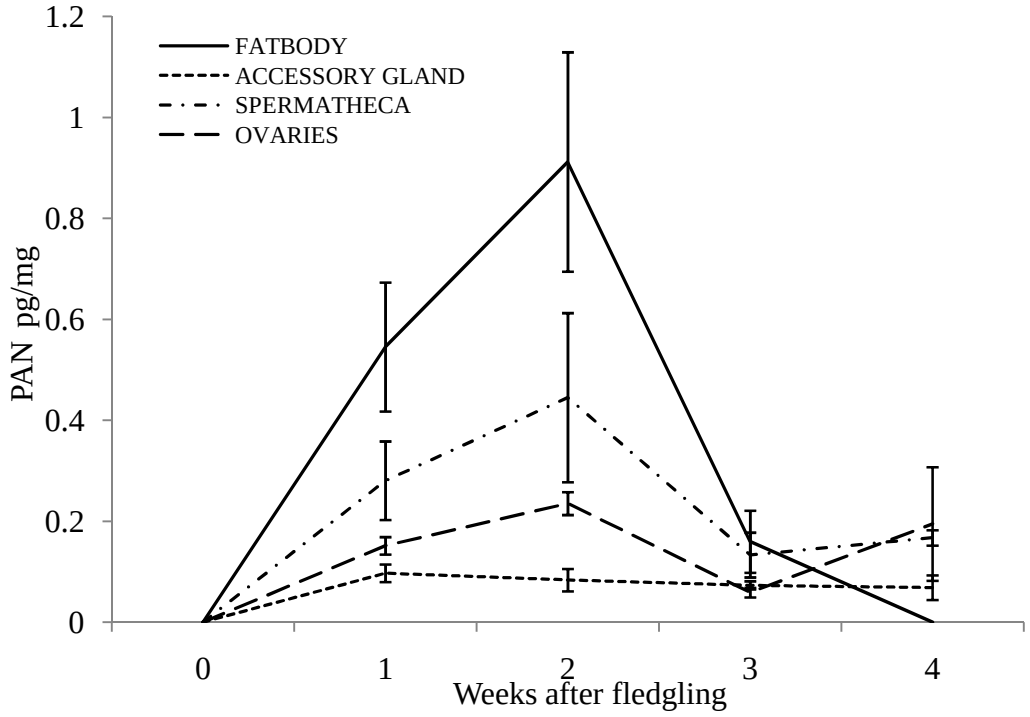


Fig 2.3 Mean phenylacetonitrile (PAN ± SE) amounts determined from differing ages of the female *Schistocerca gregaria.* (A) external and (B) internal body parts.

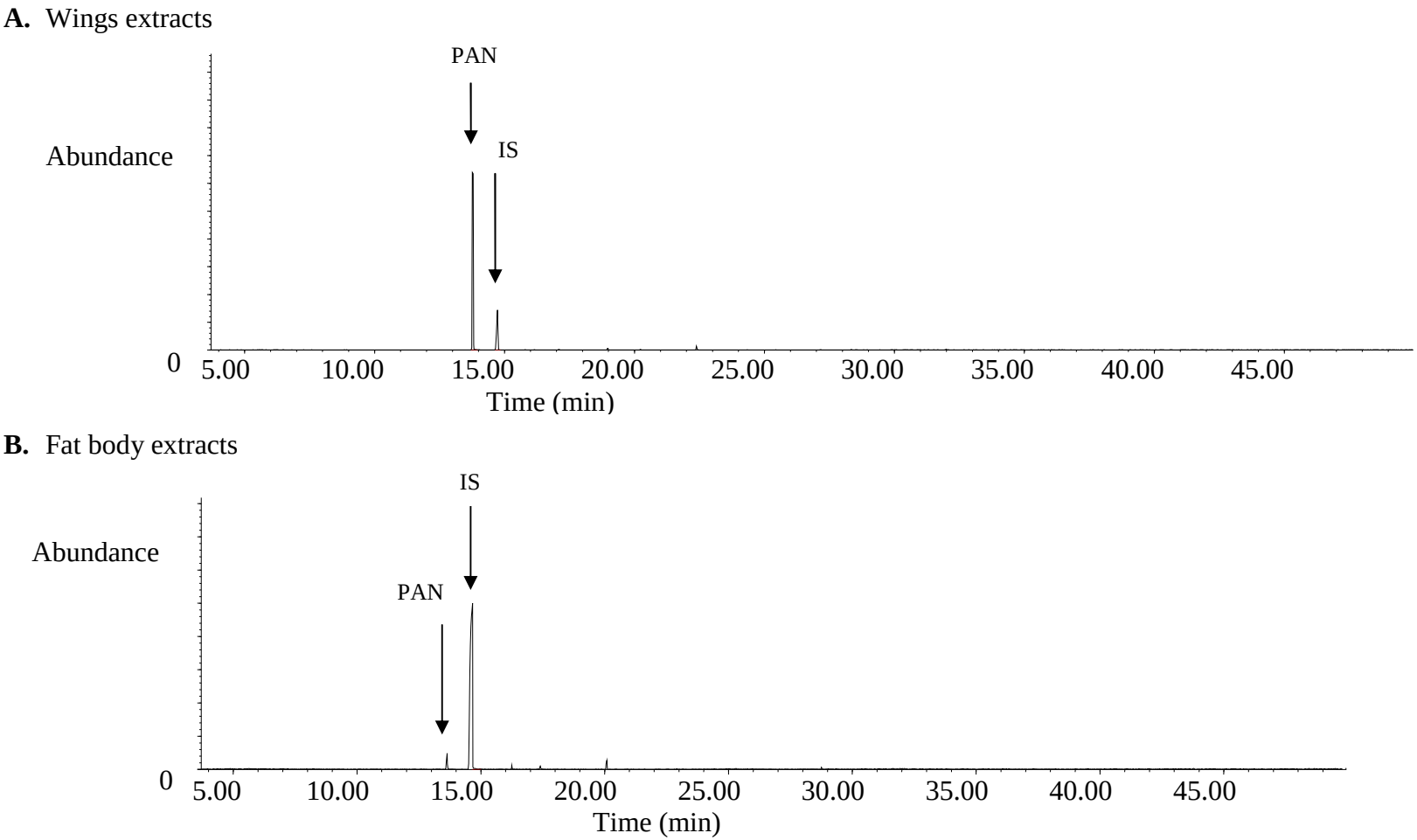


Fig 2.4 Representative GC-MS profile showing relative levels of PAN in body parts of sexually mature male at selected ion *m/z* 90 and 117. The body parts are a) wings and b) fat body extracts. The chromatograph at retention time 13.7 represents PAN while 14.7 is methyl salicylate used as the internal standard (IS), at concentration ~150 ng in both cases.

The quantification of PAN from the tissues of the desert locust was based on two calibration approaches, internal and external quantification. The standard calibration curves using synthetic PAN showed good linearity within the range of 1 - 10 ng/µl as shown in Fig 2.5. The mass areas of the peak obtained from the different concentrations of synthetic PAN standard were used to draw this curve against the concentration. The linear equation was y = 8.32x with R^2 = 0.9932. The linear trend was used for external quantification of PAN present in the extracts.

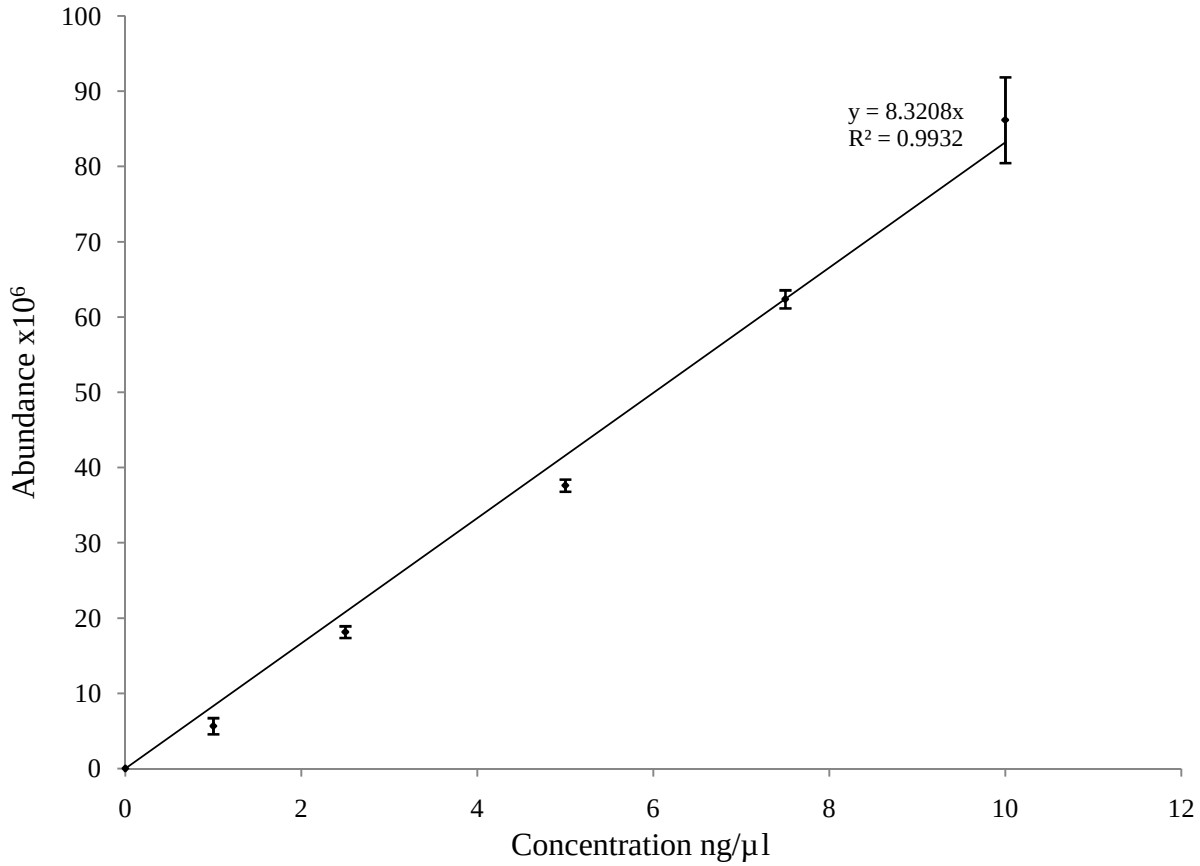


Fig 2.5 Calibration curve of synthetic PAN standard at different concentrations (0, 2.5, 5, 7.5 and 10ng/µl) used for external quantification.

Discussion

This study was used to demonstrate the ability of GC-MS to detect and quantify even small amounts of PAN in various body parts of the desert locust and to document that there were both age- and sex-related differences in amounts of PAN present in various tissues of the locust. The increase in the amount of PAN from tissues with age correlated well with the earlier findings that showed increased headspace volatile amounts with age (Mahamat et al. 1993; Ferenz et al. 1994; Torto et al. 1994; Seidelmann et al. 2000). However, PAN was present in the body parts of both males and females although tissues from males contained 20-fold more PAN compared with the females. To our knowledge, this is the first study that detects amounts of PAN in the body parts of females. Production of PAN starts at immature stages and reaches maxima at their sexual maturation stages. Earlier production of PAN is consistent with other roles (social cohesion and maturation synchrony in adults, separation of hoppers from adult from adult groups) of the pheromone (Mahamat et al. 1993; 2000; Deng et al. 1996). This is supported in our study where most PAN was present in 2-week-old females and conversely in 3-week-old males. However, the amount of PAN in 2-week-old males was significantly higher than in females of the same age. A previous report indicated that pentanoic acid, a compound associated with ‘female locust pheromone’ produced by the Comstock-Kellog glands (CKGs) was found more in 2-week old females (Njagi et al. 2002). The results of this study also show a similar trend in the presence of PAN in females, a likely indicator that females mature faster than males based on both PAN and pentanoic acid production.

In our study, the wings had the highest amounts of PAN in males which concurred with previous data (Seidelmann et al. 2003). However a remarkable feature in our study was the ubiquitous

presence of PAN in extracts of all tissues analyzed from both sexes with a greater distribution of the pheromone on the external rather than internal tissues. Seidelmann et al. (2003) reported the occurrence of veratrole, another typical component of the adult male volatile in all body parts. In flour beetles, the aggregation pheromone was shown to occur in the exocrine glands on the ventral side of the first pair of femur (Olsson et al. 2006). The results of our study suggest that the potential origin of PAN production is an external body part based on the high amounts found on the wings and legs. However, the concurrent detection of the PAN in internal tissues indicates that the pheromone may be produced in multiple locations in the body. This could be attributed to the presence of glandular epidermal cells in nearly all body parts of the desert locusts (Kendall, 1972). The broad distribution of PAN in the epidermis of the desert locust may be an important mechanism for faster release and dispersion of the pheromone. Earlier reports showed concentration-dependent responses to PAN by *S. gregaria* males (Rono et al. 2008). There was evidence of arrestment (Torto et al. 1994) of individuals closer to a PAN source at low relative doses but repellent characteristics (Seidelmann and Ferenz, 2002) at higher doses. It is possible that, accumulating high concentrations of PAN could be toxic to the locusts hence it appears the locusts have developed a mechanism to disperse it rapidly from the body tissues. This would easily be accommodated by wing fanning and would explain why wings of males contain the largest amounts of PAN.

In conclusion, our results have demonstrated that both males and females of the desert locust produce PAN. Our research has documented that virtually all tissues contain detectable amounts of PAN, findings that provide leads on tissues that should be further examined as sites of biosynthesis, transportation and distribution of PAN in the body of the locust.

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CHAPTER THREE

Volatile pheromones mediate mate location in desert locust, *Schistocerca gregaria* (Orthoptera: Acrididae)

Abstract

Although previous reports suggest that gregarious locusts find their mate easily because of the high population density through close-range recognition, the underlying mechanisms are not well known. In the present study, bioassays were carried out in a flatbed wind tunnel to determine chemical communication mediated mate finding in gregarious sexually mature adults and whether mate finding correlated with locust age and titer of phenylacetonitrile (PAN). Subsequently, the release of PAN at different time interval and temperature were analyzed and correlated. The bioassay results indicated that females were attracted to their opposite counterparts of the same age, suggesting that females were responding to both visual and chemical cues provided by males. This contradicts earlier studies that indicated that female did not respond to males. There was a possibility that males take a longer time to get stimulated. There was significant difference in PAN titers during photophase (day) and scotophase (night). The results revealed a positive correlation between temperature and PAN emission. Also, these results suggest that both sexes of gregarious desert locusts actively seek the opposite sex using chemical cues and that mate finding may be influenced by the age and levels of PAN released in both sexes of adults.

Keywords: Phenylacetonitrile, mate attraction, wind tunnel, desert locust

Introduction

A number of semiochemiclas, a more important mode of intraspecific chemical communication and also interspecific signals from desert plants have been established for various locust species (Torto et al. 1994; Niassy et al. 1999; Bashir et al. 2000). These semiochemicals trigger behavioral responses such as aggregation (Obeng-Ofori et al. 1993; Obeng-Ofori et al. 1994; Torto et al. 1994), oviposition (Norris 1970), mate location and recognition (Whitman 1990), and maturation synchrony (Mahamat et al. 1993; Bashir and Hassanali 2010). Some of these semiochemicals may act alone or in combination with acoustic, visual and tactile cues. For example, adult gregarious male desert locusts release a bouquet of volatiles including benzaldehyde, guaiacol, phenol and considerable quantities of phenylacetonitrile (PAN) for cohesion (Pener and Yerushalmi 1998; Seidelmann et al. 2003). These compounds have also been shown to accelerate maturation (Mahamat et al. 1993; Mahamat et al. 2000) in immature adults. In the case of PAN, depending upon its concentration, it may serve as an adult aggregant or courtship inhibiting pheromone (Seidelmann and Ferenz 2002; Rono et al. 2008).

Mate finding, recognition, and copulation are essential steps in successful reproduction in insects. The lack of information on reproductive behavior of adults, particularly the role of semiochemicals, could hinder development of effective detection and management strategies for many insect species that are economically important pests. Mating behavior of the desert locust indicates that under high population density such as in the gregarious form (e.g. 15 x 10^3/ha), mate finding is easy (El Bashir et al., 1993). Gregarious females are reported not to display courtship behavior indicated by their lack of movement towards the males whereas the males are relatively aggressive and appear to recognize females (Inayatullah et al. 1994). However, in low

density populations both sexes are active in the mate location process (Uvarov 1977; Byers 1991).

Sexual receptivity is female behavior that allows or helps a male to fertilize her eggs. Through this behavior, females play an active role in reproduction. Multiple signals may be used for receptivity or unreceptivity. For sexually mature conspecific females and males to mate, they must exchange signals. The signals comprise either sexual advertisement (long-range signals) or courtship (short-range signals given just prior to mating). In species using sexual advertisement, either males or females broadcast chemical or auditory signals to attract potential mates from a distance (Ringo 1996). Because even sexually mature females that advertise may still be initially reluctant to mate when males approach, all insect species that mate use some type of courtship. This process can be as simple as moving to an appropriate location. The present study was undertaken to investigate sexual receptivity of both sexes in gregarious *S. gregaria* and if there is age preference during mate attraction in relation to the PAN production. Knowledge of gregarious *S. gregaria* mating behavior may provide information for the development of management methods for the locust.

Materials and Methods

Insects

Schistocerca gregaria colony from the International Centre of Insect Physiology and Ecology (*icipe)* (originating from the Port Sudan, *icipe*, field station in Sudan) were used in this study. Mixed sexes of the desert locusts were bred under crowded conditions in aluminium cages (50 x 50 x 50 cm) in a special room (4.5 x 4.5 m) each well aerated with a duct system to maintain a

negative pressure. Conditions in the rearing room was at a 30 ±1^{o}C, 50% relative humidity (RH) and a light–dark cycle of 12:12 h (Ochieng-Odero et al. 1994). Unmated virgin gregarious-reared mature locusts (2-week-old and 3-week-old males and females) were used. During this period, the insects were fed on fresh wheat seedlings and bran daily.

Bioassay

The bioassay was performed in a Plexiglas flatbed wind tunnel (Fig. 3.1), 100 cm long x 30 cm wide x 30 cm high. Air speed inside the tunnel was 20 cm/sec. Two screen mesh cages (6 x 6 x 6 cm) were placed in the upwind ends of the tunnel. The treatment source was one adult insect either male or female placed inside one of the cages while an empty cage served as control. One test insect of opposite sex to the odour source was introduced into the centre of the wind tunnel and its behavior was observed for 30 min. The distance traversed by the test locust toward the target locust (maximum 40 cm) and time (maximum allocated time 30 min) elapsed to reach the target were recorded. The test locust that walked >30 cm upwind towards target or control odour was recorded as a response. Test locust that did not walk upwind to any odour source was recorded as no response. After 5 insects were tested the wind tunnel was cleaned with alcohol and left to dry for 5 min and the positions of the target insect and control were switched to avoid positional bias. Six experiments were conducted using mature adults of different ages: (i) response of 3-week-old female to 3-week-old male odour vs. control (ii) response of 3-week-old male to 3-week-old female odour vs. control (iii) response of 2-week-old female to 3-week-old male odour vs. control (iv) response of 3-week-old male to 2-week-old female odour vs. control response of 2-week-old female to 2-week-old male odour vs. control (vi) response of 2-week-old male to 2-week-old female odour vs. control (vii) response of 3-week-old female to 1-week-old

male odour (viii) response of 1-week-old male to 3-week-old female odour vs. control (Table 3.1). For each experiment 25 individuals were tested once only. The experiment was performed during the photophase period from 10:00 to 16:00 hrs. Five bulbs (60 W each) were placed 70 cm above the tunnel equally spaced along its length for uniform lighting. The temperature inside the tunnel during the experiments was 30 ±1 ^{o}C.

Table 3.1 Experimental set up of mating response bioassay

Odor source	Respondent
3 wk male vs. control	3 wk female
3 wk female vs. control	3 wk male
3 wk male vs. control	3 wk female
2 wk female vs. control	3 wk female
2 wk male vs. control	2 wk female
2 wk female vs. control	2 wk male
3wk female vs. control	1 wk male
1 wk male vs. control	3 wk female

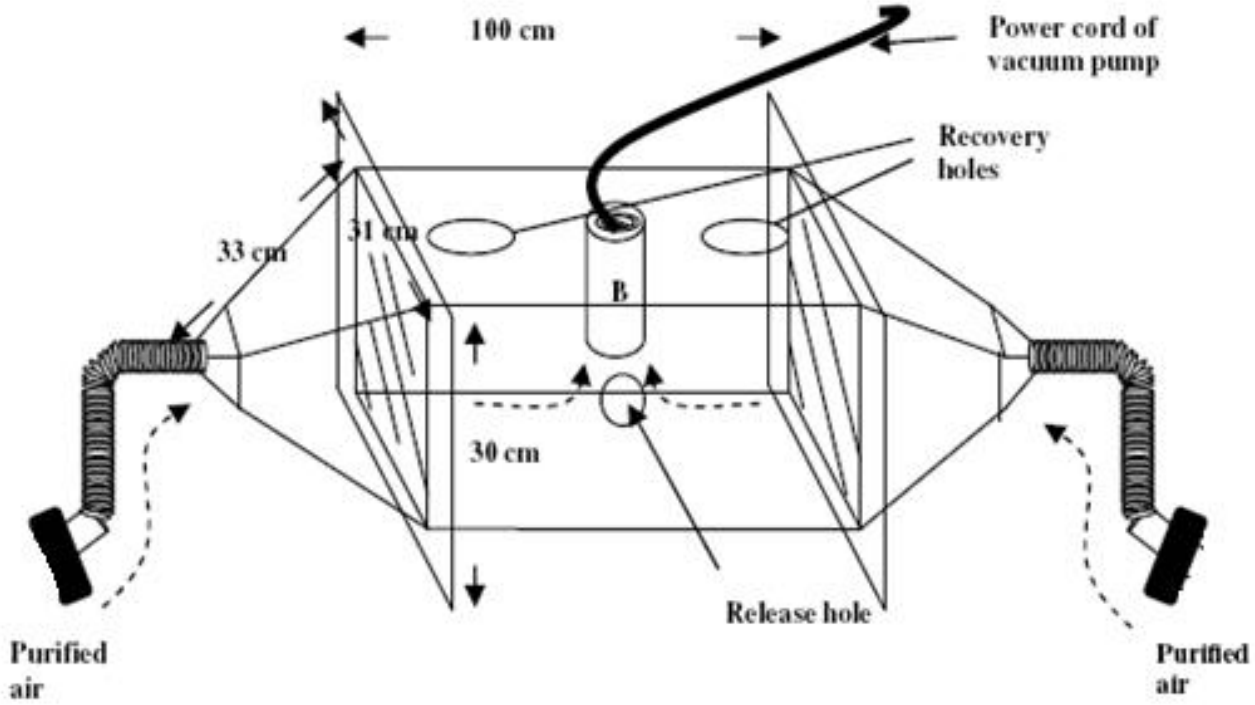

Figure 3.1 A schematic representation of a flatbed wind tunnel used for dual choice experiment.

Volatile collection and analysis

PAN emitted from gregarious-reared mature males (3 weeks after fledgling) was collected on a solvent-cleaned Super Q trap. A Talento® 800 series time switches (Grässlin, UK), time-based volatile collection system was used to collect volatiles at different time intervals from the locust. Collections were done during (i) photophase (06:01 to 18:00 hr) and scotophase (18:01 to 06:00 hr) and (ii) two-hour intervals for 24 hours. Trapped volatiles were eluted with 200 µl High-perfomance liquid chromatography (HPLC) grade dichloromethane (Aldrich Ltd). Methyl salicylate (146.75 ng) added to 40 µl of eluted volatile sample was used as an internal standard, and for quantification of PAN. An aliquot (1µl) was analyzed by gas chromatography-mass spectrometry (GC-MS) using an Agilent Technologies 7890A Series GC coupled to a 5975C inert, Triple Axis MSD.

Data Analysis

The statistical differences of test insects that reached the source of signal(s) in different treatments were analyzed using chi-square (χ^2) goodness of fit. The time it took for the insect to reach a target was analyzed using paired t-test. All the data was analyzed using R statistical package (R Development Core Team (2011). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/). The quantities of PAN released during scotophase and photophase were analyzed by paired t-test whereas the two-hour PAN quantities were analyzed using Analysis of Variance (ANOVA). A correlation test was used to correlate the temperature values and PAN quantities.

Results

Sexual receptivity of both males and females

In the mate finding bioassay, 3-week-old female (after eclosion) were significantly attracted to corresponding males of the same age: 14 females chose the cage with male while 6 females chose the empty control cage (χ^2 = 4.9, df = 1, p = 0.03). 2-week-old females were also significantly attracted to males of the same age (χ^2 = 6.7, df = 1, p = 0.01). However, neither 3-week-old males (χ^2 = 0.1, df = 1, p = 0.75) nor 2-week-old males (χ^2 = 2.5, df = 1, p = 0.11) were significantly attracted to their corresponding females of the same age. In addition, 3-week-old males were not significantly attracted to 2-week-old females (χ^2 = 0.9, df = 1, p = 0.34) while 2-week-old female were not significantly attracted to 3-week-old male (χ^2 = 2.3, df = 1, p = 0.13). 1-week-old females were not attracted to the 3-week-old female (χ^2 = 0, df = 1, p = 1.0). Nevertheless, 3-week-old females were significantly attracted to the control compared to the 1-week-old male (χ^2 = 5.4, df = 1, p = 0.02). All the results of the mate finding bioassay are shown in Table 3.2.

Table 3.2 Responses of individual males and females desert locust to odor source of opposite sex in a flat bed wind tunnel.

Treatment source	Respondent	N	Response	Response		p-value
				Odor	Control	
3 wk male vs. control	3 wk female	25	20	14 (70%)	6 (30%)	0.03
3 wk female vs. control	3 wk male	23	20	11 (55%)	9 (45%)	0.75
3 wk male vs. control	2 wk female	25	22	14 (64%)	8 (36%)	0.13
2 wk female vs. control	3 wk male	21	20	12 (60%)	8 (40%)	0.34
2 wk male vs. control	2 wk female	24	19	14 (74%)	5 (26%)	0.01
2 wk female vs. control	2 wk male	23	20	13 (65%)	7 (35%)	0.11
3wk female vs. control	1 wk male	25	20	10 (50%)	10 (50%)	1.00
1 wk male vs. control	3 wk female	25	18	5 (33%)	13 (67%)	0.10

Statistical significance differences, chi-square $p < 0.05$. N is the number of total insects used in the bioassay and response is the number of insects that chose either of the treatment.

PAN release

Effect of temperature on PAN release during photophase vs. scotophase

Results of this study reveal significant quantitative differences in PAN released by sexually mature adult gregarious desert locust during photophase and scotophase period ($t = 5.87$, $df = 3.84$, $p = 0.0047$). The mean amount of PAN per insect produced during photophase (06:01 hr to 18:00 hr) and scotophase (18:01 hr to 06:00 hr) was 17.51 ± 0.85 and 9.63 ± 1.80 ng/µl/male, respectively. The highest amount of PAN was released during the photophase compared with the scotophase. The mean temperature during photophase period was (33.06 ± 1.80^{o}C) while in scotophase period it was 25.3 ± 0.88^{o}C (Fig. 3.2). There was positive correlation ($cor = 0.88$) between the temperature and PAN production during the photophase and scotophase period ($t = 3.67$, $df = 4$, $p = 0.02$.

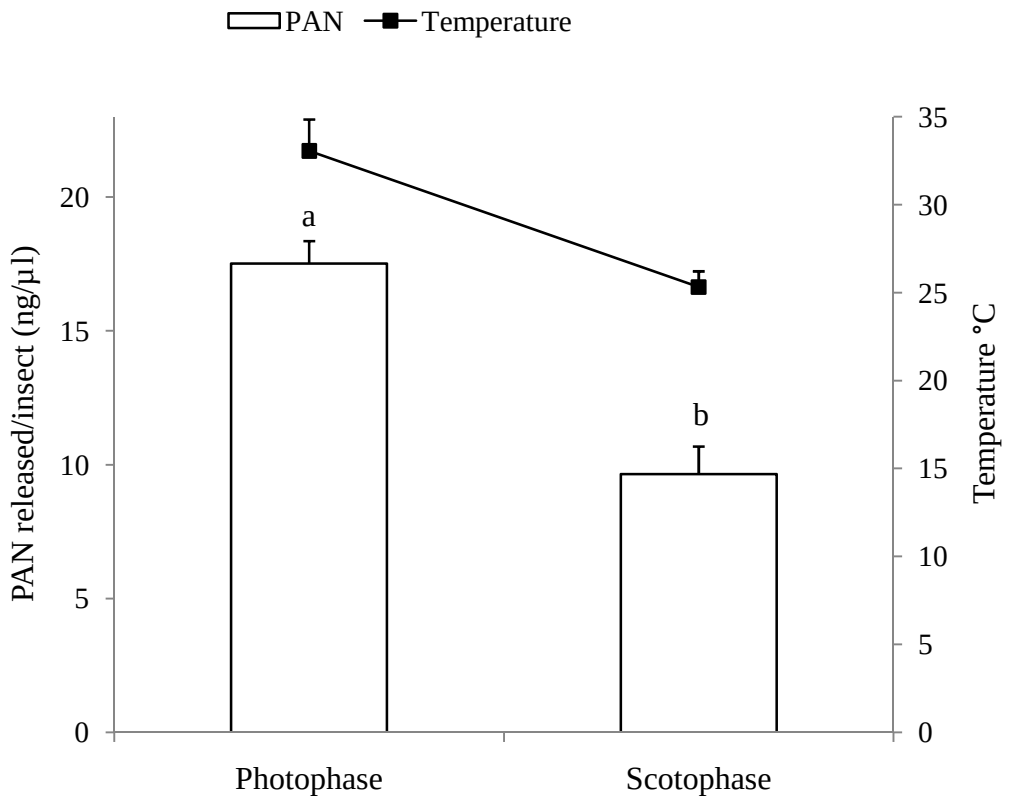

Fig 3.2 The amount of PAN released by sexually mature adult male desert locust, *Schistocerca gregaria*, at different time interval during photophase and scotophase.

Effect of temperature on PAN release in a two-hour interval

PAN quantities collected at two-hour interval in a span of 24 hr period indicated quantitative differences between 14:00–16:00 and 04:00–06:00 hr. PAN produced in the other two-hour time frame did not show any significant difference (Fig. 3.3). The highest PAN amounts were released at 10.08 ± 5.82 ng/µl/male between 1400-1600 hr followed by a gradual decrease until at 0400–0600 hr when PAN was 2.25 ± 1.30ng/µl/male. From 0400 – 0600 hr, the amount of PAN started increasing again until 1400 - 1600hr. Consequently, the temperature trend with time

was similar to that of PAN produced by the mature males. The highest mean temperature was 35.33 ± 0.67 at 1400–1600 hr whereas the lowest temperature was 25.3 ± 0.88 ^{o}C at 0400–0600 hr. There was positive correlation (cor = 0.62160) between temperature and PAN collected in the two-hour interval in a span of 24 hr in the laboratory (F = 3.073, p = 0.01).

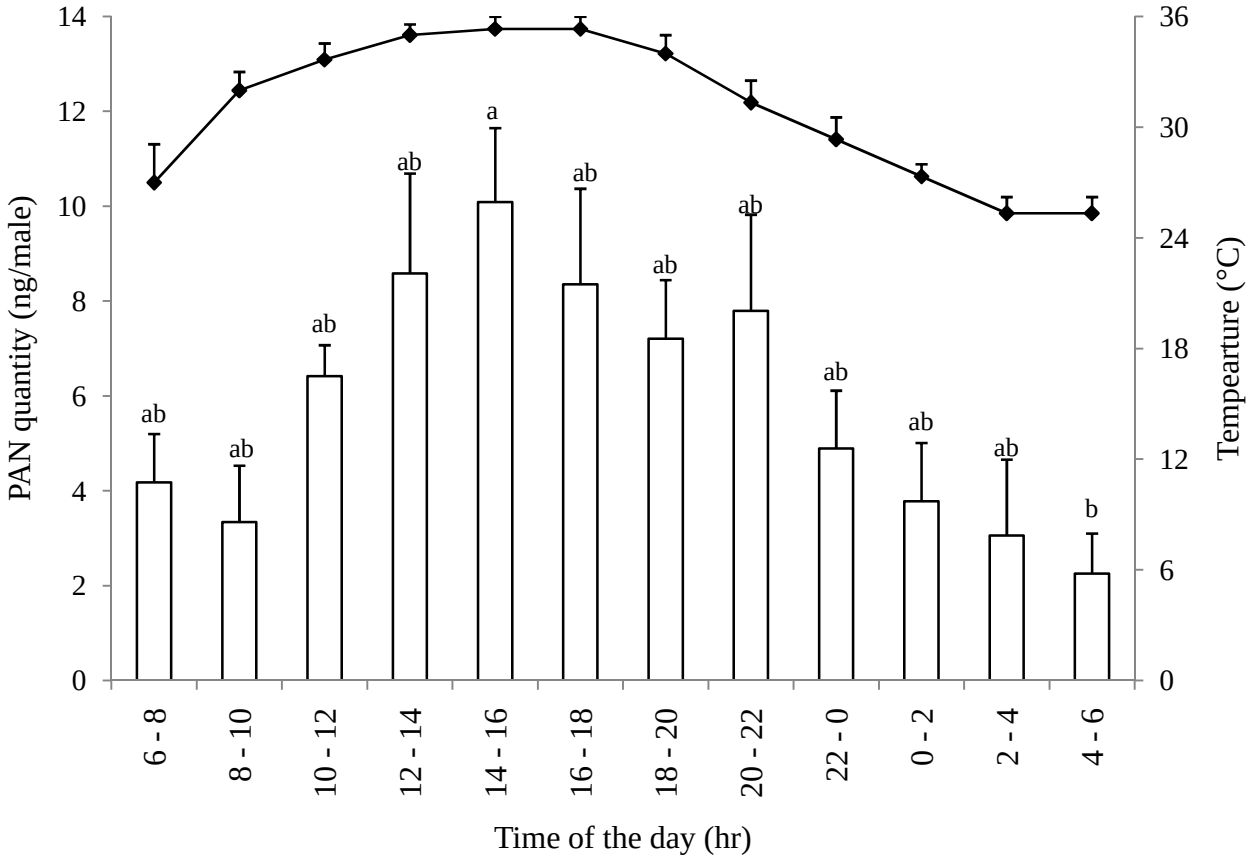

Fig 3.3 The amount of PAN released at two-hour interval by mature gregarious adult male desert locust, *Schistocerca gregaria* in a span of 24-hour.

Discussion

For successful reproduction, mate finding and recognition of both sexes of the desert locusts is imperative (Hassanali et al. 2005). In the bioassay, sexually mature females were significantly attracted to their corresponding sexually mature males. However, the sexually mature males were not significantly attracted to their corresponding female. Also, the sexually mature females preferred the control (empty cage) to the cage with immature males. The results suggest that the females were responding to both visual and chemical cues produced by the males and PAN may possibly play a role in mating behavior. This implies that the sexual attraction of the desert locusts may be under the control of both visual and chemical cues. Recent studies have detected PAN in the body parts of gregarious female adults (Amwayi et al. 2012), an indication that the males are able to respond to the trace amounts of PAN being released by the females. The bioassay results contradicts previous studies that females are relatively passive and do not move towards the male during courtship (Strong and Amerasinghe 1977). There is a possibility that the males were not significantly attracted to the females because they needed more time to be stimulated. In a previous study, gregarious reared males attempted to pair with solitarious males but not with their gregarious male counterparts nor with gregarious male - female pairs (Seidelmann and Ferenz 2002). Both the gregarious females and solitary reared males do not produce PAN in its headspace volatiles, even though females exhibited presence of low amounts of PAN in their body extracts (Amwayi et al. 2012).

Another interesting feature, mature males and females of different ages (3 weeks and 2 weeks, respectively) did not show any significant attraction. Based on PAN production, males produced the highest amount of PAN in tissue extracts at 3-weeks and females at 2-weeks (Amwayi et al.

2012). Also, PAN is released on attaining sexual maturity at around 12-14 days after fledgling (Torto et al., 1994; Seidelmann et al., 2000). Therefore, mate finding in the desert locust maybe attributed to amount of PAN but also synchronization is vital.

Previous studies have revealed that only gregarious sexually mature male desert locust produce considerable amounts of PAN compared to their female counterparts, immature adults and solitarious adults (Torto et al. 1994; Seidelmann et al. 2000). The present study reveals that these gregarious sexually mature males release significantly more PAN during photophase (day) than scotophase period (night). These results suggest that more PAN is produced when the insects are more active during the day than at night. Symmons and Cressman (2001) showed that the gregarious locusts fly frequently during the day when the temperature is high for sustained flight. The current study also relates the amount of PAN released with temperature. This indicates that the high temperature plays a role in the production of PAN based on the high titer levels obtained. There may be a relationship in PAN production and activity. However, during the 2-hr volatile collection time period, there was significant difference observed at 14:00–16:00 hr and 04:00–06:00 hr which is consistent with the photophase and scotophase data.

Pheromone communication has been demonstrated in the mating behavior of many orthopteroids groups. Otte (1970) and Whitman (1990) speculated that the ancestral stock of orthopteroids may have relied upon chemical cues for mate recognition. Mate recognition of conspecific mates of gregarious phase of desert locust may be mediated by short range or contact signals due to high density population (El Bashir et al. 1993; Hassanali et al. 2005). In addition to the release of pheromone, the sexually mature gregarious male desert locusts have a bright yellow colour

compared to their female counterparts that could assist in their mutual recognition (Ould Ely et al. 2006). Pentane body washings of gregarious-reared adult mature males of the desert locusts were investigate (Francke and Schmidt 1994). Two interesting compounds with 3-methyl-2-one substructure were located in the GC-MS; one was identified as 3, 7-dimethyl-heptacosan-2-one which bears a remarkable similarity to the sex pheromone of the German cockroach, *Blatella germanica* (Francke and Schmidt 1994; Hassanali et al. 2005).

In conclusion, the females were attracted to the males during mating bioassay and the attraction appears to be initiated by volatiles and visual cues produced by males. However, studies have shown no variation in response by crowded solitary-reared adults to their opposite counterparts in the presence or absence of the visual cues (Ould Ely et al. 2006). This study reveal that there was that no evident attraction by females to immature males (as the odour source). Mostly the mature females preferred their corresponding males of the same age only.

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CHAPTER FOUR

Molecular basis mediating aggregation behavior and pheromone release in the desert locust (*Schistocerca gregaria*; Orthoptera)

Abstract

Phenotypic expression and chemical communication are critical traits that distinguish solitarious from gregarious phases in the desert locust, *Schistocerca gregaria*. Aggregation pheromones such as phenylacetonitrile are of particular interest in desert locust chemical communication because they are only associated with the gregarious phase. The molecular events associated with phase shift, and more importantly, aggregation behaviour and pheromone release in the desert locust, however, are not well understood. Knowledge of these molecular mechanisms may be useful for developing future management strategies for this locust species including manipulation of its chemosensory systems to create mutant locusts which are incapable of swarming. Forward and reverse subtracted cDNA libraries were created to represent the upregulated genes that are associated with phase shift from gregarious to solitarious and *vice versa* of the desert locust. This was achieved using suppression subtractive hybridization (SSH) technique. Results of blastx searches using components of the subtracted cDNA library identified potential genes that may mediate gregarious traits in the desert locust.

Keywords: *Schistocerca gregaria*, PAN; biosynthesis, subtracted cDNA, gregarious traits

Introduction

The desert locust, *Schistocerca gregaria*, is predominantly known as a detrimental swarm-forming insect, which can inflict devastating damage to the agricultural production in large areas of the world (FAO website: http://www.fao.org/ag/locusts/). Interestingly, the same species can also occur in a more harmless solitarious form, which tends to avoid the company of other locusts. The two extreme forms or phases of the desert locust are referred to as phase polyphenism, showing different phenotypes having the same genotype. Other than the distinct behavioural disparity, the two phases also differ in colour, morphology, biochemistry and physiology (Uvarov 1966; Pener and Yerushalmi 1998; Simpson et al. 1999).

Chemical communication has been implicated to play an important role in the phase dynamics of the desert locust. Gregarious phase desert locusts, unlike their solitarious counterparts, produce pheromones which regulate their behavior including aggregation, courtship, maturation and oviposition and these pheromones have been fully characterized (Hassanali et al. 2005; Ferenz and Seidelmann 2003). The aggregation behavior in adults is regulated by the presence of benzene derivatives, dominated by phenylacetonitrile (PAN) in the volatiles released by mature-males (Torto et al. 1994; Seidelmann et al. 2000), while in nymphs this behavior is regulated by short-chain saturated aldehydes and their fatty acid derivatives and feacal phenols (Hassanali and Torto 1999; Obeng-Ofori et al. 1994). Male-produced pheromone (principally PAN) not only mediates aggregation behavior of gregarious adults, but also physiological traits, such as synchronous maturation and releaser and primer effects in the nymphal stages. Thus expression of genes associated with phase shift do not only ‘control release of pheromones’, but a whole series of events, starting (most probably) with release of neurotransmitters, which may be

responsible for switching on traits typical of the gregarious phase, including biosynthesis and emission of pheromones that mediate key behavioral and physiological traits of gregarious locusts, other longer-term changes that takes include morphometric and colour changes

The biosynthesis pathway of PAN in locusts is not well understood. However, two aggregation pheromonal components, guaiacol and phenol, are not produced by the locusts themselves but by gut bacteria (Dillon et al. 2000; Dillon and Charnley 2002). Recently, PAN and benzaldehyde were revealed to be biosynthetic products of phenylalanine produced in the epidermal cells of wings and legs of the desert locust (Seidelmann et al. 2003). In a previous chapter, the abundance of PAN in wings and legs of the gregarious adult males was confirmed and also shown to be present in other tissues not previously reported. Additionally, PAN was present in females but the amounts in males were significantly higher than females (Amwayi et al 2012).

Knowledge on the phase differences has increased dramatically in recent years, but the molecular mechanisms that switch between the two extreme phenotypes have yet to be fully established (Kang et al. 2004; Ma et al. 2011; Badisco et al. 2011). Understanding the molecular mechanisms regulating phase shift is important for future manipulation of the chemosensory systems to create mutant locusts. In a previous study, the only mutant defective for expression of an odorant binding protein (OBP) was the *Drosophila* mutant, LUSH *obp76a* (Kim et al. 1998). Drosophila OBO LUSH is important for the activity of the aggregation pheromone 11-*cis* vaccenyl acetate (VA). Thus, mutants lacking LUSH are insensitive to VA, both at the level of olfactory neuron activation and at the level of aggregation behavior normally elicited by this pheromone (Xu et al. 2005).

Recent studies have identified and elucidated the gene expression patterns associated with phase shift. Kang et al. (2004) revealed gene expression patterns in migratory locust in solitary-reared and crowded-reared fifth-instar *Locusta migratoria* nymphs where whole carcasses and samples of head, hind legs and gut were used to derive cDNA libraries from which EST library was produced. More recently, EST database derived from *Schistocerca gregaria* central nervous system (CNS) to design oligonucleotide microarrays were compared with the genes expressed in the CNS of gregarious and solitarious adult desert locust (Badisco et al. 2011; Ott et al. 2012). This led to the identification of 214 differentially expressed genes, of which 40% have been annotated to date, including genes encoding proteins that are associated with CNS development and modeling, sensory perception, stress response and resistance, and fundamental cellular processes.

The present study was however, based on previous studies where wings and legs of the mature gregarious desert locust males are the predominant releasing sites of PAN. Moreover, a site-specific mechanotactile stimulus was shown to be associated with outer face of hind femurs (Hägele and Simpson 2000; Roessingh et al. 1998; Simpson et al. 2001). Therefore, the current study tested the association of phase shift with differentially expressed genes on the legs of the desert locust, and possibility of up and down regulation in the phases of the desert locust. A forward and reverse subtracted cDNA library was constructed from the legs of mature gregarious male desert locust using suppression subtractive hybridization (SSH). The potential genes identified may have a releaser effect mediating gregarious traits in the desert locust.

Materials and methods

Rearing of the insects

Solitary reared locusts, *Schistocerca gregaria* from ICIPE colony were derived from a laboratory colony obtained from Dr. Steve Rodgers, Department of Zoology, Cambridge University, UK in 2009. Egg pods were incubated at 30^{o}C in an oven and on emergence of first instar nymphs of the insect were kept individually in standard aluminium cages (10 x 10 x 24cm) in visual olfaction and tactile isolation from other insects. Conditions of the insectary rearing room (1.5 x 4.5cm) were 27-32^{o}C, relative humidity < 50% and a light dark cycle of 12:12 LD maintanied using an electric timer connected to the source of power (Ochieng-Odero et al. 1994). Gregarious reared locusts, *Schistocerca gregaria* were obtained by crowding solitary locusts as described by Deng et al. (1996) where one-day-old first-instar nymphs (100) of the same stock were crowded in standard cages (50 x 50 x 50cm). Volatiles were then collected to compare phenylacetonitrile (PAN) levels between the gregarious locust crowded from solitary culture and normal gregarious locusts. All the insects were fed on fresh wheat seedlings and bran daily

Suppression subtrative hybridization (SSH)

Total RNA extraction

RNA was extracted from the legs of 3-week-old individuals of both phases of the desert locust, the solitary-reared and gregarious-reared mature locusts using TRIZOL® LS reagent (Invitrogen). In each phase, 10 individuals were used and eventually the RNA was pooled together. The legs were chopped into small pieces and placed in clean 1.5ml Eppendorf tubes. The tubes were inserted into a container with liquid Nitrogen and pulled out with a forced and crushed immediately with a pestle until a powder was formed. Phosphate buffered saline (PBS)

(750µl) was then added into the tube, vortexed for 1-2 min until the tissue was homogenized. The tissue homogenate was then centrifuged at 12,000 rpm at 4 ^{o}C for 5 min, and the supernatant transferred to a clean eppendorf tube. The supernatant (250µl) was transferred into a clean tube and 750µl of TRIZOL® LS reagent (Invitrogen) was added. The mixture was left at room temperature (RT) for 5-10min, followed by addition of 200µl chloroform to the sample. The sample was then vortexed and further incubated at RT for 5-10 min then spun at 12,000 rpm at 4^{o}C for 10 min. From the two layers formed, the top clear aqueous solution was removed carefully avoiding the lower layer and placed in a clean microcentrifuge tubes. Glycogen (1µl) and 500µl of ice cold Isopropanol were added to the sample to precipitate the extracted RNA. This was vortexed and incubated at RT for 5-10 min, then centifuged at 12,000 rpm for 10 min at 4^{o}C. A white pellet was formed at the bottom of the tube, the supernatant was discarded and 500µl of 75% ethanol added to wash the pellet. The tube was gently inverted to wash the inside of the tube. The tube was then spun for 2 minutes at 12,000 rpm at RT. The supernatant was discarded again and the tube further spun for 30 sec to remove excess liquid. 20µl of nuclease free water was added to the tube containing the pellet and left to stand for 5 min to resuspend the pellet.

RNA quality and quantity was checked by agarose gel electrophoresis and spectophotometry, respectively, before Poly (A) purification. Since TRIZOL® LS reagent (Invitrogen) extraction method only precipitates the RNA once, a second alcohol precipitation was also done on the RNA to remove residual salts. After repelleting the RNA and washing with 75% ethanol, the RNA (approx 2-400µg) was resuspended in 250µl Nuclease free water and then mixed thoroughly by vortexing to completely resuspend the pellet. An equal volume of 2X binding

solution (250µl) was also added to the RNA solution and mixed thoroughly. This was followed by binding the RNA to Oligo (dT) cellulose to isolate Poly (A) RNA using the MicroPoly(A) Purist Kit (Ambion Inc, Austin, TX).

Poly (A) RNA isolation from total RNA

Each RNA sample was added to 1 tube oligo (dT) cellulose then mixed by inversion to resuspend the resin, the presence of clumps were broken by pipetting up and down. The mixture was then heated at 65-75^{o}C for 5 min to denature secondary structures and maximize hybridization between the poly (A) sequences found in most mRNAs and poly (T) sequences on the oligo (dT) cellulose. The mixture was then incubated for 30-60 min at RT with gentle agitation to enable poly (A) binding to occur. Rocking or agitation was for maximum efficiency of poly(A) RNA binding to the oligo (dT) cellulose. The mixture was centrifuged at 4,000 g for 3 min to pellet the oligo (dT) cellulose. The oligo (dT) cellulose was washed twice with wash buffer solutions to remove non specific bound material and ribosomal RNA. After the two washes, the poly (A) RNA was recovered from the oligo (dT) cellulose by adding two 100 µl preheated The RNA storage solution, vortexing and spinning at ~ 5,000 g for 2 min to elute poly (A) RNA in a clean 1.5 ml Eppendorf tube. THE RNA storage solution strips the poly(A) from the oligo (dT) cellulose. To precipitate the eluted poly (A) RNA, 20 µl of 5M ammonium acetate, 1 µl glycogen and 550 µl absolute ethanol were added and the mixture left at -20°C overnight. RNA was recovered by centrifugation at 12,000 g for 30 minutes at 4°C and pellet re-suspended in 5 µl RNA storage solution. The quality of the poly (A) RNA was analyzed on a 0.3% agarose/ethidium bromide (0.1 µg/ml) gel run at 60 V for 2 h and concentration, were

determined by spectrophotometer. The poly (A) RNA obtained here was used for construction of a subtracted cDNA library using Suppression Subtractive Hybridization (SSH).

Construction of a subtracted cDNA library

SSH was performed using the PCR-Select cDNA subtraction kit (Clontech, Mountain view, CA, USA). cDNA was generated from poly(A) RNA obtained from both solitary males and gregarious males by reverse transcription. In the forward subtraction reaction, cDNA derived from solitary males was the 'driver' while cDNAs derived from the gregarious males was the 'tester'. In the reverse subtraction reaction, cDNA from solitary males was used as 'tester' to investigate up- and down regulation of mRNA molecules. Both cDNA samples were digested with the restriction endonuclease *Rsa* I (1.5 hr at 37°C) to generate shorter, blunt-ended ds cDNA fragments. To select for transcripts more highly expressed in the tester population, two different polymerase chain reaction (PCR) adaptors were ligated to the two portions of tester pool (Tester 1-1 and Tester 1-2) for forward subtraction, and (Tester 2-1 and Tester 2-2) for reverse subtraction. The tester pools were hybridized with excess cDNA from the driver pool. Before hybridization, the tester and driver were denatured (1.5 min at 98°C). First hybridization was performed for 8 h at 68°C with excess driver concentration. Abundantly expressed cDNAs hybridized faster and became enriched as single-strand cDNAs. Both incubations (Tester 1 & driver and Tester 2 & driver) were merged, undenatured and denatured driver was added in excess to further enrich differentially expressed cDNA single strands (incubation overnight at 68°C) to undergo second hybridization (Fig 4.1). Hybridized double-strand cDNA molecules, which are characterized by different adaptors bound to the 3' and 5' end at each strand (which also represent the differentially expressed sequences), were amplified by two serial PCRs with

two different primer pairs annealing to the adaptors. The primary PCR (PCR1) consisted of initial denaturation step for 25s at 94°C followed by 27 cycles for 10s at 94°C, 30s at 66°C, and

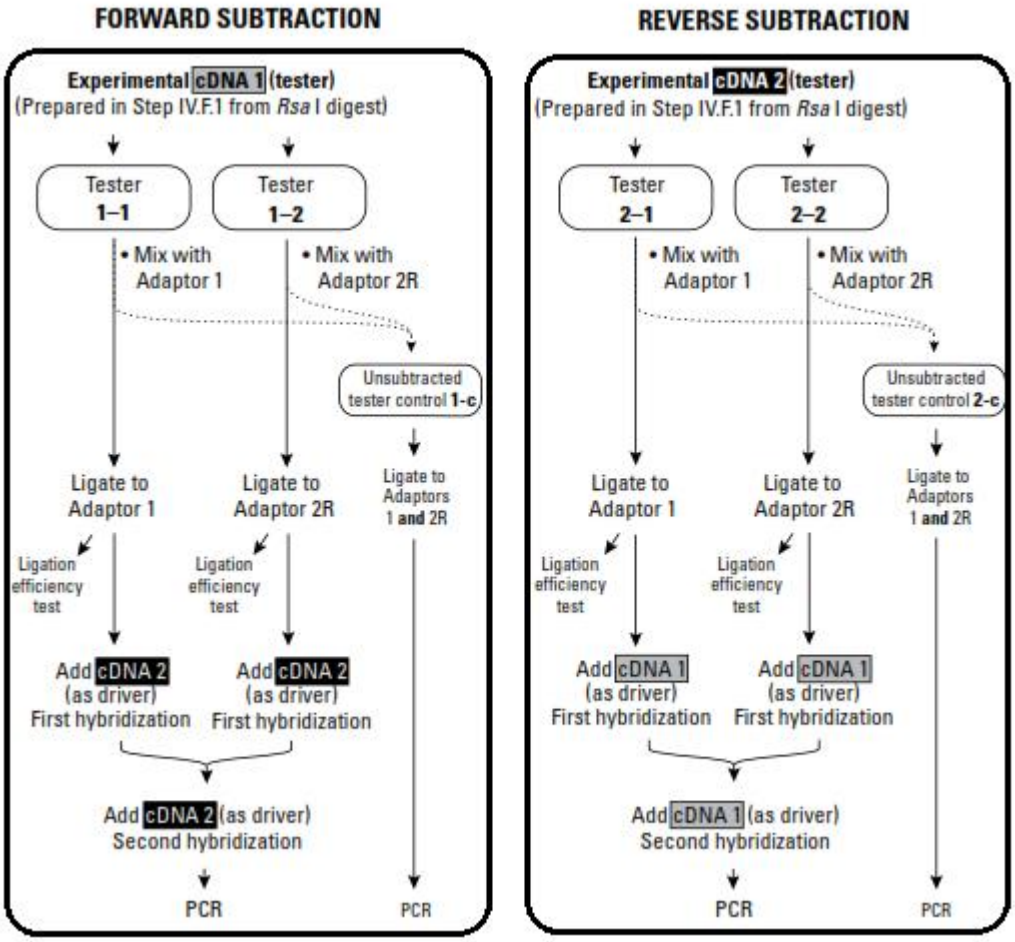


Fig 4.1 Preparing adaptor-ligated tester cDNA for hybridization and PCR. (Source: PCR select^{TM} cDNA subtraction kit user Manual).

1.5 min at 72°C using PCR primer 1 (5'- CTAATACGACTCACTATAGGGC- 3'). A secondary PCR amplification was performed using nested primers to further reduce any background PCR products and to enrich the differentially expressed sequences. PCR 2 consisted of 15 cycles of 10s at 94°C, 30s at 68°C and 1.5 min at 72°C using primers 1 and 2R. Nested primer 1 sequences were (5'-TCGAGCGGCCGCCCGGGCAGGT-3') and nested primer 2R sequences (5'-AGCGTGGTCGCGGCCGAGGT-3'). The subtracted cDNA was then subjected to evaluation of its subtraction efficiency by amplification of equal amounts of cDNA from the pre-subtractive tester and final subtracted products using *Ubi*, a housekeeping gene. The sequence of the *Ubi*-forward primer was 5'-GACTTTGAGGTGTGGCGTAG-3' and *Ubi*-reverse was 5'-GGATCACAAACACAGAACGA-3' and the expected band size was 76 bp.

Purification of the PCR products

The secondary PCR products were gel purified using the QuickClean® Gel Purification Kit (Genscript, NJ, USA). Three volumes of binding solution II were added to one volume of gel slice, and incubated at 50°C for 10 min with occassional vortexing until the gel was completely dissolved. One volume of Isopropanol was added and mixed. Approximately 600µl of the mixture was added into a QuickClean column and centrifuged for 30s at 12,000 rpm. The flow through was discarded, the column washed with 500µl of wash solution and centrifuged for 1 min at 12,000 rpm. 20µl of elution buffer was added to the colum, incubated for 1 min and the elute collected in clean 1.5 Eppendorf tubes by spinning at 12,000 rpm for 1 min. The eluted purified products were then analyzed by 1% agarose gel electrophoresis before use to confirm presence of eluted gel purified PCR product and the rest of the product was stored at -20°C untill use.

Cloning of subtracted PCR products

Ligation

The gel purified products were polyadenylated before cloning into a pGEM®-T Easy Vector (Promega, USA). This is because the 50X Advantage cDNA Polymerase Mix (BD Advantage PCR 2 kit) has proofreading activity resulting to blunt-ended products. The poly (A) tails were added into the blunt-ended PCR products by incubating 1µl 2mM dATP, 7µl of purified product, 1µl 10X PCR buffer with $MgCl_2$ and 1µl 5U/µl Taq Polymerase (both from Genscript) in a 10µl reaction mix at 70°C for 45 min. This poly (A) tailed product was then ligated into 50ng pGEM-T-vector (Promega, USA) in a 10µl reaction containing 5µl 2X Rapid ligation buffer, 1µl 50ng pGEM-T-vector, 3µl poly (A) tailed product and 1µl T4 DNA ligase (3Weiss units/µl) incubated overnight at 4°C.

Transformation

An aliquot (5µl) of ligated product was transformed in 50µl of *E. coli* JM109 cells which was incubated on ice for 20 min followed by heat shocking at 42°C for 50 sec and immediately transferred on ice for 2 min. SOC medium (900µl) was added to the transformed cells to supply required nutrients to the cells and incubated at 37°C for 2hr. Selection of transformants was accomplished by plating the cells on LB agar plates containing ampicillin (100µg/ml), Isopropylthio-D-galactoside (IPTG; 40µg/ml) and chromogenic dye 5-bromo-4-chloro-indolyl-D-galactoside (X-gal; 40µg/ml) followed by incubation at 37°C overnight for blue/white colonies assay.

Screening of transformed bacterial colonies using PCR

Only white or light blue colonies were selected from the plate. A colony PCR was done using M13 primers (F 5'- GTAAAACGACGGCCAG -3') and (R 5'- CAGGAAACAGCTATGAC -3'). Each reaction for each colony was comprised of 2.5µl of 10 x *Taq* buffer (Gene script), 0.5µl 25 mM $MgCl_2$, 0.5µl dNTPs, 0.5µl M13 forward primer (10 pmoles), 0.5µl M13 reverse primer (10 pmoles), 0.25µl Taq Polymerase (Gene Script) and 20.25 µl sterile water. Only white or light blue colony from the plate were selected and inserted into the PCR solution using a fine plastic pipette tip. The thermocycling conditions included initial denaturation at 5 min at 94 °C, followed by 30 cycles of 45s at 94 °C, 30s at 54 °C and 1 min at 72 °C. The expected colony PCR products with band size more than 250-300 bp indicated the presence of an insert in the vector, since amplification of the vector alone with M13 primers gives a 300bp. The positive clones from the colony PCR confirmation were then grown on 5 ml LB/Ampicillin broth and incubated at 37 °C overnight shaking at 150 rpm. Only milky broth overnight cultures were targeted for purification using a QuickClean 5M Miniprep Kit (GenScript, NJ, USA).

Plasmid purification

The overnight culture were transferred into a 1.5 ml microcentrifuge tube and centrifuged at 12,000 rpm for 8 min to pellet the plasmids cells. The supernatant was discarded and more of the overnight culture added onto the same tube and centrifuged again as indicated above. The resuspension buffer (100µl) with RNase A was added to the pellet to resuspend the cells by running the narrow end of the microcentrifuge tube quickly along the top of an empty microcentrifuge storage rack. This was repeated four times or more until the cells were resuspended. Lysis buffer (200µl) was then added to the mixture and this was mixed gently by inverting the tube 4-6 times. This was followed by the addition of 300µl of neutralizing buffer to

stop the lysis reaction. The tube was mixed gently by inverting the tube 4-6 times then spun at 12,000 rpm for 5 min. The supernatant was transferred into a clean column by decanting then the column was spun at 12,000 rpm for 30s. The flow through was discarded and 500µl of wash solution was added into the column, and centrifuged at 12,000 rpm for 30s. Once again the flow-through was discarded. Another centrifugation at 12,000 rpm for 60s was done to remove residual wash solution. The column was transferred into a clean 1.5 ml microcentrifuge tube, 50µl of elution buffer added to the center of the column membrane, the column let to stand at room temperature for one minute and finally spun at 12,000 rpm for 1 min. The purified plasmid collected in the fresh Eppendorf tube was run on a 1% agarose/ethidium bromide gel electrophoresis to determine quality and presence of the insert.

*Digestion with Eco*RI

To ensure that each positive excised plasmid contained one type of insert, the colonies were analyzed by restriction enzyme digestion. In this step, the plasmid product was digested with *Eco*RI restriction enzyme. *Eco*RI restriction enzyme has restriction sites on both ends of the p-GEM®-T vector. Thus, cutting the plasmid on the *Eco*RI restriction sites was able release the insert from the plasmid DNA. Each digestion reaction comprised of 15µl nuclease free water, 2µl of 10X FastDigest green buffer (Fermentas, USA), 1µl of FastDigest *Eco*RI restriction enzyme (Fermentas, USA) and 2µl of plasmid DNA. The reaction components were incubated at 37C°C for 1 hr and run on 1% agarose/ethidium bromide gel electrophoresis.

Sequencing and homology search

Services for sequencing of the products was outsourced from Commercial supplier (Macrogen, South Korea). The sequences were edited using the BioEdit software. Sequence homology searches were performed with default parameters against the non-redundant database for each unigene sequence using BLASTn (http://blast.ncbi.nlm.nih.gov/) and to the gene ontology database. The vector sequences and adaptor sequences ligated during the subtraction hybridization were eliminated manually by the VecScreen option in the NCBI. Sequence alignments of the inserts were further performed with the ClustalW software package and phylogenetic analysis carried out using Molecular Evolutionary Genetics Analysis (MEGA 5.05).

Results

RNA and Poly (A) RNA quality

The total RNA and Poly (A) RNA extracted from the legs of mature (3-week-old after fledgling) solitary- and gregarious-reared male are shown in Fig 4.2. The 28S and 18S ribosomal RNA (rRNA) bands were more sharp and discrete in the total RNA. On the other hand, the subsequent isolated Poly (A) RNA from total RNA was found to be intact and its quality on the agarose gel revealed that it appeared as a diffuse smear.

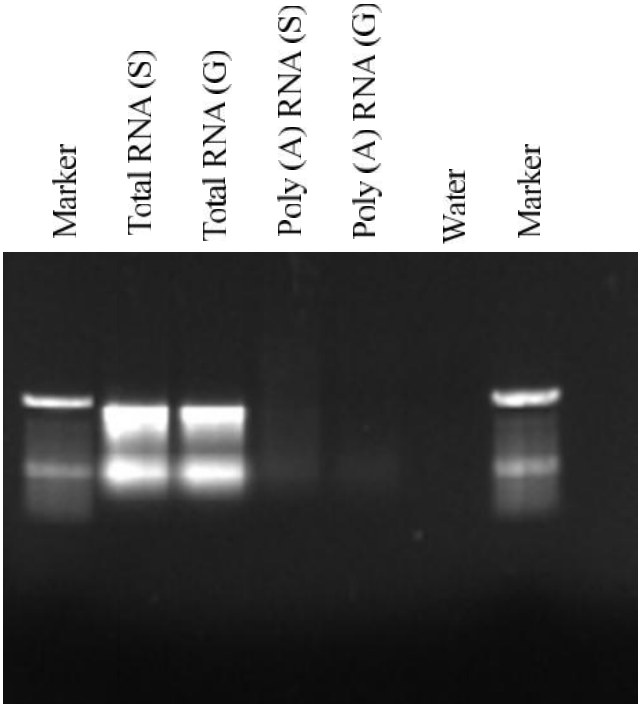
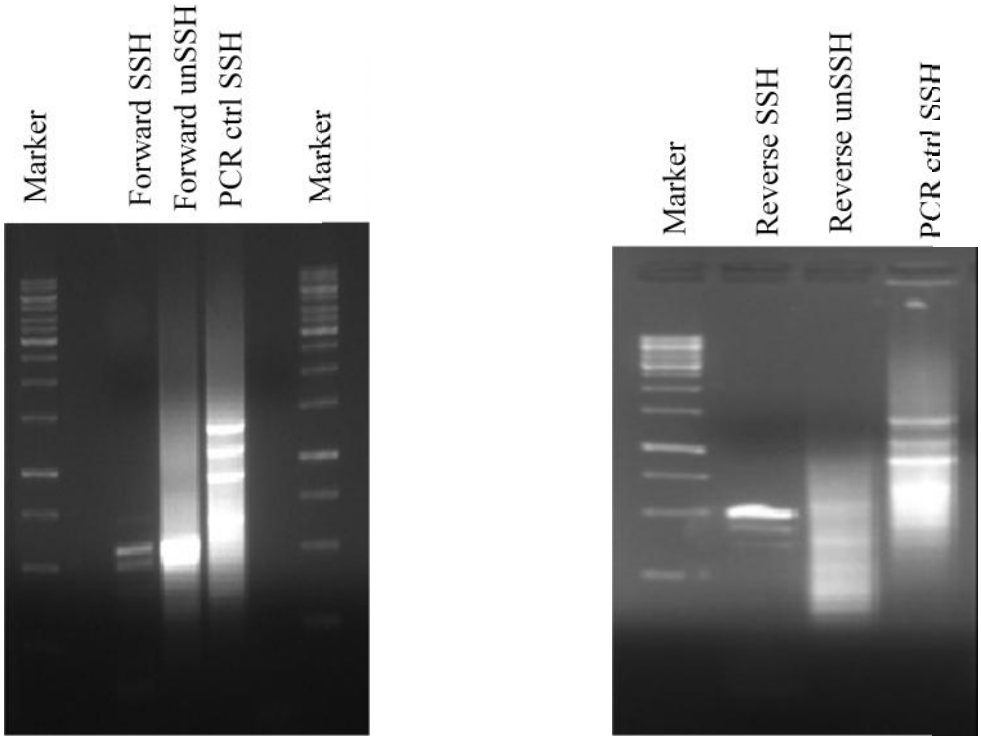


Fig 4.2 Total RNA and poly (A) RNA from solitary (S) and gregarious locusts (G). 5µl of denatured total RNA and poly RNA were resolved on a 0.3% agarose gel stained with ethidium bromide (M) 100 bp ladder (Fermentas, USA).

Construction of the subtracted cDNA Library

There was a clear difference in banding pattern between the subtracted cDNA library with the unsubtracted cDNA library on a 1% agarose gel. The banding patterns also show that the subtracted cDNA library was successfully constructed (Fig 4.3).



a) Forward subtracted cDNA library b) Reverse subtracted cDNA library

Fig 4.3 SSH PCR product analysis. PCR products amplified following a) forward subtraction (Fs) and reverse subtraction (Rs) subtraction ranging in size from 250-750bp. Marker is 1kb ladder (Fermentas, USA).

Verification of the Subtractive Library

As a first step to validating the libraries, the efficiency of the subtraction reactions was tested using the *Ubi*, a housekeeping gene for adult desert locusts. The unsubtracted cDNA samples PCR products for *Ubi* were clearly detected within 30 amplification cycles. By contrast, in the subtracted sample *Ubi* PCR products were detected until 35 cycles had been performed (Fig. 4.4) and, thus, we concluded that the subtraction reaction had been performed successfully.

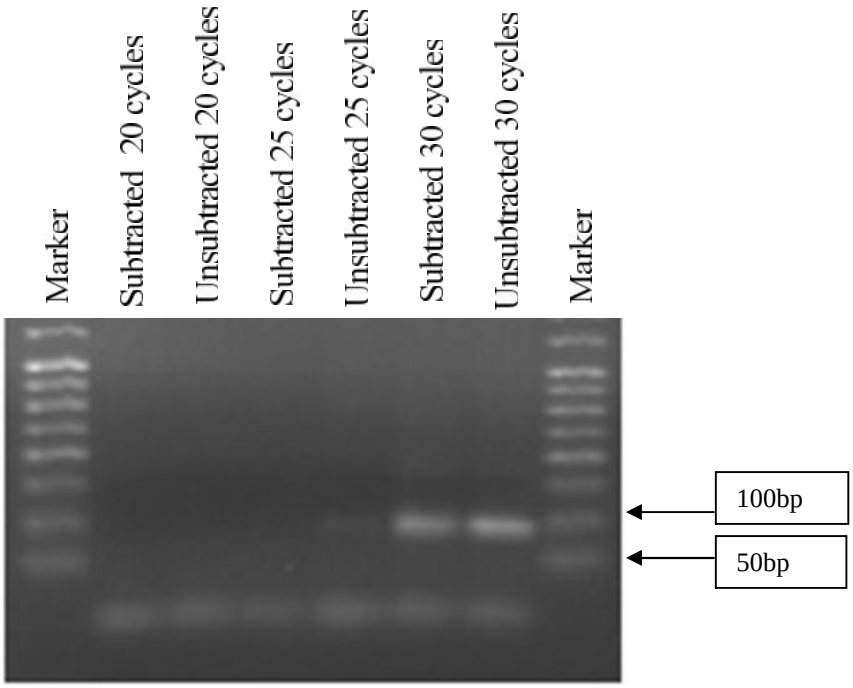
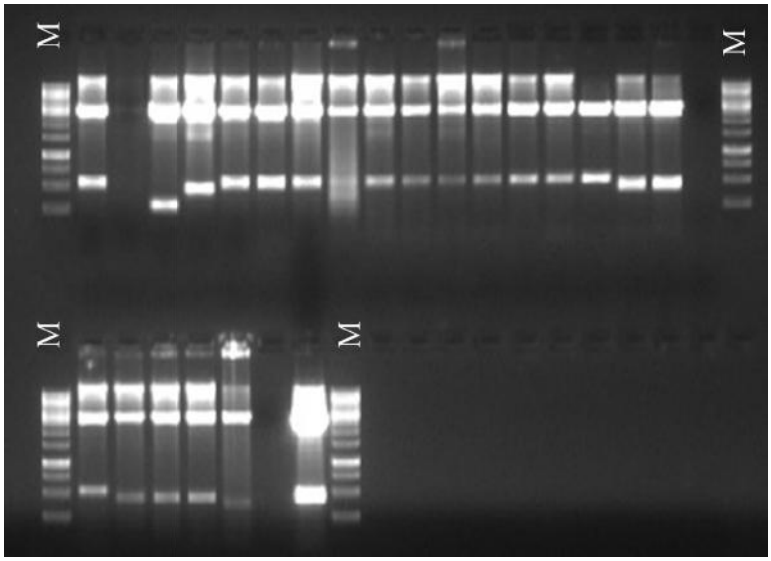


Fig 4.4 Confirmation of SSH efficiency analyzing the amount of the desert locust ubiquitin gene (Ub) present in both the subtracted cDNA and unsubtrated cDNA using increasing numbers of PCR cycles. The marker was 50 bp ladder marker.

*Restriction digestion using Eco*RI *enzyme*

Restriction enzymes have proved to be invaluable for the physical mapping of DNA. The approximate positive clones were 32 and 28 in forward subtracted (FS) and reverse subtracted (RS) clones respectively. After restriction digestion with *Eco*RI enzymes, the DNA inserts were released from the excised plasmid (Fig 4.5). From this study, all the positive clones had DNA insert of different sizes ranging from 250-550 bp. DNA inserts of same band size were assumed to be identical clones of the same sequence.

A.



B.

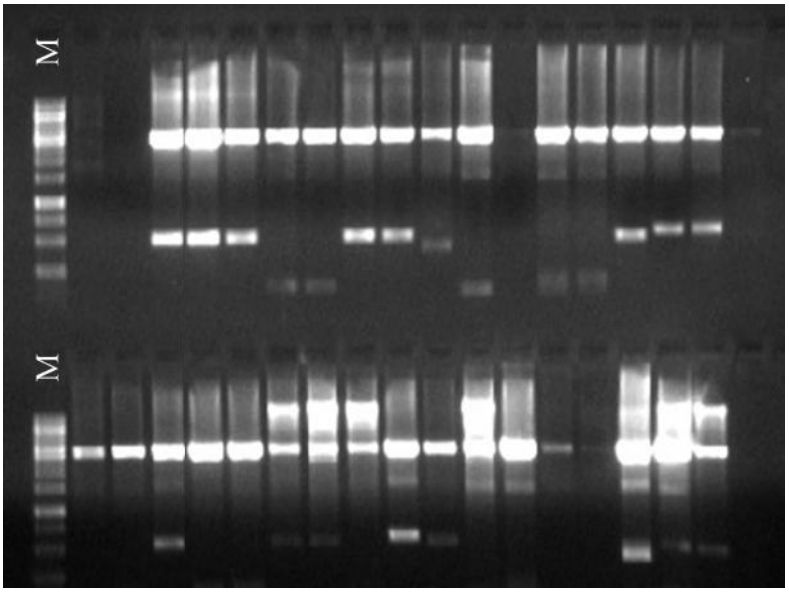


Fig 4.5 *Eco*RI restriction enzyme digestion analysis of positive clones isolated using subtractive cDNA screening (a) RS *Eco*RI digestion clones (b) FS *Eco*RI digested clones. M (Marker) used is1kb ladder (Fermentas, USA).

Sequence analysis of subtracted cDNA library

Sequence homology searches of the clones sequences from the SSH cDNA library were performed. The vector portion, Adaptor 1 and 2R and other uninformative sequences after the homology searches were excluded. A total of 14 out of 20 clones were selected for further analysis using bioinformatics tools after vector screening. Sequence homology searches were performed with default parameters against the non-redundant database for each sequence using blastn (NCBI) which gave 6 consensuses (55%) with no significant similarity to known genes from reported sequences in the database. The remaining predicted 14 consensuses (45%) showed significant homology to non-redundant GenBank database maintained at NCBI hitting *Schistocerca gregaria* genes (Table 4.1 and 4.2).

Table 4.1 Putative up-regulated genes hits from forward subtracted clones of the desert locust.

Subtracted clone	Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FS4	AY605952.1	Schistocerca gregaria voucher CUEM 1251-R5 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	830	830	96%	0.0	98%
	AY605951.1	Schistocerca gregaria voucher CUEM 1251-R6 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	830	830	96%	0.0	98%
	DQ309428.1	Schistocerca gregaria 16S ribosomal RNA gene, partial sequence; tRNA-Val gene, complete sequence; and 12S ribosomal RNA gene, partial sequence; mitochondrial	830	830	96%	0.0	98%
	GQ491031.1	Schistocerca gregaria gregaria mitochondrion, complete genome	824	824	96%	0.0	98%
	AY605945.1	Schistocerca cancellata voucher CUEM 1251-P1 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	802	802	96%	0.0	97%
FS10	AY605952.1	Schistocerca gregaria voucher CUEM 1251-R5 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	821	821	99%	0.0	98%
	AY605951.1	Schistocerca gregaria voucher CUEM 1251-R6 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	821	821	99%	0.0	98%
	DQ309428.1	Schistocerca gregaria 16S ribosomal RNA gene, partial sequence; tRNA-Val gene, complete sequence; and 12S ribosomal RNA gene, partial sequence; mitochondrial	821	821	99%	0.0	98%
	GQ491031.1	Schistocerca gregaria gregaria mitochondrion, complete genome	815	815	99%	0.0	98%
	AY605945.1	Schistocerca cancellata voucher CUEM 1251-P1 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	793	793	99%	0.0	97%
FS11	AY605952.1	Schistocerca gregaria voucher CUEM 1251-R5 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	649	649	100%	0.0	98%
	AY605951.1	Schistocerca gregaria voucher CUEM 1251-R6 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	649	649	100%	0.0	98%
	DQ309428.1	Schistocerca gregaria 16S ribosomal RNA gene, partial sequence; tRNA-Val gene, complete sequence; and 12S ribosomal RNA gene, partial sequence; mitochondrial	649	649	100%	0.0	98%
	GQ491031.1	Schistocerca gregaria gregaria mitochondrion, complete genome	643	643	100%	0.0	98%
FS18	AY605951.1	Schistocerca gregaria voucher CUEM 1251-R6 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	929	929	99%	0.0	98%
	DQ309428.1	Schistocerca gregaria 16S ribosomal RNA gene, partial sequence; tRNA-Val gene, complete sequence; and 12S ribosomal RNA gene, partial sequence; mitochondrial	929	929	99%	0.0	98%
	GQ491031.1	*Schistocerca gregaria gregaria* mitochondrion, complete genome	929	929	99%	0.0	98%
	AY605952.1	Schistocerca gregaria voucher CUEM 1251-R5 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	918	918	99%	0.0	98%
	AY605945.1	Schistocerca cancellata voucher CUEM 1251-P1 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	880	880	99%	0.0	96%
	AY605942.1	Schistocerca quisqueya voucher CUEM 1251-Q1 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	876	876	99%	0.0	96%

Table 4.2 Putative up-regulated genes hits from reverse subtracted clones of the desert locust.

Subtracted clone	Accession	Description	Max score	Total score	Query coverage	E value	Max ident
RS1	AY605952.1	Schistocerca gregaria voucher CUEM 1251-R5 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	830	830	96%	0.0	98%
	AY605951.1	Schistocerca gregaria voucher CUEM 1251-R6 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	830	830	96%	0.0	98%
	DQ309428.1	Schistocerca gregaria 16S ribosomal RNA gene, partial sequence; tRNA-Val gene, complete sequence; and 12S ribosomal RNA gene, partial sequence; mitochondrial	830	830	96%	0.0	98%
	GQ491031.1	Schistocerca gregaria gregaria mitochondrion, complete genome	824	824	96%	0.0	98%
	AY605945.1	Schistocerca cancellata voucher CUEM 1251-P1 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	802	802	96%	0.0	97%
RS3	AY605951.1	Schistocerca gregaria voucher CUEM 1251-R6 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	383	383	90%	3e-103	99%
	DQ309428.1	Schistocerca gregaria 16S ribosomal RNA gene, partial sequence; tRNA-Val gene, complete sequence; and 12S ribosomal RNA gene, partial sequence; mitochondrial	383	383	90%	3e-103	99%
	GQ491031.1	Schistocerca gregaria gregaria mitochondrion, complete genome	377	377	90%	1e-101	99%
	AY605952.1	Schistocerca gregaria voucher CUEM 1251-R5 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	377	377	90%	1e-101	99%
RS4	AY605951.1	Schistocerca gregaria voucher CUEM 1251-R6 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	704	704	99%	0.0	99%
	DQ309428.1	Schistocerca gregaria 16S ribosomal RNA gene, partial sequence; tRNA-Val gene, complete sequence; and 12S ribosomal RNA gene, partial sequence; mitochondrial	704	704	99%	0.0	99%
	GQ491031.1	Schistocerca gregaria gregaria mitochondrion, complete genome	699	699	99%	0.0	99%
	AY605952.1	Schistocerca gregaria voucher CUEM 1251-R5 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	693	693	99%	0.0	99%
	AY605945.1	Schistocerca cancellata voucher CUEM 1251-P1 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	660	660	99%	0.0	99%
RS6	AY605951.1	Schistocerca gregaria voucher CUEM 1251-R6 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	824	824	99%	0.0	98%
	DQ309428.1	Schistocerca gregaria 16S ribosomal RNA gene, partial sequence; tRNA-Val gene, complete sequence; and 12S ribosomal RNA gene, partial sequence; mitochondrial	824	824	99%	0.0	98%
	GQ491031.1	Schistocerca gregaria gregaria mitochondrion, complete genome	819	819	99%	0.0	98%
	AY605952.1	Schistocerca gregaria voucher CUEM 1251-R5 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	813	813	99%	0.0	98%
	AY605945.1	Schistocerca cancellata voucher CUEM 1251-P1 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	774	774	99%	0.0	96%
RS7	AY605951.1	Schistocerca gregaria voucher CUEM 1251-R6 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	843	843	99%	0.0	99%
	DQ309428.1	Schistocerca gregaria 16S ribosomal RNA gene, partial sequence; tRNA-Val gene, complete sequence; and 12S ribosomal RNA gene, partial sequence; mitochondrial	843	843	99%	0.0	99%
	GQ491031.1	Schistocerca gregaria gregaria mitochondrion, complete genome	837	837	99%	0.0	99%
	AY605952.1	Schistocerca gregaria voucher CUEM 1251-R5 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	832	832	99%	0.0	98%

	AY605945.1	Schistocerca cancellata voucher CUEM 1251-P1 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	793	793	99%	0.0	97%
RS10	AY605951.1	Schistocerca gregaria voucher CUEM 1251-R6 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	839	839	99%	0.0	99%
	DQ309428.1	Schistocerca gregaria 16S ribosomal RNA gene, partial sequence; tRNA-Val gene, complete sequence; and 12S ribosomal RNA gene, partial sequence; mitochondrial	839	839	99%	0.0	99%
	GQ491031.1	Schistocerca gregaria gregaria mitochondrion, complete genome	833	833	99%	0.0	98%
	AY605952.1	Schistocerca gregaria voucher CUEM 1251-R5 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	828	828	99%	0.0	98%
	AY605945.1	Schistocerca cancellata voucher CUEM 1251-P1 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	789	789	99%	0.0	97%
RS12	AY605951.1	Schistocerca gregaria voucher CUEM 1251-R6 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	843	843	99%	0.0	99%
	DQ309428.1	Schistocerca gregaria 16S ribosomal RNA gene, partial sequence; tRNA-Val gene, complete sequence; and 12S ribosomal RNA gene, partial sequence; mitochondrial	843	843	99%	0.0	99%
	GQ491031.1	Schistocerca gregaria gregaria mitochondrion, complete genome	837	837	99%	0.0	99%
	AY605952.1	Schistocerca gregaria voucher CUEM 1251-R5 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	832	832	99%	0.0	98%
	AY605945.1	Schistocerca cancellata voucher CUEM 1251-P1 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	793	793	99%	0.0	97%
RS15	AY605951.1	Schistocerca gregaria voucher CUEM 1251-R6 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	839	839	99%	0.0	99%
	DQ309428.1	Schistocerca gregaria 16S ribosomal RNA gene, partial sequence; tRNA-Val gene, complete sequence; and 12S ribosomal RNA gene, partial sequence; mitochondrial	839	839	99%	0.0	99%
	GQ491031.1	Schistocerca gregaria gregaria mitochondrion, complete genome	833	833	99%	0.0	98%
	AY605952.1	Schistocerca gregaria voucher CUEM 1251-R5 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	828	828	99%	0.0	98%
	AY605945.1	Schistocerca cancellata voucher CUEM 1251-P1 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	789	789	99%	0.0	97%
RS24	AY605951.1	Schistocerca gregaria voucher CUEM 1251-R6 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	845	845	99%	0.0	99%
	DQ309428.1	Schistocerca gregaria 16S ribosomal RNA gene, partial sequence; tRNA-Val gene, complete sequence; and 12S ribosomal RNA gene, partial sequence; mitochondrial	845	845	99%	0.0	99%
	GQ491031.1	Schistocerca gregaria gregaria mitochondrion, complete genome	839	839	99%	0.0	99%
	AY605952.1	Schistocerca gregaria voucher CUEM 1251-R5 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	833	833	99%	0.0	99%
	AY605945.1	Schistocerca cancellata voucher CUEM 1251-P1 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	795	795	99%	0.0	97%
	AY605942.1	Schistocerca quisqueya voucher CUEM 1251-Q1 NADH dehydrogenase subunit 1 gene, partial sequence; tRNA-Leu, 16S ribosomal RNA, and tRNA-Val genes, complete sequence; 12S ribosomal RNA gene, partial sequence; mitochondrial	791	791	99%	0.0	97%

The amplified fragments were sequenced bi-directionally and consensus sequences generated using BioEdit program. BLAST search was done using blastx against non redudant (NR).

Following the confirmation of the size of the clones by restriction digestion, a total of 20 subtracted clones were sequences bi-directionally, aligned to the non-redundant (nr) nucleotide database (GenBank) at the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov:80/BLAST/) using the BLAST algorithm for functionally annotation. All consensus sequences size range between 233 bp to 528 bp. The blast searches identified several potential gene homologs specific to *Schistocerca gregaria* with high E-values (Table 4.1 and 4.2). Most of the gene hits were related to those involved in energy metabolism. The phylogenetic relationship between the forward and reverse subtracted clones from the sexually adult desert locust is shown in Fig. 4.6.

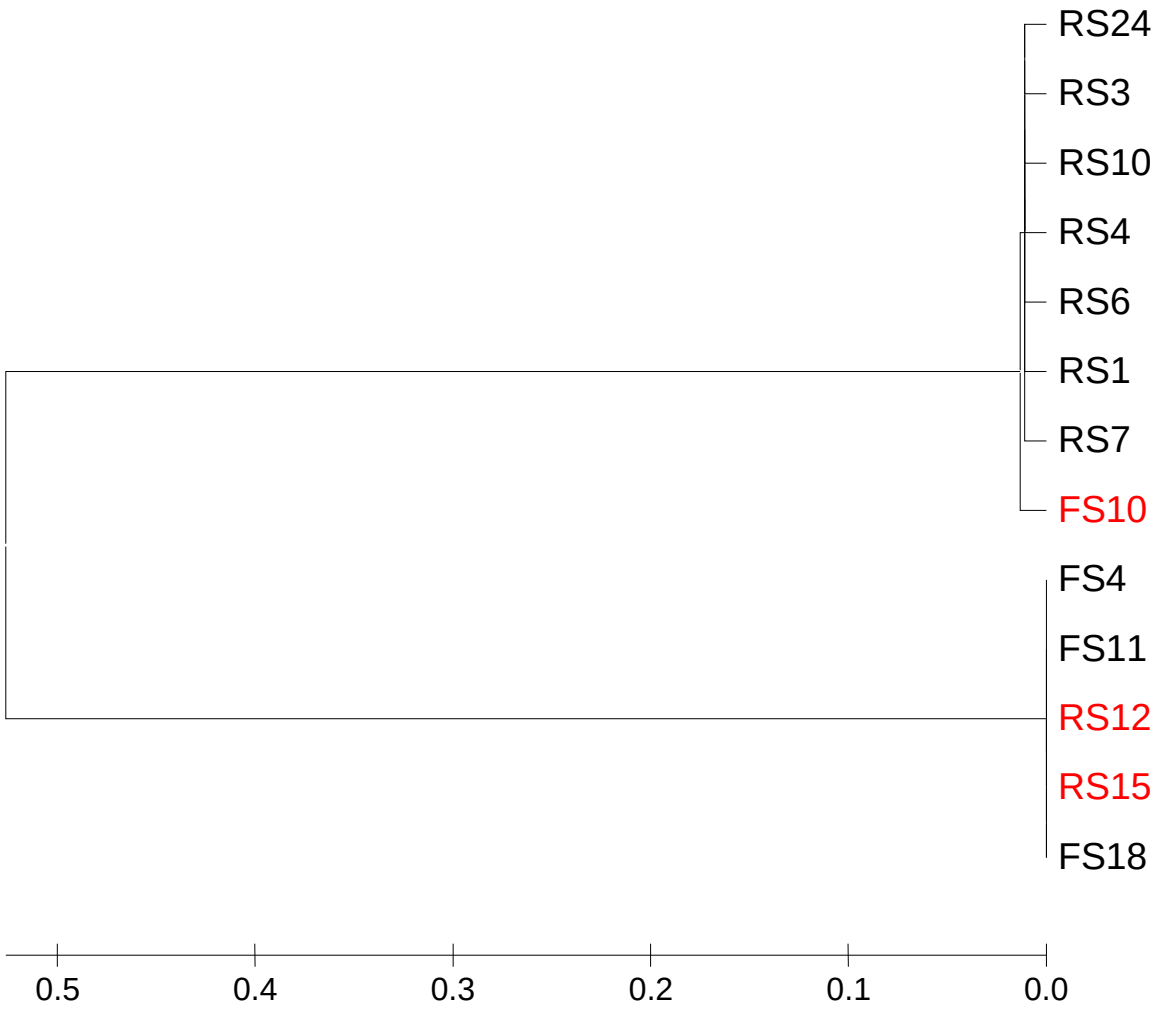


Fig 4.6 Phylogenetic comparison of the desert locust, *Schistocerca gregaria* forward subtracted (FS) and reverse subtracted (RS) cDNA clones. Clones abbreviated FS were obtained from the forward subtraction while RS from reverse subtraction. The tree was constructed from an alignment with MEGA 5.05

Discussion

The present study was aimed at identifying genes that are associated with phase transition between the gregarious and solitarious desert locust using suppression subtractive hybridization (SSH) technique. This approach can elucidate differentially expressed genes in one population but not in another (Hara et al. 1991; Diatchenko et al. 1996; Sneesby 2003; James et al. 2010). Our study demonstrated the successful construction of a subtracted cDNA library and predicted subtracted differentially expressed genes present in the gregarious adult males but not in solitarious adult males of the desert locust. Interestingly, profiles of these genes point towards possible roles in energy regulating metabolic processes with the key genes being NADH dehydrogenase subunit 1 and mitochondria related genes in comparison to genes regulating signal transduction reported by Badisco et al. (2011). Furthermore, the genes encoding NADH dehydrogenase subunit 1 showed an upregulation in gregarious locusts and downregulation in solitarious locusts.

Mitochondria organelles are involved in oxidative phosphorylation (OXPHOS), a competent metabolic pathway that uses energy released by the oxidation of nutrients to produce ATP (Ventura et al. 2002; Perier et al. 2005). OXPHOS complex I plays a central role in the regulation of ATP production, intermediary metabolism, and apoptosis (Ventura et al. 2002; Perier et al. 2005). The first enzyme of the respiratory electrons chain in the mitochondrion is NADH dehydrogenase. Therefore, there is a possibility that this enzyme may be involved in the desert locust dramatic alteration from the solitarious to gregarious phase of the desert locust. Earlier studies have reported the behavioural disparity between the two extreme phases of the desert locust (Deng et al. 1996; Hassanali et al. 2005). The gregarious phase is characterized by increased activity and locomotion, social cohesion, upright posture and gait, body coloration and

a broad dietary range whereas the solitarious phase occur at low population densities, actively avoid conspecifics are cryptic in appearance and behavior; walk with a slow, creeping gait and have restricted dietary preferences (Deng et al. 1996). It seems logical that the gregarious locusts require and use more ATP because of the more behaviourally active nature compared to the solitarious phase of the desert locust. In human muscle, both mitochondrial ATP production rate (MAPR) and mitochondrial enzyme activities were shown to increase with endurance training and to decrease with detraining (Wibom et al. 1992). The fact that gregarious phase of the desert locust are more active than their solitary counterparts could explain the need for more NADH dehydrogenase for increased energy metabolism requirements.

Previous studies showed that PAN was produced in the appendages of the desert locusts (Seidelmann et al. 2003). Appendages like the wings and legs are controlled by muscles and therefore may require the increased production of ATP that is necessary for muscle contraction. A glycolytic enzyme, phosphoglucose isomerise (pgi) has been related to flight physiology during dispersal in butterflies (Hanski et al 2004; Haag et al 2005). PAN is present in the air-borne volatiles of the gregarious reared adult males but absent in the solitarious reared adult males (Torto et al. 1994; Njagi et al. 1996; Seidelmann et al. 2000). It is likely that NADH dehydrogenase may be involved in the production of PAN. Based on the structure of PAN, two amino acids (phenylalanine and tyrosine), which have a benzene ring, are the likely precursor during PAN biosynthesis. However, previous studies have shown that the probable precursor for PAN is phenylalanine by incubating the tissues of wings and legs with radiolabelled ^{14}C-phenylalanine and ^{14}C-tyrosine (Ferenz and Seidelmann 2003; Seidelmann et al. 2003). ^{14}C-PAN was produced when ^{14}C-phenylalanine but not when ^{14}C-tyrosine was used as a substrate. This implies that tyrosine was not likely to be involved in the biosynthesis of PAN. However, the

pathway involved in the biosynthesis of PAN still remains unclear. None the less two probable reactions have been postulated. In the first, phenylalanine is decarboxylated to form phenethylamine (Tieman et al. 2006), which subsequently dehydrogenated to form PAN. The second probable pathway is that phenylalanine is converted to hydroxyphenylalanine, then to phenylacetoldoxime and then PAN is produced (Ferenz and Seidelmann 2003). Based on the results of our study, the involvement of NADH dehydrogenase gene indicates the first as the most probable pathway of PAN production.

Rogers et al. (2003) indicated that solitarious locusts showed rapid behavioural phase changes in response to tactile stimuli directed to their hind femora. Wings and legs of the adult desert locusts have been shown to be the main release sites for PAN (Seidelmann et al. 2003). We speculate that increased activity of the appendages of the locust leads to PAN release and aggregation. This suggests that a complex series of molecular mechanisms switches on and off during PAN release and aggregation behaviour. Our study represents a preliminary step towards gaining a better understanding of the molecular mechanisms at work in the two phases of the desert locusts. Studies on the molecular mechanisms of PAN release and the reversible change between the two phases of the desert locust is an important focal area of research on aggregation of the desert locust.

In conclusion, this study presents results of experiments using the subtracted cDNA library derived from the legs of the two extreme phases of *S. gregaria*. The successfully identified and obtained *S. gregaria* subtracted cDNA libraries constitute an important new source of information that will be instrumental in further unraveling the molecular principles of phase

polyphenism. Further studies on the functionality of the genes will provide valuable information on the molecular mechanisms of aggregation in the desert locust and pheromone release.

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CHAPTER FIVE

Analysis of expression levels of genes controlling aggregation behavior in the desert locust (*Schistocerca gregaria*)

Abstract

Knowledge on the extensive differences on the phenotypic expression of the two phases of the desert locust, *Schistocerca gregaria* has grown dramatically in recent years. However, the molecular mechanism involved in the gene expression has not yet been fully understood. In a previous chapter, differentially expressed genes regulating aggregation behavior in the desert locust were identified from a subtracted cDNA library of gregarious and solitarious adult reared male locusts. The genes identified in the subtracted cDNA library were pointing towards energy metabolism and mitochondrial related genes. A quantitative analysis of the candidate genes encoding NADH dehydrogenase were performed using real time PCR (RT-qPCR). Primers sequences of 2 genes were selected and designed. Forward subtracted cDNA clone (FSC) targeting genes in gregarious population but not in solitarious population and reverse subtracted cDNA clone (RSC) from reverse subtracted cDNA clones targeting genes in solitarious population but not in gregarious population. The results of this study showed that FSC was upregulated in the gregarious phenotypes of the desert locust and down regulated in the solitarious phenotypes of the desert locust. However, RSC which was targeting genes from the solitarious phenotype was downregulated in the solitarious and upregulated in the gregarious phenotypes of the desert locust. These results suggest that NADH dehydogenase subunit 1 gene may be contributing to phase-dependent regulation in the desert locust.

Keywords: *Schistocerca gregaria*, gene expression, RT-qPCR, up/down regulation

Introduction

Phase transition in the desert locust (*Schistocerca gregaria*) is an important characteristic attributed to population density and favourable abiotic conditions (Uvarov 1966; Pener and Simpson 2009). Efforts are being exploited to understand the transition of the two phases of the desert locust using approaches such as behavioral and molecular techniques. Behavioral assays have indicated that mechanical, visual and contact stimulation influence the phase change from solitarious to gregarious phases (Simpson et al. 1999; Hägele and Simpson 2000; Rogers et al. 2003). Other studies have also showed that sensory stimuli generated by the presence of other locusts can induce changes in the titers of several neurotransmitters (Rogers et al. 2004; Lester et al. 2005). Thus, the central nervous system (CNS) plays a crucial role in the gregarization effects. The molecular mechanisms in the CNS that lead to switch between the two extreme phenotypes of the desert locusts is yet to be fully established (Kang et al. 2004; Ma et al. 2011; Badisco et al. 2011). There is speculation that the complex phenotypes revealed in the desert locust are a result of multiple genetic traits controlled by environmental input (Ott et al. 2012).

Report by Seidelmann et al. (2003) indicated that phenylacetonitrile, a major component of the aggregation pheromone is released by the wings and legs. These body parts are involved in movement and locomotion of the desert locusts and require the utilization of energy. Based on these results, a subtracted cDNA library was constructed in Chapter 4 and the genes regulating metabolism were implicated as the potential genes in comparison to genes regulating signal transduction reported by Badisco et al. (2011). NADH dehydrogenase subunit I, one of the candidate genes is an enzyme involved in oxidative phosphorylation, a metabolic pathway that uses energy released by the oxidation of nutrients to produce ATP. There is a possibility that

gregarious locusts require and use more ATP than the solitarious counterparts. The gregarious phase is characterized by increased activity and locomotion, an upright posture and gait, coloration, a broad dietary range, and, most critically, attraction to other locusts whereas the solitarious locusts occur at low population densities and actively avoid conspecifics; cryptic in appearance and behavior; walk with a slow, creeping gait; and have restricted dietary preferences (Deng et al. 1996). Also, NADH dehydrogenase may be involved in the pathway of PAN biosynthesis. In this chapter, gene expression analysis was used to investigate quantitative difference in the levels of NADH dehydrogenase gene in cDNA obtained from the two phases of the desert locust.

Using RT-qPCR technique, reliable expression levels of a gene can be investigated. It has been broadly applied to microarray verification, pathogen quantification, cancer quantification, transgenic copy number determination and drug therapy studies (Klein 2002). Here, this technique was used to verify the expression of the differentially expressed genes from the subtracted clones. To learn more about the expression profiles of upregulated genes from the forward and reverse subtracted cDNA libraries, real time PCR quantitation studies were performed to determine the transcript levels by comparing the expression level of target gene versus the reference (Pfaffl 2001).

$$\text{Relative expression} = \frac{\text{Concentration of gene of interest}}{\text{Concentration of reference gene}}$$

Materials and methods

Rearing of the insects

Solitary-reared locusts, *Schistocerca gregaria* from ICIPE colony were derived form a culture obtained from the Dr Steve Rodgers of Cambridge University, Department of Zoology lab colony in UK in the year 2009. Egg pods were incubated at 30 ^{o}C in an oven and on emergence of first-instar nymphs the insects were kept individually in standard aluminium cages (10 x 10 x 24 cm) in visual olfaction and tactile isolation from other insects. In the insectary rearing room (1.5 cm x 4.5 cm) temperature was maintained at 27-32 ^{o}C, less than 50% relative humidity and a light dark cycle of 12:12 LD maintanied using an electric timer connected to the source of power (Ochieng-Odero et al. 1994). On the other hand, gregarious-reared locusts, *Schistocerca gregaria* were obtained by crowding solitary locusts as described by Deng et al. (1996) where one-day-old first-instar nymphs of the same stock were crowded in standard cages (50 x 50 x 50 cm). Volatiles were then collected to compare phenylacetonitrile levels between the gregarious locust crowded from solitary culture and normal gregarious locusts. Both scenario insects were fed on fresh wheat seedlings and bran daily.

Total RNA extraction

One insect per cage from both the solitarious and gregarious desert locust acted as a replicate. Five cages from each phases were used giving a total of 5 replicates per phase. The insects were killed by freezing them at -20 ^{o}C for 20 min. The legs were then chopped into small pieces and placed in clean 1.5 ml Eppendorf tubes. The tubes were inserted into a container with liquid Nitrogen and crushed immediately with a pestle until a powder was formed. 750µl phosphate buffered saline (PBS) was then added into the the tube, vortexed for 1-2 min until the tissue homogenized. The tissue homogenate was then centrifuged at 12,000 rpm at 4 ^{o}C for 5 min, the

supernatant transferred to a clean Eppendorf tube. 250µl of the supernatant was aliquoted to a clean tube and 750µl of TRIZOL® LS reagent (Invitrogen) was added. The mixture was left to stand at room temperature (RT) for 5-10 min, followed by addition of 200µl chloroform to the sample. The sample was then vortexed and further incubated at RT for 5-10 min then spun at 12,000 rpm at 4 ^{o}C for 10 min. From the two layers formed, the top clear aqueous solution was removed carefully avoiding the lower layer and placed in a clean microcentrifuge tubes. 1µl of glycogen and 500µl of ice cold Isopropanol were added to the sample to precipitate the extracted RNA. This was vortexed and incubated at RT for 5-10 min, then centifuged at 12, 000 rpm for 10 min at 4 ^{o}C. A white pellet was formed at the bottom of the tube, the supernatant was discarded and 500µl of 75% ethanol added to wash the pellet. The tube was gently inverted to wash the inside of the tube, and spun for 2 min at 12,000 rpm at RT. The supernatant was discarded again and the tube further spun for 30 s to remove excess liquid. 20µl of nuclease free water was added to the tube containing the pellet and left to stand for 5 min to resuspend the pellet.

RNA quality and quantity was checked by agarose gel electrophoresis and spectophotometry, respectively, before Poly (A) purification. Since TRIZOL® LS reagent (Invitrogen) extraction method precipitates the RNA once, a second alcohol precipitation was also done on the RNA to remove residual salts. After repelleting the RNA and washing with 75% ethanol, the RNA was resuspended in 20µl Nuclease free water and then mixed thoroughly by vortexing to completely resuspend the pellet. The RNA was stored at -20 ^{o}C for shorter storage or -80 ^{o}C for longer storage. The RNA integrity was assessed prior to cDNA synthesis. First, the RNA was denatured at 75 ^{o}C for 5 min in a thermocycler followed by ethidium bromide stained agarose gel

electrophoresis. The presence of both the 18S and 28S rRNA as sharp bands after electrophoresis of total eukaryotic RNA indicated that the RNA was intact. The 28S rRNA band should be approximately twice as intense as the 18S rRNA. Any smearing of rRNA bands is an indication of degraded mRNA. If this occurs, a new sample of total RNA should be prepared. Concurrently, the RNA quantity was determined using a spectrophotometer and the ratio at 280/260nm also gave the quality of the RNA. A ratio of 1.7-1.9 showed good quality DNA.

cDNA synthesis

cDNA was generated from the total RNA extracted from solitarious and gregarious desert locusts using RevertAid^{TM} First strand cDNA Synthesis Kit (Fermentas, USA). During the first strand synthesis, 2µl of RNA template (approx 1 µg), 1µl of oligo $(dT)_{18}$ primer and 9 µl of nuclease free water were added in a sterile nuclease Eppendorf tube kept on ice. The tube with the above contents was mixed gently, spun down briefly and incubated at 65 ^{o}C for 5 min. After this, the mixture was briefly spun and returned back on ice. Another mixture containing (4µl of 5 X reaction buffer, 1µl RiboLock RNase inhibitor and 2µl of 10mM dNTPs mix and 1µl RevertAid M-MuLV reverse transcriptase (200 U/µl)) was prepared and 10µl of this mixture was added to the previous tube kept on ice. The tube was mixed gently, spun down and incubated at 42 ^{o}C for 60 min followed by heating at 70 ^{o}C for 5 min to terminate the first strand synthesis. The second strand was synthesized by adding to the 20µl of the first strand mixture, 8µl of 10 X reaction buffer, 1µl RNAse H and water topped to 100µl. This was followed by gently vortexing and brief centrigation and incubation for 15 ^{o}C for 2 hr. 12.5 u of T4 DNA polymerase was added and the mixture further incubated at 15 ^{o}C for 5 min. The reaction was terminated by adding 5µl of 0.5 M EDTA pH 8.0. The cDNA synthesized was used for gene expression studies using real

time PCR with gene specific primers designed from the obtained subtracted clones sequences in Chapter 4.

Primer design

The primers were designed manually from the subtracted clones. All the forward subtracted and reverse subtracted clones were aligned together using ClustalW and the conserved regions were used to design the primers. The melting temperature (Tm) and any overlapping of the designed primers were checked using PCR primer Stats. Sequence information for the primers is provided in Table 5.1

Table 5.1 Sequences of the designed primers.

Primer	Forward primer	Reverse primer	Amplicon
FSC	5'-ATGATGTTTTGTTGGGGTGA-3	5'-CTTAGGATAGAAACCGACCT-3'	289bp
RSC	5'-CTTAGGATAGAAACCGACCT-3'	5'-ATGTTTTGTTGGGGTGACAT-3'	286bp

The forward subtracted cDNA (FSC) forward and reverse primers were designed for the forward subtracted cDNA library while reverse subtracted cDNA (RSC) forward and reverse primers from the reverse subtracted cDNA library. The primers were compared with homologous sequences in BLASTn (http://blast.ncbi.nlm.nih.gov/) to confirm if they were homologous to the previous found gene hits. The primers were then used in real time PCR gene quantification and expression.

Polymerase Chain Reaction

Prior to the real time PCR, optimization of the primers was done using the standard PCR to get the optimal amplification conditions. PCR reactions had a total volume of 25µl. The reaction mix comprised of: 1µl DNA template, 0.8µl of 2.5mM dNTPs, 2.5µl of 10X GenScript PCR buffer, 1 µl of 25mM $MgCl_2$ (Promega), 0.5µl of forward primer (10 pmoles) and 0.5µl reverse primer (10 pmoles), 17.6 µl sterile H_2O and 0.25 units *Taq* polymerase (GenScript). Temperature cycling was performed on a GeneAmp PCR system 9800 (Applied Biosystems) thermocycler. The PCR thermocycling was done using the following program: 2 min initial denaturation step at 94 ^oC, followed by 35 cycles of 15 s at 94 ^oC, 2 min at 60 ^oC, 2 min at 72 ^oC, a final extension step at 72 ^oC for 7 min and a holding step at 15 ^oC after these cycles. The 5µl of the PCR product was run on 1% agarose gel electrophoresis stained with ethidium bromide. The optimal conditions defined the optimal primer annealing temperature to be used in the real time PCR process.

Real Time PCR

For verification of the differential expression levels of 2 selected genes, semi-quantitative RT-PCR was carried out. Primers were designed using Primer3 software and synthesized by Inqaba Technologies, South Africa. All sequence information for the primers is provided in Table 5.1. RT-qPCR was conducted on a Rotor gene Q (Qiagen, USA). Ubiquitin was used as an internal control for the procedure. Five replicates from independently isolated RNA samples of each phase of the desert locust were used and each of the reactions was further performed in triplicates. The PCR reaction mixture was composed of 6.7 µl water, 2 µl of 5X HOT FIREPol® Evagreen HRM mix dye, 0.4 µl of forward primer, 0.4 µl reverse primer and 0.5 µl of template cDNA from either gregarious or solitarious desert locusts. The thermocycling reaction consisted

of an initial denaturation step for 15 min at 95 ^{o}C followed by 55 cycles for 20 s at 95 ^{o}C, 30 s at 60 ^{o}C, and 30 s at 72 ^{o}C. High resolution melting (HRM) and cycling curves were generated by the machine. The take off or cycle threshold (Ct) and amplification values for the reference (UbiQ) and target (FSC and RSC) were used for analysis of relative expression using REST2009 package (Pfaffl 2001; Pfaffl et al. 2002)

Results

PCR optimization

It was necessary to determine the optimum cycling parameters required for the PCR amplification of the primers designed. FSC and RSC primers representing clones of genes in the forward and reverse subtracted cDNA library, respectively. This was mainly used to save on the expensive reagents such as Evagreen and SYBR green dye required in real time PCR. Figure 5.1 illustrates the optimum PCR products with very intense bands at 35 cycles. The same PCR conditions were used in the real time PCR quantification process.

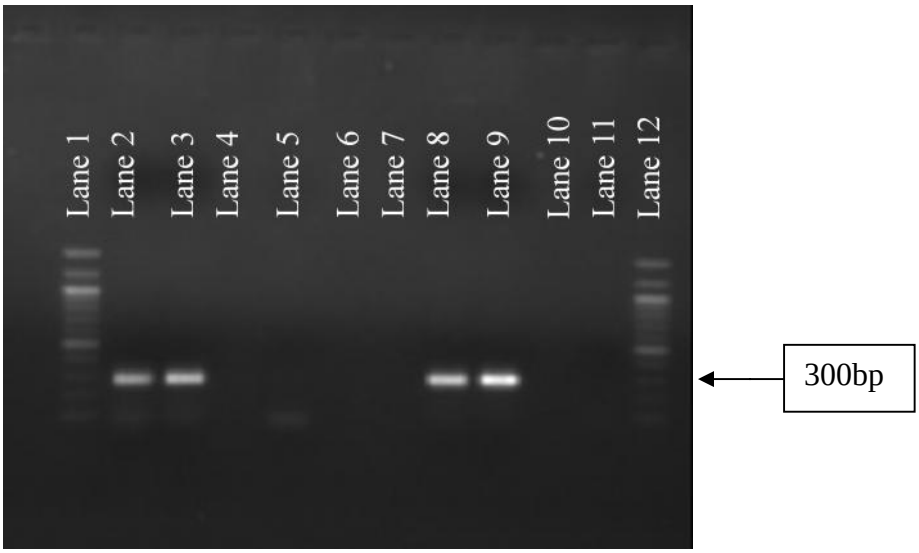

Figure 5.1 PCR amplicon of the FSC and RSC genes respectively. Lane 1 and Lane 12 represent 100bp ladder (Fermentas, USA), Lane 2 and Lane 3, gregarious and solitarious cDNA, respectively, amplified with FSC gene; Lane 4 was the FSC negative control; Lane 8 and Lane 9 gregarious and solitarious cDNA, respectively, amplified with RSC gene; L11 was RSC negative control.

Gene expression studies

The RT-qPCR for the FSC and RSC were normalized against a reference gene (Ubiquitin gene). The relative expression of Ubiquitin as the reference gene was constantly 1.00 whereas FSC and RSC had a relative expression of 2.82 ± 1.03 and 2.88 ± 1.12, respectively, when solitarious cDNA was the control group while the treatment group was the gregarious cDNA both showing an up regulation expression for gregarious phenotypic phase of the desert locust. When the control and treatment groups were reversed FSC and RSC gave a relative expression of 0.36±0.11 and 0.35±0.14, respectively, both showing a down regulation expression for the solitarious phase (Figure 5.2).

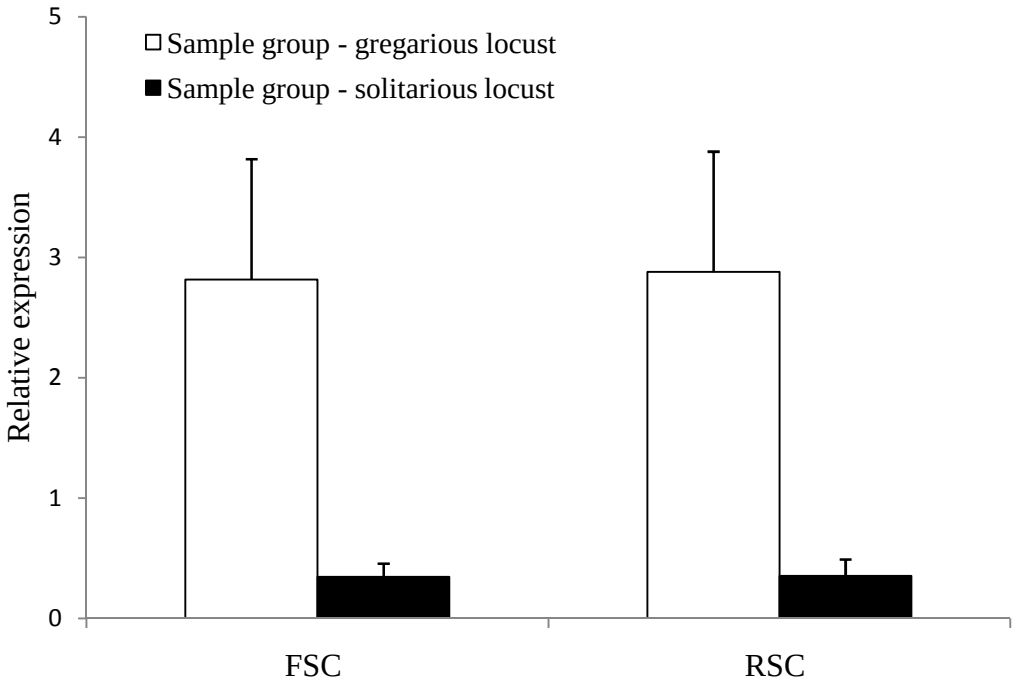

Figure 5.2 Relative expressions of FSC and RSC genes in gregarious cDNA and solitarious cDNA.

Discussion

In this study, gene expression levels in gregarious and solitary locusts using RT-qPCR showed quantitative differences. FSC representing genes present in gregarious but not in solitarious desert locust was up and down regulated in gregarious and solitarious locust, respectively. Surprisingly, RSC which was targeting genes in solitarious desert locust was down regulated in the solitarious while upregulated in gregarious phenotype of the desert locusts. These primers encode the NADH dehydrogenase 1 and mitochondrial related genes. Therefore, this study suggests that NADH dehydrogenase is upregulated in gregarious phase of the desert locust. This could be explained by the more behaviorally active nature of the gregarious phase while the

cryptic solitarious phase of the desert locust (Uvarov 1966; Deng et al. 1996; Hassanali et al. 2005). Also, a previous study indicates that solitarious locusts show rapid behavioural phase change in response to tactile stimulation directed to the hind femora (Rogers et al. 2003). Wings and legs of the adult desert locust have been shown to be the main release sites for PAN (Seidelmann et al. 2003). Therefore, there is possibility that if activity is applied to the legs and wings of solitarious locusts, aggregation behaviour and PAN release can be observed. This may imply that a complex series of molecular mechanisms switches on and off during PAN release and aggregation behaviour. This study represents a first step towards gaining a better understanding of the molecular mechanisms at work in the two phases of the desert locusts. More studies on the molecular mechanisms of PAN release and the reversible change between the two phases of the desert locust may play important part of research on aggregation of the desert locust.

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CHAPTER SIX

Concluding remarks

The desert locust (*Schistocerca gregaria*) aggregation and pheromone release dynamics and behaviour have been previously extensively studied (Torto et al. 1994; Seidelmann et al. 2000; Hassanali et al. 2005). Some of the key pheromones of the gregarious phase have been characterized. There is evidence of stage-specific aggregation pheromones that mediate grouping behaviour in nymphal and adult stages of the gregarious phase (Torto et al. 1996). Gregarious male desert locusts release a bouquet of volatiles when they become sexually mature, among them large amounts of PAN and some other aromatic compounds such as veratrole, anisole and benzaldehyde (Luber et al. 1993; Ferenz et al. 1994; Torto et al. 1994; Seidelmann et al. 2000). Recent studies reveal that the epidermal cells on the wings and legs of the desert locust were the releasing sites of PAN and that phenylalanine is the precursor for PAN biosynthesis (Seidelmann et al. 2003). However, the site of production and the biosynthetic pathway of PAN from the precursor still remain unclear. The basis of the present study was to further investigate the origin and biosynthesis of PAN.

Previous chapters in this thesis describe the composition of PAN in body parts of both sexes of the desert locust, sexual receptivity in relation to PAN composition, genetic analysis and quantitative gene profiling of aggregation and pheromone release in the desert locust. This chapter (a) summarizes the key findings for each of the preceding chapters by bringing together the chemical ecology, behavior and molecular basis of the aggregation behavior and pheromone release in *S. gregaria*, and (b) highlights areas for future research and the potential use of the knowledge acquired to develop new methods that can be used to control desert locust outbreaks.

PAN composition in various body parts

The chemical composition of PAN in both adult males and females of the two phases of the desert locust is described in Chapter 1. The quantitative difference in PAN amounts from various body parts of both males and females of both phases of the desert locust were determined using gas chromatography – mass spectrometry (GC-MS). Findings here showed that PAN was in nearly all body parts of both sexes of the adult gregarious phase in varying amounts. Previously, only gregarious sexually mature males produced considerable amounts of PAN from headspace volatile collection (Torto et al. 1994; Seidelmann et al. 2000). This is the first study that reports the presence of PAN in tissues of adult female desert locusts, although ~20-fold less than in male tissues. Production of PAN is consistent with other roles such as social cohesion and synchronous maturation etc.

Sexual receptivity in relation to PAN

The development of a mating disruption technique to control the frequent desert locust outbreaks is crucial. The latest outbreak in 2004 was the largest known since those of 1986 – 89. It affected the whole Sahel, from Senegal and Mauritania to the Red Sea, moved offshore from the Atlantic Ocean to the Canary and Cape Verde Islands and reached the Mediterranean Sea (http://www.fao.org). The role of PAN in mate finding in both sexes of the desert locusts was investigated. PAN released by sexually mature gregarious reared males at different time interval was determined and correlated with temperature. There was significant difference in PAN titres produced during photophase (day) and scotophase (night). In both photophase and scotophase, the amount of PAN released had a strong correlation with temperature. An attempt was made to understand the mate finding responses in a bioassay conducted in a flatbed wind tunnel.

Significant responses were observed in test insect (male or female) to odors of their conspecific insects of the same age. This may imply that there was synchronous effect (Mahamat et al. 1993; Mahamat et al. 2000). Both visual and olfactory cues appear to affect the responses in both sexes.

Differentially expressed genes

The existence of the two extreme phases of the desert locust is an example of phenotypic plasticity and the two different phenotypes are encoded by the same genome (Wilmore and Brown 1975; Uvarov 1977; Pener and Yerushalmi 1998). Increases in locust population density lead to the expression of phenotypically plastic trait that transform solitarious phase individuals into gregarious swarming phase locusts (Pener 1991; Pener and Yerushalmi 1998; Simpson et al. 2001). The suppression subtractive hybridization was employed as a method to evaluate gene profiles in the two phases of the desert locust. A successful subtracted library was obtained and genes differentially expressed in the gregarious phase but not in the solitarious desert locust and *vice versa* were identified. Potential candidate genes identified may be involved in the releaser functions of PAN and gregarious traits of the desert locust.

Gene expression studies

Reserve transcription combined with the polymerase chain reaction (RT-PCR) has proven to be a powerful method to quantify gene expression (Klein 2002). The genes identified in this study provide a strong starting point for further studies of the underlying molecular differences in solitarious and gregarious forms of the adult desert locust. In particular, the subtracted cDNA library represent differentially expressed genes in gregarious but not in solitary desert locust and

vice versa. There is therefore need for further investigation of the functions of these genes in the aggregation behavior for future development of locust control strategies. One approach that is viable is to exploit the techniques of RNA interference to knock down the genes triggering the aggregation behavior in the desert locusts.

Based on the results of tissue distribution of PAN in the body matrix of the desert locust by chemical analysis and with the aid of the designed primers that gave quantitative differences between solitarious and gregarious phases of the desert locust, the relative expression of the gene in different tissues of the desert locusts can be explored using real time PCR. The levels of the differentially expressed gene may confirm the high amounts of PAN in wings and legs (Seidelmann et al. 2003; Amwayi et al. 2012) and also the ubiquitous presence of the pheromone in nearly all body parts of the desert locust. Also, the quantity of the gene(s) in the body matrix that is up or down regulated during aggregation process can be studied.

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