

**ON-HOST BEHAVIOURAL INTERACTIONS BETWEEN ADULT *Rhipicephalus appendiculatus* AND CHARACTERISATION OF THE MEDIATING PHEROMONES**

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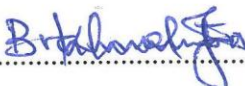
**NOVEMBER, 2017**

**DECLARATION**

I hereby declare that this is my original work and has not been presented for the award of a degree or any other award in any other University.

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## **DEDICATION**

This work is dedicated to my adorable daughter Breanna Faith Nasimiyu, my lovely wife Gordilla Nafula, my parents Daniel Khaemba and Felistus Nasimiyu, my siblings Cornelius, Ferdinand, Aggrey and Teresa.

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**ABBREVIATIONS AND ACRONYMS**

AAAP	Attraction aggregation attachment pheromone
ANOVA	Analysis of variance
ASP	Attractant sex pheromone
DRC	Democratic Republic of Congo
DVB	Divinylbenzene
ECF	East Coast fever
GC-MS	Gas chromatograph coupled to mass spectrometer
GNP	Genital sex pheromone
ICIPE	International Centre of Insect Physiology and Ecology
MSP	Mounting sex pheromone
PDMS	Polydimethylsiloxane
SNK	Student Newman Keuls
SPME	Solid phase micro-extraction
TBDs	Tick borne diseases

**ABSTRACT**

*Rhipicephalus appendiculatus* (Brown Ear tick) is an efficient vector of *Theileria parva* the aetiological agent of East Coast fever (ECF) in cattle. Currently, ECF threatens about 28 million cattle in eastern, central and southern Africa. Control of this tick has largely depended on synthetic acaricides. The use of these chemicals for tick control has however, been compromised by increased cases of tick resistance, high cost of the acaricides and concerns over environmental pollution as a result of extensive use of the acaricides. There have been calls therefore, for alternative tick control approaches that minimise or eliminate the use of synthetic acaricides. Pheromone aided tick control can be one such an approach. Effective use of pheromones in controlling ticks however, demands an understanding of the ticks' behaviour and the identity of the pheromone(s) mediating these behaviours. This study sought to understand the on-host behavioural interactions between adult *R. appendiculatus* ticks and to characterise the pheromone(s) mediating these behaviours. The findings show that female *R. appendiculatus* attach readily on the host even in the absence of male ticks. After feeding for at least 4 days, female ticks not only attract sexually mature males for copulation but also attract and induce the attachment of unfed males. GC-MS analyses of SPME trapped tick volatiles from 5 days fed *R. appendiculatus* females showed the presence of phenol, *p*-cresol and 2,6-dichlorophenol in the ratio 11:5:6. All the three phenols and their blends were found attractive to unfed male *R. appendiculatus* ticks in a two choice climbing assay. *P*-cresol at a concentration of 2.5ng/ $\mu$ l exhibited the highest relative percentage attractancy ( $50.41 \pm 1.88$ ) among the individual compounds tested. A blend consisting of 5.5ng/ $\mu$ l phenol and 2.5ng/ $\mu$ l *p*-cresol exhibited the highest relative percentage attractancy ( $50.90 \pm 1.77$ ) among the blends tested. 2,6-dibromophenol, an analogue of the identified phenols was also found attractive to the unfed male ticks with a concentration of 5.0 ng/ $\mu$ l exhibiting the highest relative percentage attractancy ( $37.90 \pm 2.76$ ) among the 2,6-dibromophenol doses tested. Behavioural interactions in this tick species could thus be simply mediated by phenols. Male ticks seem to play a critical role on the repletion time and engorged mass of the female ticks. The time and the physiological state of the males (partially fed or unfed) at the time they join females on the host had an impact on the females' engorged mass and repletion time. Females that attached on the host the same day with males attained an engorged mass of  $402.60 \pm 37.30$  mg and reached repletion in  $10.47 \pm 0.36$  days. 5-Days feeding females that were accompanied on the host by 5-days fed males attained an engorged mass of  $237.22 \pm 22.28$  mg and reached repletion in  $11.17 \pm 0.23$  days while those 5-days feeding females that were accompanied by unfed males attained an engorged mass of  $387.90 \pm 32.42$  mg reaching repletion in  $13.40 \pm 0.34$  days. These findings lay some ground work for deploying the brown ear tick's pheromone in its control especially so by targeting the male tick which appears to play a critical role in the successful feeding of the female.

## CHAPTER ONE

### INTRODUCTION

#### 1.1 Background information

Ticks and tick borne diseases (TBDs) are a major constraint to livestock production and improvement (Norval *et al.*, 1992) and have considerable economic importance especially to rural people affecting not only their food supply, but also their daily income and other agricultural activities (Minjauw & McLeod, 2003). Estimates of annual global losses associated with ticks and TBDs by Marcelino *et al.*, 2012, ranged between US\$ 13.9 billion and US\$ 18.7 billion. While the threat of ticks and TBDs to livestock is truly a global problem, tropical and sub-tropical countries are the most affected (Bram, 1983). In Africa, TBDs are considered the most important animal disease problem (Young *et al.*, 1988). This is particularly so because most of the tick species transmitting disease-causing pathogens to domestic animals are found in Africa (Walker *et al.*, 2003).

*Rhipicephalus appendiculatus*, a species confined to parts of eastern, central and south-eastern Africa is one of the most economically important tick species in Africa (Walker *et al.*, 2000). The tick owes its economic importance to the fact that it is the most efficient vector of *Theileria parva*, the aetiological agent of East Coast fever (ECF) in cattle. ECF is a rapidly fatal lympho-proliferative disease (Bishop *et al.*, 2004) that has been reported in 11 countries across eastern, central and southern parts of Africa. The disease puts about 28 million cattle in the region at risk and is responsible for 1 million deaths of cattle per year (Gachohi *et al.*, 2012). Much of the economic losses as a result of the disease are concentrated on small-scale resource-poor households (Minjauw & McLeod, 2003).

Control of ECF relies on three methods: vaccination, chemotherapy and tick control. Vaccination against *T. parva* is currently done by the infection and treatment method. Once correctly immunised and subjected to continued low levels of natural challenge, an animal becomes immune for life (Minjauw & McLeod, 2003). The adoption of this method is however, limited by the need for an effective cold-chain facility to transport live parasite sporozoite, occasional failure to achieve protection immunity (Minjauw & McLeod, 2003) and the fact that inoculated animals may act as carriers of the ECF parasite (De Deken *et al.*, 2007).

Chemotherapeutic agents like parvaquone, buparvaquone and halofuginone are available to treat *T. parva* infections. These drugs are however, expensive and treatment is only effective if administered early enough to limit the development of the schizont stage of the parasite and subsequent damage to the immune system (Minjauw & McLeod 2003; Katzer *et al.*, 2010).

Tick control has therefore been the most practical and widely used method to control ECF. Traditionally, tick populations were controlled by burning pasture or clearing of bushes to destroy ticks' micro-habitats (Manna *et al.*, 2001). Synthetic acaricides however, form the centre of control and eradication efforts because they offer relatively quick and cost-effective suppression of tick populations (Abbas *et al.*, 2014). Long term and extensive use however, has led to acaricide resistance and heightened health and environmental concerns (Raynal *et al.*, 2013). Consequently, efforts to develop integrated tick control strategies that make use of tick semiochemicals have attracted considerable interest (Sonenshine, 2004; Maranga *et al.*, 2006; Hassanali *et al.*, 2008).

Semiochemicals are information bearing chemicals that mediate behavioural interactions between organisms. In ticks, these chemicals are host-derived (kairomones), aiding ticks to locate their hosts, and con-specific tick-derived (pheromones), facilitating interactions between ticks of the same species. Application of semiochemicals in tick control takes advantage of the behaviour mediated by these compounds. A clear understanding of the ticks' behaviour and knowledge of the identity/ composition of the behavioural mediating chemical signal is thus of paramount importance.

Studies on the *R. appendiculatus* pheromones reported the presence of phenol and *p*-cresol in fed virgin females (Wood *et al.*, 1975) and 2,6-dichlorophenol (2,6-DCP) in both male and female ticks (McDowell & Waladde, 1986). The three phenolic compounds were found attractive to male ticks in a T-tube bioassay (Wood *et al.*, 1975). Consequently, the compounds were suggested to act as sex pheromones in this tick species (Wood *et al.*, 1975; McDowell & Waladde, 1986).

According to Sonenshine (2006) a pheromone may consist of a single compound or a mixture of different compounds in a distinct ratio characteristic of that species. The findings of Wood *et al.* (1975 and McDowell & Waladde (1986) suggest that *R. appendiculatus* pheromone is a mixture of compounds. Often, when the pheromone is a mixture, the different compounds guide specific components of the mediated behaviour in a precise sequence of events (Sonenshine, 2006). Alternatively, the compounds may work as a blend with one or two compounds having a synergistic or additive effect on the other compound(s). None of these possibilities has been investigated in this tick species.

Isolation of pheromones from ticks has always been done by solvent extraction or effluvial collection (Sonenshine, 1991). By solvent extraction, hundreds or thousands of ticks are immersed in a large excess of an organic solvent for a number of hours. The extract obtained is further purified before concentrating and analysing it (Wood *et al.*, 1975; McDowell & Waladde, 1986). In effluvial collection, purified air is passed over a number of ticks restricted in a glass chamber and subsequently passed through a volatile trap consisting of an adsorbent material such as Porapak Q for say 24 hours. The trapped volatiles are then eluted using an organic solvent, concentrated and analysed. In both methods an organic solvent must be used. With organic solvents becoming an environmental concern however, focus is shifting to extraction techniques that minimize or does not use an organic solvent. Solid phase micro-extraction (SPME) is such a technique. It is a simple and effective adsorption/absorption and desorption technique that eliminates the need for solvents and combines sampling, isolation and enrichment in one step (Tobiszewski *et al.*, 2012). No study has reported the isolation of *R. appendiculatus* pheromone using SPME.

## **1.2 Problem Statement and Justification**

ECF still remains a threat to livestock production and improvement despite efforts to control the tick vector. Control of the tick vector has heavily relied on conventional chemical acaricides and repellents (Kemunto *et al.*, 2014). However, ticks have increasingly become resistant to these chemicals limiting their usefulness. Extensive use of these chemicals has also raised health and environmental concerns (Raynal, 2013). Consequently, there has been an increasing demand for an alternative tick control strategy that is both eco-friendly and cost-effective. Semiochemicals aided tick control strategy has been deemed a suitable alternative since there would be minimum or no use of conventional chemical acaricides or repellents (Sonenshine, 2004; Maranga *et al.*, 2006; Hassanali *et al.*, 2008)

Application of semiochemicals in tick control takes advantage of the behaviour they mediate. An understanding of the ticks' behaviour and knowledge of the info-chemicals mediating these behaviours is thus vital. Research findings in this study will not only help in understanding the on-host behavioural interactions in the adult *R. appendiculatus* ticks but also lay down ground work for deploying the tick's pheromone in its control.

### **1.3 Hypotheses**

- i. *R. appendiculatus* feeding females attract and facilitate the attachment of males.
- ii. Behavioural interactions between adult *R. appendiculatus* ticks at the feeding stage are mediated by a multi-component pheromonal signal.

### **1.4 Objectives**

#### **1.4.1 General objective**

To carry out on-host behavioural studies on the adult *R. appendiculatus* ticks at the feeding stage and characterise the candidate pheromone(s) mediating these behaviours.

#### **1.4.2 Specific objectives**

- i. To determine the percentage attachment of groups of adult *R. appendiculatus* ticks on rabbits and establish whether female *R. appendiculatus* ticks have an influence on the attachment of males.
- ii. To determine the time during the feeding period when female *R. appendiculatus* ticks are most attractive to the males.
- iii. To trap tick volatiles from the most attractive fed female *R. appendiculatus* ticks by solid phase micro-extraction (SPME) technique.
- iv. To carry out GC-MS analysis of the SPME trapped volatiles in order to identify candidate pheromonal signals.

- v. To carry out 2-choice climbing assay to evaluate behavioural responses of male *R. appendiculatus* ticks to individual and blends of pheromonal candidates.
- vi. Determine whether 2, 6-dibromophenol an analogue of 2, 6-dichlorophenol, is attractive to male *Rhipicephalus appendiculatus* ticks.

### **1.5 Scope and limitation of the study**

The study was limited to the adult *R. appendiculatus* ticks under laboratory conditions.

Larval and nymphal stages were not studied.

### **1.6 Significance of the study**

This study was geared towards characterisation of the pheromones mediating behavioural interactions in the adult *R. appendiculatus*. These findings will lay some ground work for deploying the pheromone of this tick in developing an integrated and effective tick control strategy. Findings will be disseminated to relevant stakeholders through peer reviewed journals.

## CHAPTER TWO

### LITERATURE REVIEW

#### 2.1 Ticks

Ticks are obligate, non-permanent ectoparasites of terrestrial vertebrates. All species are exclusively hematophagous in their feeding stages (Klompen *et al.*, 1996). Among the arthropods, ticks have attracted considerable interest particularly as important vectors of a wide variety of pathogens such as viruses, rickettsiae, bacteria and protozoa to their vertebrate hosts. These pathogens are aetiological agents of tick-borne diseases that are a major constraint to the livestock development predominantly in (sub) tropical areas of the world (Jongejan & Uilenberg, 2004).

About 896 tick species in three families are reported (Guglielmone *et al.*, 2010). These families include the monotypic Nuttalliellidae containing the single entity *Nuttalliella namaqua*, Argasidae or ‘soft ticks’ (due to their flexible leathery cuticle) comprising 193 species and Ixodidae or ‘hard ticks’ (by virtue of their hard dorsal shield) comprising 702 species. Little is known about the family Nuttalliellidae (Oliver, 1989). Although Argasid ticks play a significant role as vectors of diseases, it is especially so in poultry. Ixodidae family is thus not only dominant in the total number of species ( $\approx 80\%$ ) but also in the number of species with greatest economic and veterinary importance (Jongejan & Uilenberg, 1994; Hopla *et al.*, 1994; Minjauw & McLeod, 2003).

##### 2.1.1 Ixodidae

###### 2.1.1.1 Morphology

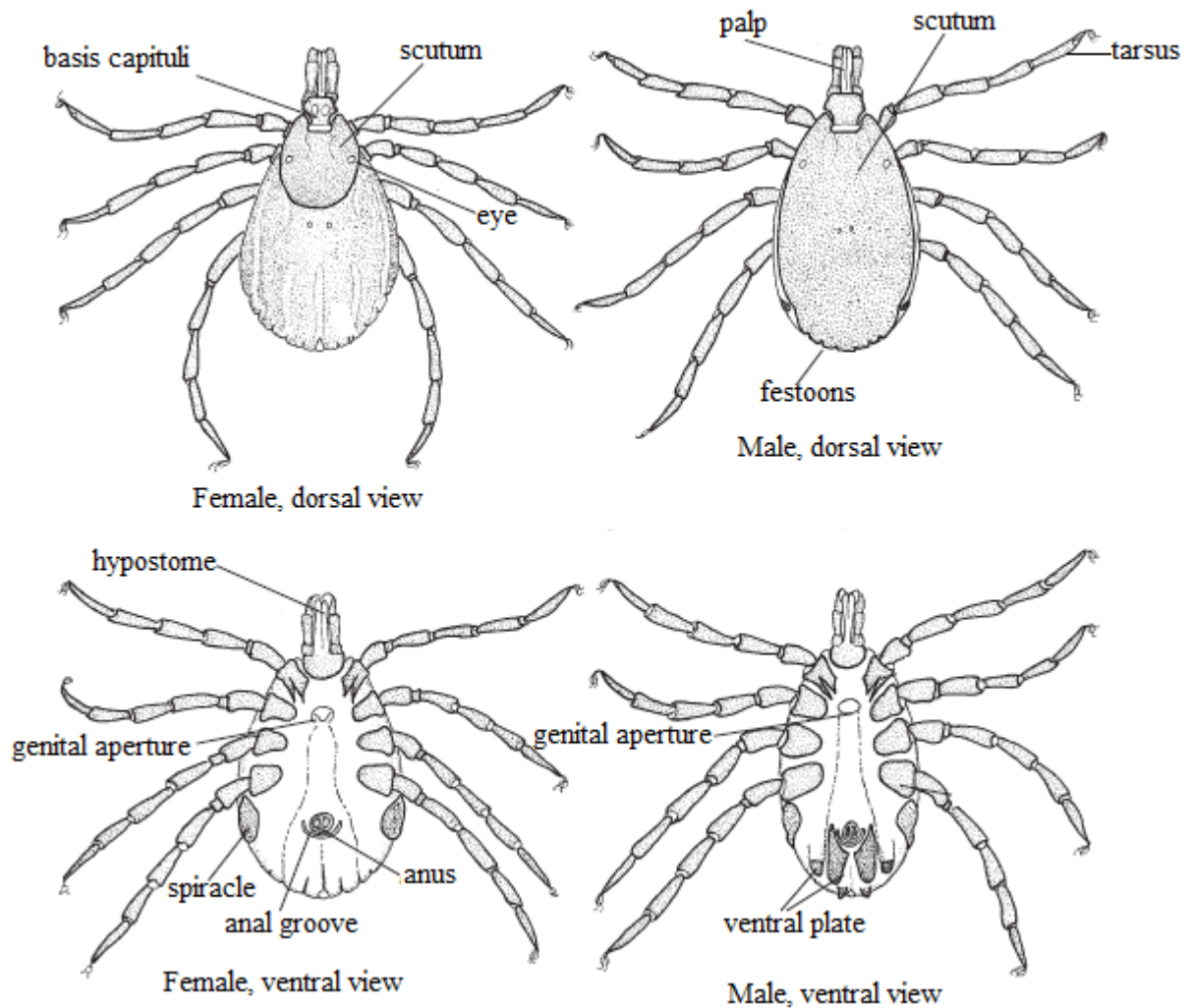
The body of a tick is divided into two main parts: the smaller anterior capitulum (gnathosoma) and the larger posterior part containing the internal organs called opisthosoma. The capitulum consists of a basis capituli onto which the mouthparts are mounted. The

mouthparts include a pair of four-segmented palps, a pair of chelicerae within a sheath and the blade shaped hypostome containing re-curved teeth (Walker *et al.*, 2003).

Ixodid ticks are characterised by a sclerotized dorsal shield called the scutum. This completely covers the dorsal surface of the body in males but is merely a smaller shield behind the capitulum in females and immature (larvae and nymphs). Their eyes, when present, are located near the lateral margin of the scutum (Walker *et al.*, 2000).

Larvae are six-legged while nymphs and adults are eight-legged. Located on the tarsi of the first pair of legs is a highly specialized aggregation of sensory structures (Haller's organ) which contains olfactory receptors that enable the ticks to respond to remote volatile chemicals from hosts and from other ticks (Sonenshine *et al.*, 1986).

Sexual dimorphism is only apparent in the adults. Although larvae and nymphs resemble females, they lack a genital opening. In adults the genital opening, the gonopore, is situated ventrally behind the gnathosoma, usually at the level of the second pair of legs (Wall & Shearer, 2001). Figure 2.1 shows the general morphology of male and female ixodid tick.



**Figure 2.1:** General morphology of male and female ixodid ticks (Walker *et al.*, 2003).

### 2.1.1.2 Feeding

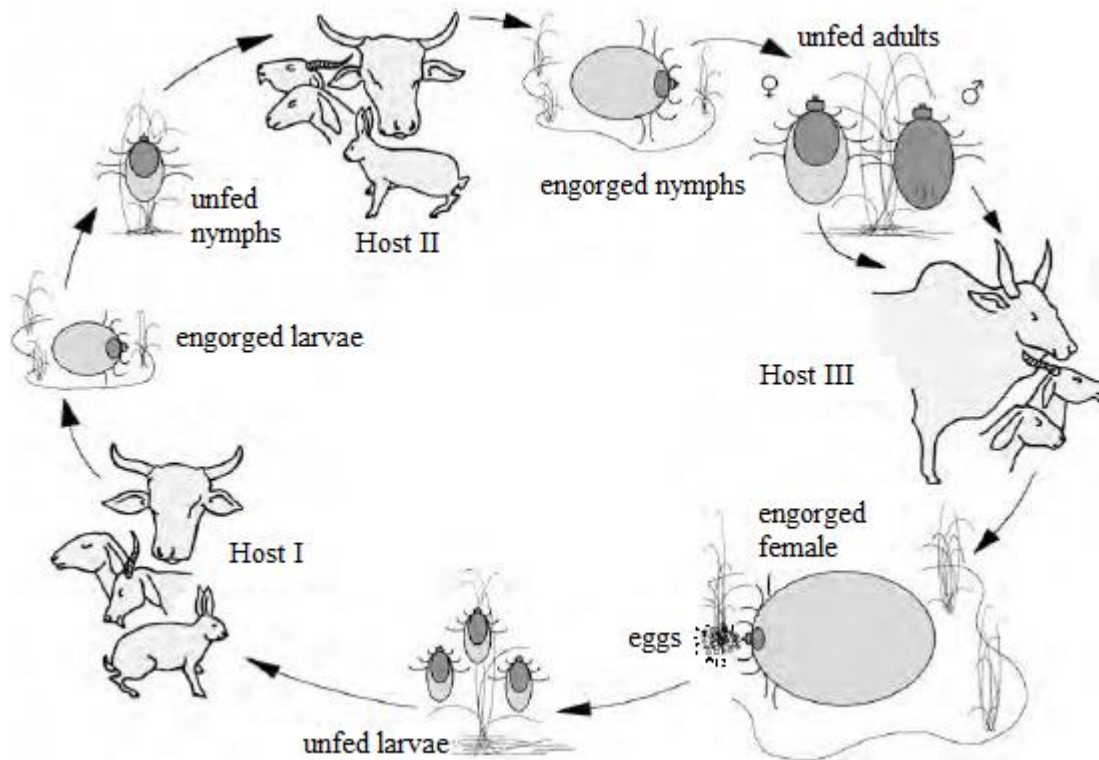
Feeding is a vital part of the life of all ticks. It is the cause of the damage they do to their host and the means by which disease organisms are transmitted (Waladde & Rice, 1982; Latif & Walker, 2004). Once on the host at its predilection site, the tick breaks the dermis of the host using the chelicerae. The hypostome is then shoved into the resulting wound and securely anchors the tick onto the host using the back-ward directed teeth (Wall & Shearer, 2001; Krenn & Aspöck, 2012). This mechanical damage of the host's skin would ordinarily lead to formation of a haemostatic plug by activation of the coagulation cascade and vessel contraction, as well as to inflammatory responses. Such host's responses would disrupt tick

feeding and cause rejection of the tick with detrimental consequences to the tick's viability and reproduction. The ticks counter such host's responses by secreting multifunctional saliva into the wound. The saliva contains a "cocktail" of compounds that inhibit blood coagulation, platelet aggregation, and inflammatory responses, promotes vasodilation, and suppresses the host's immune responses (Kazimírová, 2008).

The feeding of Ixodid ticks is slow since their cuticle needs to grow before it can expand to take a very large blood meal (Latif & Walker, 2004). They therefore remain attached for a long time. In most of them, this attachment is maintained by a cement protein in their saliva that hardens around their mouthparts and cements the tick in place (Wall & Shearer 2001;Kazimírová, 2008). As feeding progresses, the rate of salivary fluid secretions increases greatly, enabling the Ixodid tick to concentrate the blood meal by returning excess water and ions to the host (Sauer *et al.*, 1995). This large amount of saliva fluid is the principal avenue for pathogen transmission (Wall & Shearer, 2001).

### **2.1.1.3 Life cycle**

The life cycle of Ixodid ticks consists of four stages: an embryonated egg followed by three active parasitic stages (larvae, nymphs and adult) (Oliver, 1989). They take only one blood meal as larvae, nymphs and adults then develop to the next stage, which takes weeks, months or even years depending on the ambient temperature. This inter-stadial period is usually passed off the host for more than 90% Ixodid species that undergo the three host life cycle. The relatively few two- and one-host ticks (e.g. *Hyalomma anatolicum excavatum* and *Boophilus microplus*, respectively) remain on the host for one or both of the inter-stadial periods (Randolph, 2004). In all the three life cycles, replete, mated females drop from their host, find a sheltered location, and subsequently oviposit hundreds or thousands of eggs (Sonenshine *et al.*, 2002). Figure 2.2 shows the life cycle of a three host tick.



**Figure 2.2:** Life cycle of a three host tick (Speybroeck *et al.*, 2003).

## 2.2 Genus *Rhipicephalus*

Of the 13 genera in the family Ixodidae (Keirans, 1992) the genus *Rhipicephalus* is one of the largest. The number of species in this genus has been increasing with proceeding studies. While Hopla *et al.*( 1994) reports 75 species in this genus, Walker *et al.*( 2000) recognizes 76 species (74 *Rhipicephalus* species and 2 sub-species) and Guglielmone *et al.*( 2010) records 82 species. It is however, noted that *Rhipicephalus* is mainly an Afro-tropical genus with only one species, *R. sanguineus* (brown dog tick) occurring worldwide (Hopla *et al.*, 1994; Walker *et al.*, 2000).

Species in this genus have the following morphological features in common: their hypostome and palps are short and their basis capituli is usually hexagonal; they have eyes, festoons and, in the males, adanal plates. With the exception of four species, *R. dux*, *R. humeralis*, *R.*

*maculatus* and *R. pulchellus*, they are inornate, i.e. the adults do not have a colour pattern on the scutum, hence their common name 'the brown ticks' (Walker *et al.*, 2000).

Most rhipicephalids are three-host ticks but some species in the genus such as *Rhipicephalus eversti eversti* and *Rhipicephalus eversti mimeticus* are two-host ticks (Walker *et al.*, 2000).

### **2.2.1 *Rhipicephalus appendiculatus***

*Rhipicephalus appendiculatus* is undoubtedly the most economically important African tick species in the genus *Rhipicephalus*. It owes this prominence to the fact that it is the most efficient vector of *Theileria parva*, the causative organism of classical East Coast fever in cattle. The specific name *appendiculatus* from Latin “*appendo*” meaning “to hang” doubtless refers to the appearance of the engorging adults (Walker *et al.*, 2000). It is also known as the brown ear tick because of its colour and preference for feeding on the ear of the mammalian host (Walker *et al.*, 2003).

It is morphologically similar to *R. zambeziensis*, *R. duttoni* and *R. nitens*. It is a three-host tick (Lounsbury, 1904) and cattle are the preferred domestic hosts for all its stages of development (Walker, 1974; Norval & Lightfoot, 1982). The preferred wild hosts of all stages of development are the African buffalo, eland, various species of tragelaphine antelope and waterbucks (Norval *et al.*, 1982; Horak *et al.*, 1992).

#### **2.2.1.1 Geographical distribution of *Rhipicephalus appendiculatus***

The distribution of *R. appendiculatus* is confined to parts of eastern, central and south-eastern Africa (figure 2.3) and has been recorded in 15 countries in the region extending from the Central African Republic and southern Sudan southwards through Zaire, Uganda, Kenya, Burundi, Rwanda, Tanzania, Zambia, Malawi, Mozambique, Zimbabwe, and Botswana, into

South Africa where it occurs in the Transvaal, Natal, and the coastal areas of the Eastern Cape Province (Andrew *et al.*, 1994; Walker *et al.*, 2000). Records outside this region are thought to be misidentification, probably of *R. duttoni*, *R. nitens* and *R. zambenziensis*. Its distribution is patchy even in those countries in which it occurs most commonly with its occurrence being influenced by climate, vegetation and host availability (Andrew *et al.*, 1994; Walker *et al.*, 2000). It is found at altitudes ranging from just above sea level to 2000 m, although some records show its presence above 2400 m. Rainfall for the region in which it is most prevalent varies between 500 mm and 2000 mm annually (Walker *et al.*, 2000).



**Figure 2.3:** Distribution of *R. appendiculatus* ticks (Walker *et al.*, 2003).

#### 2.2.1.2 Disease pathogens associated with *Rhipicephalus appendiculatus*

*R. appendiculatus* is known to transmit a number of disease causing pathogens: *Theileria parva lawrencei* from African buffalo to cattle, causing Corridor disease in the latter animals (Neitz, 1955; De Vos, 1981); *Theileria parva bovis* of cattle and *Theileria taurotragi* of eland

and cattle (Lawrence & MacKenzie, 1980; Fivaz *et al.*, 1989); *Ehrlichia bovis* of cattle (Matson, 1967; Norval, 1979) as well as the viruses causing Nairobi sheep disease and Kisenye sheep disease (Montgomery, 1917). It is however, its effectiveness in transmitting *Theileria parva*, the causative agent of East Coast fever (ECF) in cattle that gives it a great global economic importance (Bishop *et al.*, 2008).

### **2.3 East Coast fever**

ECF is caused by *Theileria parva*, a protozoan parasite transmitted by *R. appendiculatus*. The disease was first recognized in southern Africa in 1902 (Lawrence, 1992) and subsequently found to be widely distributed in eastern and central Africa (Norval *et al.*, 1992). It is reported in 11 countries in the region: Kenya, Uganda, Tanzania, Burundi, Rwanda, Malawi, Mozambique, southern Sudan, Democratic Republic of Congo (DRC), Zambia and Zimbabwe (Lawrence, 1992).

ECF is characteristically a disease causing high morbidity and high case fatality (> 90%) in susceptible adult cattle of all breeds. In endemic situations the disease causes large losses in calves of exotic Taurine breeds and low to negligible losses in calves of indigenous zebu or sanga breeds (Norval *et al.*, 1992). The disease thus limits the introduction of the most productive exotic breeds of cattle hampering the development of livestock industry considerably (Gachohi *et al.*, 2012).

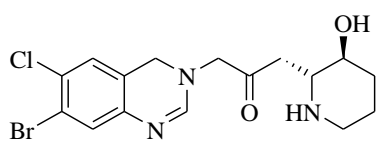
It is estimated that 28 million cattle in the affected region are under threat of ECF with losses of up to 1 million animals being reported annually. These losses are concentrated mainly on resource-poor farmers (Gachohi *et al.*, 2012).

### 2.3.1 Control of East Coast fever

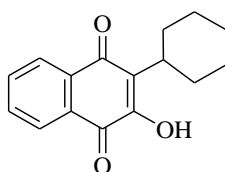
Control of ECF is based on three main methods: chemotherapy, vaccination and tick control.

#### 2.3.1.1 Chemotherapy

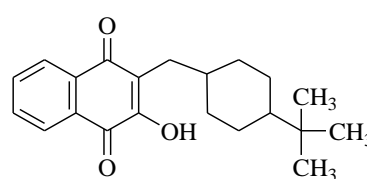
There are currently three effective drugs to treat ECF: halofuginone lactate (**1**), parvaquone (**2**), and an analogue of parvaquone, buparvaquone (**3**) (Norval *et al.*, 1992; Minjauw & McLeod, 2003).



**1**



**2**



**3**

The availability of a chemotherapeutic means of controlling ECF is a significant development. However, the use of these drugs is limited because they are too expensive for most African farmers and effective therapy is only possible where there is rapid and accurate diagnosis (Norval *et al.*, 1992). Furthermore, none of these drugs eliminates carrier infections induced by *Theileria* species (Minjauw & McLeod, 2003).

#### 2.3.1.2 Vaccination

Vaccination against *T. parva* is currently done by the infection and treatment method. The method consists of a live infective sporozoite challenge with simultaneous administration of chemotherapeutic drugs (Vercruysse *et al.*, 2007). Recovery from *T. parva* infection results in an excellent immunity to homologous or related stock of the parasite lasting for over 36

months in the absence of re-infection. With regular natural exposure to the parasites however, the immunity persists for the entire life of the animal (Minjauw & McLeod, 2003).

The involvement of live parasites requires the use of a cold chain to safely deliver the vaccine to the farmers. This is often a challenge especially where infrastructure is poor (Uilenberg, 1999). Breakdown of the cold chain either during transportation or immunization compromises the viability of the sporozoites. Consequently, immunized animals may fail to develop protective immunity (Minjauw & McLeod, 2003).

The existence of different immunological types of the parasite and the fact that immunity is strain or stock specific (Minjauw & McLeod, 2003) makes it challenging to come up with a single stock of a vaccine to be used in all the ECF affected areas. For wider protection, a cocktail of stocks like the “Muguga stock” is used (Minjauw & McLeod, 2003). Most countries however, avoid using a cocktail since immunized animals might become carriers of the stocks used. This would possibly introduce new immunological strains that might subsequently infect animals immune to local strains of the parasite (Minjauw & McLeod, 2003). There is however, no scientific evidence to support these fears (De Castro *et al.*, 1997).

### **2.3.1.3 Tick control**

Tick control is a key tool in tick borne disease management. Existing tick control methods rely heavily on chemical acaricides (Kemunto *et al.*, 2014). The acaricides are either directed against the free living stage of ticks in the environment or against the parasitic stage on the host (Muhammad *et al.*, 2008). Traditionally, acaricides were applied to cattle with a hand sprayer, spray races or through immersion of animals in dipping vats. Recently, treatment

possibilities include the use of pour-on products, injectables, acaricide impregnated ear tags and pheromone/acaricide impregnated devices attached in different ways on the host (George *et al.*, 2004).

The use of chemical acaricides dates back to the use of arsenical solutions in the 1890s. These solutions were first used in 1893 in South Africa and in 1895 in Australia to control ticks on cattle (George *et al.*, 2004). The US adopted arsenic as a recommended tick control agent in 1910 (Graham & Hourrigan, 1977).

The evolution of resistance of ticks to arsenicals, the narrow limits between the effective concentrations for tick control and the toxic concentration for cattle, and concerns about toxic residues in animal tissues led to the replacement of arsenicals with synthetic organic acaricides (Graham & Hourrigan, 1977). These organic acaricides include organochlorines, organophosphates, carbamates, pyrethroids and macro-cyclic lactones. Progressively, however, ticks have developed resistance to almost all these classes of acaricides limiting their usefulness. Furthermore, extensive use of these chemicals have increasingly led to environmental and health concerns. Consequently, efforts by cattle producers to manage ticks and tick borne diseases using these chemicals have been frustrated (George *et al.*, 2004). To address the question of resistance and environmental concerns, a reduction of the number of acaricide treatments to the minimum has been suggested (Roush, 1993). Tick control strategies that incorporate tick semiochemicals minimises the use of chemical acaricides (Sonenshine, 2004).

## **2.4 Tick semiochemicals**

In ticks, as in most animals, behaviour is guided by information bearing chemicals known as semiochemicals (Latha, 2012; Sonenshine, 2004). These chemicals are secreted external to the animal body and, when perceived, direct a specific behavioural response such as food or mate location (Sonenshine, 2004). The behavioural response can be elicited by a mixture of compounds secreted in an ordered hierarchy or by a single compound. If a mixture, the compounds are often released in a specific proportion for maximal behavioural response to be attained (Sonenshine, 2004).

Semiochemicals are classified based on the behaviour they mediate (Latha, 2012; Sonenshine, 2004). Two such classes of semiochemicals are fundamental to the behaviour and ecology of ticks. These are kairomones and pheromones.

### **2.4.1 Kairomones**

In order to obtain a blood meal for their nutritional needs, ticks need to detect and locate their preferred host (Latha, 2012). Host derived chemical cues known as kairomones facilitate host detection and location by ticks. Kairomones are thus information bearing chemical signals released by individuals of one species and detected by individuals of another species for the benefit of the recipient (Sonenshine *et al.*, 2003).

The most notable host cue is carbon dioxide, a host's respiration product. Carbon dioxide functions predominantly as a non-specific, general excitant and can activate ticks from distances as great as 30 metres (Norval *et al.*, 1989). Other host derived cues that guide ticks to their hosts include water vapour, lactic acid, mammalian skin lipids, ammonia, urea and volatile fatty acids emanating from ruminants (Yoder & Stevens, 2000).

Once stimulated, the ticks respond to the differences in the concentration gradient that leads them to the source of the chemical cue. Upon contact with the host, they are guided to their predilection sites by compounds emanating from those sites. *R. appendiculatus* ticks which prefer feeding in the cattle ear have been found attracted by cattle ear odour and repelled by cattle anal odour. On the other hand, *R. eversti* ticks that prefer feeding around the anal region have been found attracted by anal odour and repelled by ear odour. This clearly indicates that compounds emanating from a tick's predilection site plays a role in aiding the tick locate that site (Wanzala *et al.*, 2004).

#### **2.4.2 Pheromones**

Pheromones are info-chemicals emitted by one individual to influence the behaviour of other individuals of the same species (Sonenshine, 2006). They are majorly classified into two categories: primer and releaser pheromones. Primer pheromones are slow acting compounds that initiate complex physiological events affecting development and/or reproduction. This class of pheromones is especially important in the social insects such as bees, wasps and ants (Vargo & Husley, 2000; VanderMeer *et al.*, 2002). Only one example of primer pheromone has been reported in ticks (Khalil, 1984).

Releaser pheromones are fast-acting compounds that elicit immediate behavioural responses by the recipient (Vander Meer *et al.*, 2002). Most tick pheromones fall into this category and are further subdivided into assembly, aggregation and sex pheromones (Sonenshine, 2006).

Diverse chemicals serve as pheromones. These may range from highly volatile substituted phenols to non-volatile contact pheromones such as ecdysteroids and cholesteryl esters (Sonenshine, 2006).

A pheromone may consist of a single compound or a mixture of compounds in a distinct ratio characteristic of that species. Often, these different compounds guide specific components of the behaviour in a precise sequence of events. Other species may have the same compounds but in different ratio, thereby facilitating species-specific recognition (Sonenshine, 1991). Whether alone or in combination, these chemical signals are effective in regulating assembly, aggregation and mate-searching behaviours as well as mate discrimination, copulation, and other activities that function at the population level of a species (Sonenshine, 2006).

#### **2.4.2.1 Arrestment (assembly) pheromones**

These pheromones serve to concentrate off-host tick populations in optimal micro-environments (Gothe, 1987). Ticks encountering surfaces containing the pheromone cease ambulatory activity and remain akinetic, with numerous individuals in direct contact with each other (Sonenshine, 1985).

Although best known in soft ticks, assembly pheromones have also been reported in a number of hard ticks including *Ixodes ricinus* (Graf, 1978), *I. holocyclus* and *Aponomca concolor* (Treverrow *et al.*, 1977), *Hyalomma dromedarii* (Leahy *et al.*, 1981), *Rhipicephalus appendiculatus* and *Amblyomma cohaerans* (Otieno *et al.* 1985), *R. evertsi* (Gothe & Neitz, 1985) and *I. scapularis* (Allan & Sonenshine, 2002). In most of these tick species, purines, compounds liberated in tick excreta, have been shown as the major components of the assembly pheromone. These compounds are relatively stable with limited volatility (Gothe, 1987) and as such are not attractants. The clustering behaviour of ticks in the environment is thus thought to be influenced in part by some volatile compounds. Ammonia emanating from tick faeces has been reported to attract free living, unfed adult and even nymphs to the source

of the stimuli (Grenacher *et al.*, 2001). Contact with purines, especially guanine and xanthine, triggers an arrestment response and causes the ticks to cease activity forming a cluster (Sonenshine, 2004).

Assembly pheromone in *R. appendiculatus* has been reported to be active only during the dry weather. This is thought to facilitate tick survival in the few optimal environments during this period (Sonenshine, 2004).

#### **2.4.2.2 Attraction–Aggregation–Attachment Pheromone (AAAP)**

This pheromone attracts unfed ticks from the natural environment to a tick infested host inducing formation of tick feeding clusters. The pheromone has been reported solely in feeding males of certain species of the genus *Amblyomma* (Sonenshine, 2004; Kaufman, 2006). These species include *Amblyomma hebraeum*, *A. variegatum*, *A. lepidum*, *A. gemma*, and *A. marmoreum* (Sonenshine, 2004).

While male ticks in these species attach on the host irrespective of the presence of females, the females are always reluctant to attach on a host that doesn't harbour feeding males (Kaufman, 2006). After feeding for at least 5 days, the males attract conspecific males, females and even nymphs inducing them to attach in close proximity. This results in the formation of tight feeding clusters (Sonenshine, 1985). Although AAAP is not a sex pheromone since it attracts both sexes, it optimizes chances of intra-specific mating (Sonenshine, 2004).

AAAP is a multi-component pheromone, consisting primarily of three organic volatiles (*o*-nitrophenol, methyl salicylate and nonanoic acid). The concentration ratios among the three substances, and the presence or absence of additional volatile components, vary among

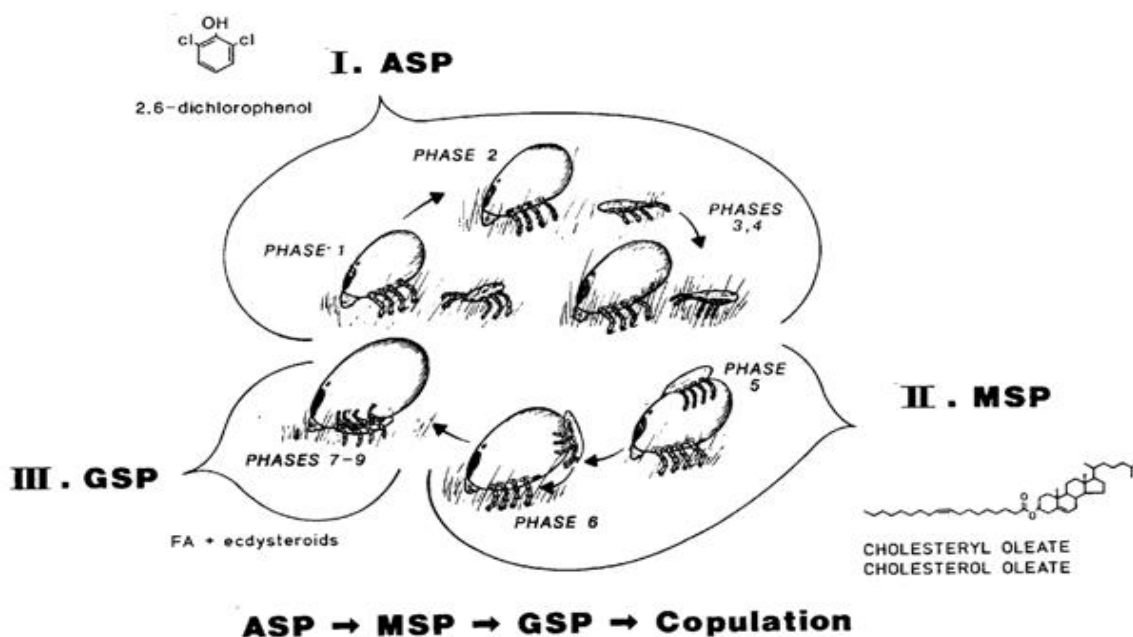
species. This variability possibly accounts for some species-specific aggregation when multiple species are feeding on the same host, even though each pheromone component may attract all species to some extent (Kaufman, 2006).

#### **2.4.2.3 Sex pheromones**

Sex pheromones are compounds or mixtures of compounds secreted by individuals of one sex to attract individuals of the opposite sex (Latha, 2012). In ticks, these compounds are produced by females. Sex pheromones play a key role in guiding mating behaviour and are thus an essential element in the preservation of species integrity.

Rather than a simple event comprising one or two steps, mating is a complex sequence of discrete behavioural events arranged in a temporal sequence, each of which mediated by different pheromones. This hierarchy of sexual selection procedures is the basis of the high degree of species-specific selection that maximizes con-specific mating (Sonenshine, 2004).

Metastriate ticks are sexually immature when they emerge from the nymphal moult and remain so until they attach on a host and begin feeding. Once feeding begins, spermatogenesis and oogenesis are initiated, and sex pheromone secretion soon follows. Three types of sex pheromones have been described: attractant sex pheromone (ASP), mounting sex pheromone (MSP), and genital sex pheromone (GNP). Each of these pheromones mediates a different aspect of the mating process (Sonenshine, 2004). Details of the pheromone-guided mating behaviour are provided in figure 2.4.



**Figure 2.4:** A general model of behavioural events during courtship in metastriate ticks.

**Phase 1**, feeding female secretes volatile attractant sex pheromone, 2,6-dichlorophenol, exciting males feeding nearby. **Phase 2**, sexually excited male detaches and commences searching behaviour in response to sex attractant odour. **Phases 3 and 4**, sexually excited male orients to and walks toward the pheromone-emitting source and locates the pheromone-secreting female. These first four phases are mediated by 2,6-dichlorophenol. **Phase 5**, the male contacts the candidate female and detects the presence of cholesteryl oleate, the mounting sex pheromone. **Phase 6**, the male recognizes the female as a suitable mate and climbs onto the female's dorsal surface, turns posteriorly and crawls over the posterior end of the female's opisthosoma (tip-over behaviour) and onto the ventral surface.

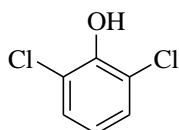
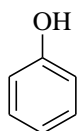
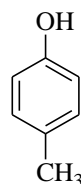
This behaviour constitutes the male mounting response. **Phases 7–9**, the male locates the female gonopore, positions itself under the female with legs intertwined, and inserts its chelicerae into the female's genital pore. Identification of the gonopore and genital probing with the chelicerae is mediated by the genital sex pheromone, comprising a mixture of long chain fatty acids and ecdysteroids. Following successful identification of the female as a con-specific mate, the spermatophore is formed and copulation ensues. **ASP** = attractant sex pheromone; **FA** = fatty acids; **GSP** = genital sex pheromone; **MSP** = mounting sex pheromone (Sonenshine, 2004).

The most reported ASP is 2, 6-dichlorophenol. The compound has been reported in 6 genera of ticks including 15 different species. MSP are mainly cholesteryl esters. They enable the male to recognize the female as a possible mating partner. Variations in the composition of the cholesteryl esters on the females of different species facilitate species recognition and determine whether courtship continues. The little known GNP has been reported in closely related *Dermacentor variabilis* and *D. andersoni*. Identification of this pheromone minimizes the occurrence of inter-specific mating when individuals of both species infest the same host

(Sonenshine, 2004; Sonenshine, 2006). GNP in the two species comprise of long chain saturated fatty acids and ecdysteroids. Species specific concentration of these compounds makes males of the two species discriminate con-specific mates (Allan *et al.*, 1989).

### 2.5 *Rhipicephalus appendiculatus* pheromones

In 1975, Wood and his team reported the presence of phenol (**5**) and *p*-cresol (**6**) in the virgin fed female ticks of the *R. appendiculatus* (Wood *et al.*, 1975).

**4****5****6**

The two compounds were found attractive to the male *R. appendiculatus* ticks in T-tube bioassays hence assigned the role of sex pheromone. Although Wood's team did not detect 2,6-dichlorophenol (**4**) in the female *R. appendiculatus* ticks, the compound was found to be attractive to the males of the same species in their T-tube bioassays. In his electrophysiological studies on *R. appendiculatus* and *Amblyomma variegatum* in 1982, Waladde reported that certain cells of particular olfactory sensillae responded to low doses of 2,6-dichlorophenol. This prompted McDowell and Waladde to reassess *R. appendiculatus* for the presence of 2,6-dichlorophenol (Mcdowell & Waladde, 1986). They detected the compound in both males and females of the *R. appendiculatus* ticks. The precise role of this halogenated phenol was not clear although they suggested that it acts as a sex pheromone just like the phenol and *p*-cresol in this species. An indication that a multi-component system mediates the behaviour of the adult *R. appendiculatus* was thus evident.

## 2.6 Pheromone isolation techniques

Pheromone isolation is a key stage in pheromone identification. The isolation technique used determines, to a greater extent, the subsequent analytical technique, especially in terms of sample introduction into the gas chromatograph (GC) (Agelopoulos & Wadhams, 2000).

Classical pheromone isolation technique is solvent extraction. In isolating 2,6-dichlorophenol, the sex pheromone of the lone star tick, *A. americanum*, Berger (1972) immersed 50,000 two-six weeks old adult ticks in one litre of benzene. 1.5 g of the residue including much of non-pheromonal material was obtained after filtration and drying. The active compound(s) were then to be separated from this residue. Since the pheromone was phenolic, the initial purification step involved suspending the residue in an alkali, removal of neutral lipids by partitioning with hexane and acidification of the alkaline solution with carbon dioxide followed by benzene extraction (Golub & Weatherston, 1984).

Later in 1975, Wood *et al*, isolated phenol and *p*-cresol from 6 day fed virgin female *R. appendiculatus* ticks by immersing 7000 ticks in ether. The purification steps were similar to those of Berger. Tick washings do, in most cases, provide a sample containing the compounds present in the pheromone glands, but may show qualitative and quantitative differences from the naturally emitted semiochemical blend. These qualitative and quantitative differences are likely to affect the ratio in which the semiochemicals are released yet ratios are a fundamental aspect of many semiochemical systems (Agelopoulos & Wadhams, 2000).

The problems associated with solvent extraction can be overcome by using an air entrainment system. Here, the organism emitting the semiochemical is contained in a glass chamber

through which a stream of highly purified air is passed. The volatiles produced are swept from the system and collected in a trap usually a porous polymer, activated charcoal or a cryogenic trapping system. The volatiles can then be recovered from the trap by either solvent or thermal desorption. The advantages of using such a system are that not only are the semiochemicals isolated in proportions emitted by the organism, but also the extracts obtained are free from contaminants associated with straight solvent extraction and are thus directly amenable to analysis by GC. In addition since the sampling is not destructive, it can be used to investigate the time course of semiochemical production (Agelopoulos & Wadhams, 2000).

A relatively more recent technique for isolating pheromones is the solid phase micro-extraction (SPME). Developed for the extraction and concentration of a wide range of volatile and semi-volatile organic compounds from various matrices such as air, water and soil (Agelopoulos & Wadhams, 2000), SPME found its first application in the analysis of insect air-borne volatile pheromones in 1995 (Chu *et al.*, 2005).

SPME is a solvent free adsorption/desorption technique that eliminates the need for complicated apparatus for concentrating volatile or non-volatile compounds. The technique takes advantage of equilibrium extraction and selective sorption from the sample matrix onto a fibre coating. Once the fibre is exposed to the sample, analytes with high affinity for the sorbent are selectively extracted. Whatever is extracted by the fibre is then thermally desorbed into a GC injector. Typically, the micro-extraction process is considered complete when the analyte concentration reaches equilibrium in the sample matrix and the fibre coating. The amount of analyte extracted onto the fibre coating is linearly proportional to the

analyte concentration in the sample. This forms the basis for quantification using SPME (Pawliszyn, 2012).

The choice of the fibre depends on the analyte of interest. Non-polar analytes are most effectively extracted with a non-polar fiber coating and polar analytes are most effectively extracted with a polar coating (Vas & Vékey, 2004). Some of the fibres used are shown in table 2.1.

**Table 2.1: Solid phase micro-extraction (SPME) fibres** (Pawliszyn, 2012)

<b>Fibre coating</b>	<b>Film thickness (<math>\mu\text{m}</math>)</b>	<b>Polarity</b>	<b>Coating method</b>	<b>Compounds to be analysed</b>
Polydimethylsiloxane (PDMS)	100	Non-polar	Non-bonded	Volatiles
PDMS	30	Non-polar	Non-bonded	Non-polar semi-volatiles
PDMS	7	Non-polar	Bonded	Medium- to non-polar semi-volatiles
PDMS–divinylbenzene (DVB)	65	Bipolar	Cross-linked	Polar volatiles
Polyacrylate (PA)	85	Polar	Cross-linked	Polar semi-volatiles (phenols)
Carboxen–PDMS	75	Bipolar	Cross-linked	Gases and volatiles
Carbowax–DVB	65	Polar	Cross-linked	Polar analytes (alcohols)
Carbowax–DVB	70	Polar	Cross-linked	Polar analytes (alcohols)
DVB–PDMS–Carboxen	50/30	Bipolar	Cross-linked	Odours and flavours

SPME has several major advantages in that it is rapid, non-destructive, does not use organic solvents and allows sequential samples to be taken. However, it's not without drawbacks, particularly when used for quantification, since adsorption on to the fibre is related to the

chemical properties of the compound, with some compounds being adsorbed more readily than others. The time needed for a compound to equilibrate with the fibre is related to the structure of the compound, and experimental conditions such as temperature and humidity can affect the adsorptive capacity of the fibre (Agelopoulos & Wadhams, 2000).

## **2.7 Application of pheromones and other semiochemicals in tick control**

Pheromones alone cannot control ticks. Rather, they must be used in combination with a toxicant. The earliest attempt to use tick pheromones to assist in tick control was by Gladney *et al.* (1974). These workers combined an extract of aggregation-attachment pheromone from male Gulf Coast ticks, *A. maculatum*, with an acaricide (isobenzan) and deposited it onto a bovine. Female ticks were lured to the treated site, attached nearby and were killed. The efficacy was however, short lived and soon became apparent that to be effective, the pheromone must be delivered continuously by means of a slow-release device (Sonenshine, 2004, 2006). In addition, the pheromone-acaricide delivery system must be optimized to exploit those characteristics of the target species e.g. host location behaviour or mating behaviour where its use would be effective (Sonenshine, 2004).

Pheromones can be used to disrupt mating among metastriate Ixodid ticks by confusing the males. Saturating an infested environment with excess attractant sex pheromone confuses the males making them unable to discriminate differences in concentration and locate feeding females (Sonenshine, 2006). The longer the males wander in search for mates in the presence of acaricides, the greater the likelihood that they will acquire a lethal dose of the acaricide and die. In exploiting this, Sonenshine *et al.* (1985), dispensed a water emulsion of 2,6-dichlorophenol and an acaricide impregnated into gel microcapsules onto tick infested dogs (Sonenshine *et al.*,1985). Microscopic observations showed numerous microcapsules

attached to the hair of these animals, providing continuous release of the active ingredients for up to 3 weeks. Most of the male ticks were killed by the treatment, and the few surviving females laid less than 10% of the eggs produced by the controls.

Adult *R. appendiculatus* ticks have been shown to use push-pull semio-chemistry to locate the bovid ear. While the bovid ear odour attracts them, they are repelled by the bovid anal odour (Wanzala et al., 2004). Hassanali *et al.* (2008) have thus proposed the use of attractant semiochemicals in control of *R. appendiculatus* ticks. The team suggested that characterization of the attractant semiochemicals may allow the development of a push-pull tactic that combines the use of a source of a synthetic or botanical tick repellent at the ear and an attractant-baited trap treated with fungal pathogen or acaricide located on the back of the animal (Hassanali *et al.*, 2008). These attractant semiochemicals maybe a combination of a kairomone blend generated at cattle ears and pheromones emitted by feeding ticks.

## CHAPTER THREE

### MATERIALS AND METHODS

#### 3.1 Experimental Ticks and chemicals

The ticks were obtained from colonies at the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi. They were reared and maintained in 10 ml glass vials plugged with cotton wool. The vials containing the ticks were kept inside desiccators over saturated potassium chloride solution to maintain a relative humidity of about 85%. The desiccators were placed in an incubator set at a temperature of  $26\pm 1^{\circ}\text{C}$  (Pinter *et al.*, 2002).

All chemicals used were of analytical grade. Phenol (99.9% purity) was obtained from Serva Feinbiochemicals, potassium chloride (99%) was obtained from Chemoquip limited, 2,6-dibromophenol (97%) HPLC grade was obtained from Fluka analytical, 2,6-dichlorophenol (99.9%) and dichloromethane (99.8%) HPLC grade were obtained from Sigma Aldrich.

#### 3.2 Experimental hosts

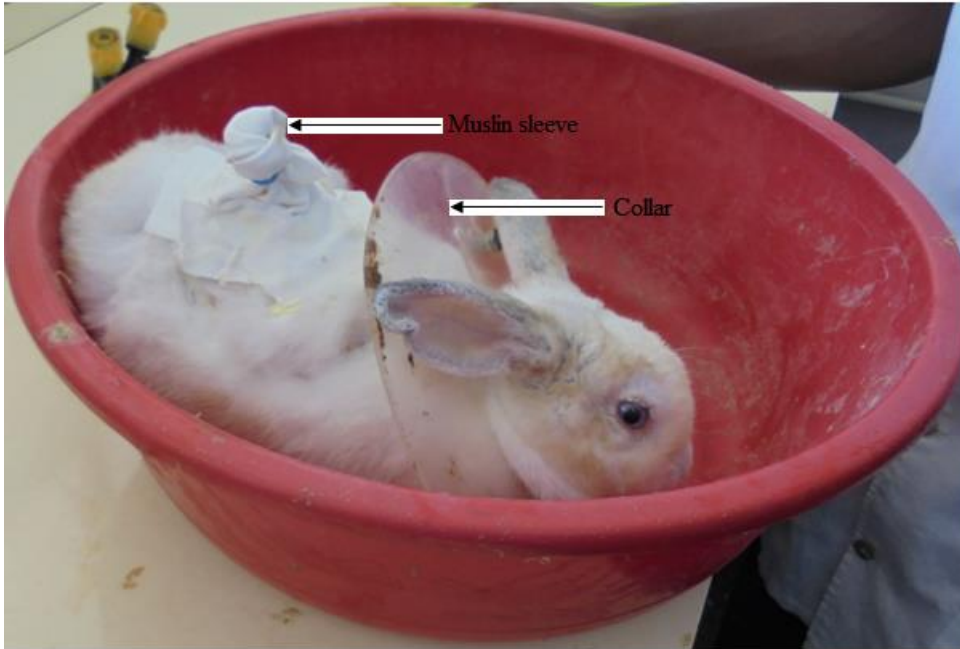
Rabbits were used as experimental hosts for the ticks since they are easy to handle and non-laborious to maintain (Bonnet & Liu, 2012). They were kept in metallic cages in a well ventilated room under natural light regime. They were fed on commercial pellets, cabbage, carrots and water *ad libidum*.

#### 3.3 Feeding of ticks on the host

Ticks were fed on rabbits as described by Bonnet & Liu, (2012) with some modifications.

The back of a tick naive rabbit was clean shaved using a pair of scissors. A muslin sleeve was glued around the clean-shaven region using Conta (an adhesive) as shown in figure 3.1. A plastic collar was then tied around the neck of the rabbit to prevent it from removing the

muslin sleeve. Five hours after the Conta had hardened and its odour disappeared, adult *R. appendiculatus* ticks (sex dependent on the experiment being done) were introduced into the muslin sleeve and its end tied with a rubber band to restrict the ticks within the shaved region.



**Figure 3.1:** A rabbit used as a host for ticks.

All the unfed adult ticks used were three months post-moulting. The mean mass  $\pm$  SE for the unfed females was  $2.36 \pm 0.25$  mg while that of unfed males was  $3.68 \pm 0.2$  mg.

### 3.4 On-host behavioural studies

#### 3.4.1 Attachment success of ticks on the host

Studies were carried out to establish the role, if any, played by one sex of *R. appendiculatus* ticks on the attachment success of ticks of the opposite sex. To do this, five sets of experiments were carried out. In all the experiments, ticks were introduced at 13:00 hrs and observed after every 24 hours.

#### **3.4.1.1 Attachment of male *R. appendiculatus* ticks in the absence of females**

Three tick naive rabbits were prepared as described in (3.3) above. 15 unfed male ticks were introduced on each rabbit and observed after every 24 hours for five days. The number of ticks that had cumulatively attached on each day was recorded. The day of introduction was taken as day 0. The percentage attachment was computed using equation (3.0).

$$\text{Percentage attachment} = \frac{T_a}{T_i} \times 100 \dots \dots \dots \text{Equation (3.0)}$$

Where  $T_a$  = total number of ticks attached on the day of observation and  $T_i$  = total number of ticks introduced on day 0.

#### **3.4.1.2 Attachment of female *R. appendiculatus* ticks in the absence of males**

Three tick naive rabbits were used in this experiment. On each rabbit, 15 unfed female *R. appendiculatus* ticks were introduced and observed after every 24 hours for 5 days. The number of ticks that had cumulatively attached on each day was recorded and percentage attachment computed using equation (3.0).

#### **3.4.1.3 Attachment of males or females in the presence of females or males**

Three tick naive rabbits were prepared as described in (3.3). On each rabbit, 15 unfed male *R. appendiculatus* ticks and 15 female *R. appendiculatus* ticks were introduced. Observations were made after every 24 hours for 5 days. The number of male and female ticks that had cumulatively attached on each day was separately recorded. Percentage attachment for each sex of ticks on each day was computed using equation (3.0).

#### **3.4.1.4 Attachment of unfed males in the presence of feeding females**

Results in experiments discussed in sections 3.4.1.1 to 3.4.1.3 informed the design of experiments 3.4.1.4 and 3.4.1.5.

15 unfed male ticks were introduced on a rabbit harbouring at least 4 days feeding females. The introduced male ticks were observed after every 24 hours for 2 days and the number that had cumulatively attached on each day recorded. Percentage attachment was calculated using equation (3.0).

#### **3.4.1.5 Attachment of partially fed males in the presence of feeding females**

15 partially fed males that had forcefully removed from their host after feeding for about 5 days were introduced on a rabbit harbouring at least 4 days feeding females. The introduced male ticks were observed after every 24 hours for 2 days. The number of ticks cumulatively attached on each day was recorded and percentage attachment computed using equation (3.0). Three rabbits were used in this experiment.

#### **3.4.2 Attachment/ feeding pattern**

While carrying out experiments in (3.4.1), the attachment and feeding pattern of both male and female *R. appendiculatus* ticks was also monitored. The number of ticks that attached and fed in clusters was recorded and percentage aggregation computed using equation (3.1). Similarly, the number of ticks that attached and fed solitarily was recorded and percentage solitary computed using equation (3.2).

$$\text{Percentage aggregation} = \frac{T_c}{T_a} \times 100 \dots \dots \dots \text{Equation (3.1)}$$

Where  $T_c$  = total number of ticks in clusters on the day of observation and  $T_a$  = total number of ticks attached on the day of observation.

$$\text{Percentage solitary} = \frac{T_s}{T_a} \times 100 \dots \dots \dots \text{Equation (3.2)}$$

Where  $T_s$  = total number of ticks feeding solitarily on the day of observation and  $T_a$  = total number of ticks attached on the day of observation.

### 3.4.3 Role of males on the feeding of females

Experiments in sections 3.4.1.3 to 3.4.1.5 were further monitored to establish the onset of copulation, the repletion time of the females and the engorged mass ratio of the replete female. The number of engorged females that voluntarily dropped from the host in each experiment was recorded. The day on which the engorged females voluntarily dropped was likewise recorded to help determine the repletion time. Masses of the engorged females were determined using a PG 503 Mettler Toledo balance. Engorged mass ratio of the female ticks was then calculated using equation (3.4).

$$\text{Engorged mass ratio} = \frac{M_{fe}}{M_{uf}} \dots \dots \dots \text{Equation (3.4)}$$

Where  $M_{fe}$  = mass of fully engorged female and  $M_{uf}$  = mass of unfed female.

### 3.5 Feeding female ticks for trapping of volatiles

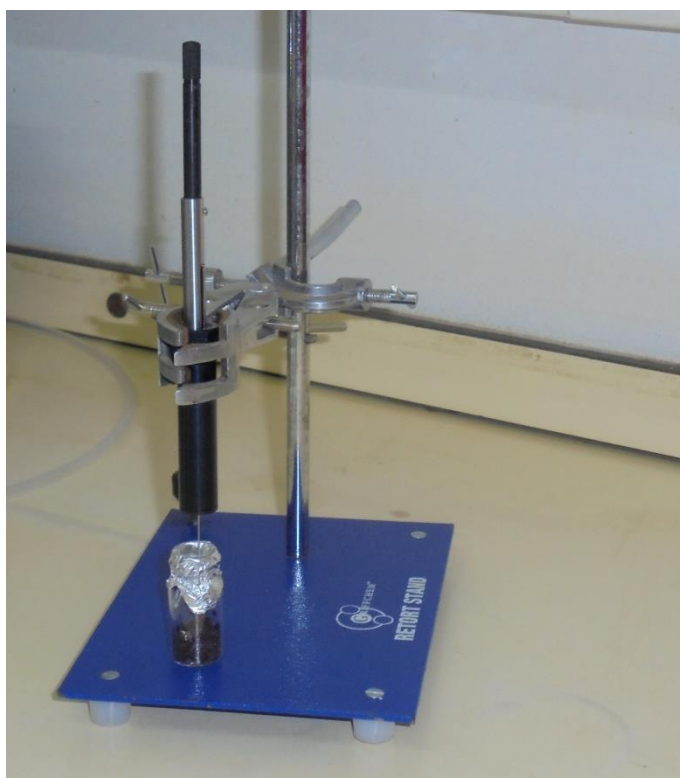
Results in experiment in section 3.4.1.3 indicated that female *R. appendiculatus* ticks become attractive to males after feeding for at least 4 days and the attraction is at its peak on day 5 (supported by earlier studies by Wood *et al.*, 1975). A total of 100 female *R. appendiculatus* ticks were thus fed on 5 tick naive rabbits for 5 days. The ticks were then forcefully removed from their hosts for volatile trapping.

### 3.6 Volatile trapping using solid phase micro extraction (SPME) technique

Volatiles from the 5 day fed female *R. appendiculatus* ticks were trapped by solid phase micro-extraction (SPME) technique using Polydimethylsiloxane-divinylbenzene (PDMS/DVB) fibre as described by Lambropoulou & Albanis, (2002). Prior to trapping, the SPME fibre was conditioned in a GC injector for 30 minutes at 240<sup>0</sup>C.

100 five day fed female ticks were placed in a 4 ml vial that had been thoroughly cleaned using dichloromethane and oven dried at 100<sup>0</sup>C for 10 minutes. The end of the vial was

carefully wrapped with a clean aluminium foil. The needle of the SPME unit was pierced through the aluminium foil into the sample vial. By depressing the plunger of the SPME unit, the PDMS/DVB fibre was exposed into the headspace (space within the vial above the ticks) to adsorb the analyte onto the fibre coating. The SPME unit was held in position using a clamp (figure 3.2). After two hours, the fibre was retracted into the needle, and the needle withdrawn from the sample vial. A control was run simultaneously with the sample. In the control, the sample vial did not contain ticks.



**Figure 3.2:** Extraction of female tick volatiles using SPME apparatus

### 3.7 SPME desorption procedure

The septum of the GC inlet was pierced with the needle of the SPME that had been used for extraction. The fibre was exposed to thermally desorb the analyte and deliver it into the GC column. The desorption temperature was 250°C. After 5 minutes, the fibre was retracted and the needle withdrawn from the GC inlet.

### 3.8 Analyses of SPME trapped ticks' volatiles

The SPME trapped volatiles were analysed by GC-MS on a 7890A gas chromatograph (Agilent Technologies, Inc., Santa Clara, CA, USA) linked to a 5975C mass selective detector (Agilent Technologies, Inc., Santa Clara, CA, USA). The GC was fitted with a HP-5ms low bleed capillary column (30 m × 250 µm × 0.25 µm) in a splitless mode, with helium at a flow rate of 1.25 ml/min serving as carrier gas. The column oven temperature was programmed from 40<sup>0</sup>C to 260<sup>0</sup>C. The initial temperature was maintained for 3 minutes then the temperature increased at 5<sup>0</sup>C/min to 260<sup>0</sup>C where it was held for 3 minutes. The mass spectra information was acquired in a full scan mode. The mass selective detector was held at a maximum ion source temperature of 250<sup>0</sup>C while the quadrupole temperature was 200<sup>0</sup>C. Electron impact (EI) mass spectra were obtained at the acceleration energy of 70 eV. The GC-MS was coupled to a computer with MS data library. The compounds were thus identified by comparing their chromatographic retention time and fragmentation pattern with reference spectral in the MS data library published by National Institute of Standard and Technology (NIST).

### 3.9 Preparation of solutions for assays

Dichloromethane was used as a solvent in the preparation of solutions that were used in the bioassays. When preparing blends, the individual compounds were mixed in the ratio in which they were found in the GC-MS analysis.

A blend of phenol, *p*-cresol and 2,6-dichlorophenol was prepared by accurately weighing 11 mg of phenol, 5 mg of *p*-cresol and 6 mg of 2,6-dichlorophenol and dissolving them in 1000 µl of dichloromethane in a thoroughly cleaned 2 ml amber vial. The resulting solution was taken as the stock solution. Serial dilutions of the stock solution were carried out to obtain

concentrations of (11 ng phenol, 5 ng *p*-cresol, 6 ng 2,6-dichlorophenol)/ $\mu$ l, (5.5 ng phenol, 2.5 ng *p*-cresol, 3 ng 2,6-dichlorophenol)/ $\mu$ l and (1.1 ng phenol, 0.5 ng *p*-cresol, 0.6 ng 2,6-dichlorophenol)/ $\mu$ l respectively.

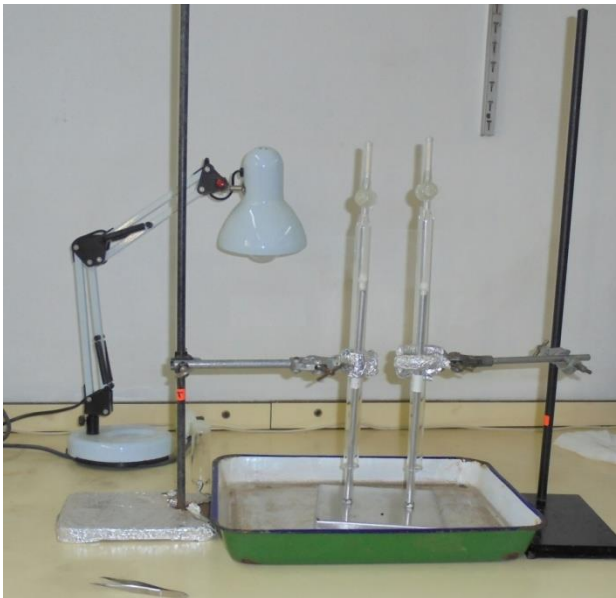
A blend of phenol and *p*-cresol was prepared by accurately weighing 11 mg phenol and 5 mg *p*-cresol and dissolving the mixture in 1000  $\mu$ l of dichloromethane to make a stock solution. Required concentrations for bioassays were obtained by serially diluting the stock solution. A blend of phenol and 2,6-dichlorophenol contained 11 mg phenol and 6 mg 2,6-dichlorophenol in 1000  $\mu$ l of dichloromethane while the blend of *p*-cresol and 2,6-dichlorophenol contained 5 mg *p*-cresol and 6 mg 2,6-dichlorophenol in 1000  $\mu$ l of dichloromethane. Stock solutions of individual identified phenols were prepared by dissolving 11 mg of phenol, 5 mg of *p*-cresol and 6 mg of 2,6-dichlorophenol each in 1000  $\mu$ l of dichloromethane.

2,6-dibromophenol was not identified in the ticks but was used in the bioassays as an analogue of the three identified phenols. Its stock solution was prepared by dissolving 10 mg in 1000  $\mu$ l of dichloromethane. All solutions were placed in 2ml amber vials, wrapped in an aluminium foil and stored at  $-32^{\circ}\text{C}$  until required for bioassays.

### **3.10 Two choice climbing assays**

A two choice climbing assay set-up (figure 3.3) consisted of an aluminium base (15 cm  $\times$  15 cm  $\times$  1.5 cm). On the base, two aluminium rods (0.6 cm diameter  $\times$  24 cm long) were mounted 7 cm apart. A glass tube (0.8 cm diameter  $\times$  25 cm long) was slipped over each of the aluminium rods and the end of each glass tube plugged with moist cotton wool. The cotton wool was used to arrest the ticks that climbed up to the tip of the glass tubes. A strip of filter paper (Whatmann No. 7, 1 cm wide) was stapled to form a collar around each glass tube

12 cm above the aluminium base. One collar was treated with 50  $\mu$ l of test odour solution (solution delivered onto the collar using 100  $\mu$ l Hamilton gastight syringe) and the other collar was treated with 50  $\mu$ l of the solvent (dichloromethane) to serve as a control. The solvent was allowed to evaporate for about 10 minutes. The tubes containing the collars were then shielded with wider tubes (3 cm internal diameter  $\times$  30 cm long), 3 cm above the aluminium base to facilitate relatively uniform vertical gradient of the test odours. These outer tubes were held in place by a clamp. The apparatus was then placed in a metal tray (24 cm  $\times$  40 cm) containing a pool of water such that the upper surface of the aluminium base was above the water level. This prevented ticks from escaping from the aluminium base.



**Figure 3.3:** A set up of a two choice climbing assay apparatus

Prior to each day's bioassays, *R. appendiculatus* male ticks were kept at high relative humidity for 12 hours. This was done by inverting a 10 cm long glass vial containing the ticks and whose end was plugged with cotton wool above a saturated solution of potassium chloride.

Male unfed ticks (6) were placed on the aluminium base and observed for 60 minutes. After the 60 minutes, the ticks on the treated and control arms were counted and recorded. The used

glass tubes were thoroughly cleaned with tap water and oven dried at 100<sup>0</sup>C for 10 minutes before re-using them. The assay for a given dose of test odour was repeated 6 times using a different set of male ticks. The results for the 6 repeats were pulled to form one replicate for a given dose of test odours. Three such replicates were carried out for each test odour dose. Relative percentage attraction was calculated using equation 3.5 (Wanzala *et al.*, 2004).

$$\text{Relative percentage attraction} = \frac{N_t - N_c}{N_t + N_c} \times 100 \dots \dots \dots \text{Equation (3.5)}$$

Where  $N_t$  = number of ticks on the treated arm and  $N_c$  = number of ticks on the control arm.

### 3.11 Statistical analyses

Comparisons of means of more than two sets of data were carried out by one way ANOVA. Means that were significantly different were separated using Student-Newman-Keuls (SNK) test. Where comparisons of means of two sets of data were required, Student's T-test was applied.

## CHAPTER FOUR

### RESULTS AND DISCUSSIONS

#### 4.1 On-host behavioural studies

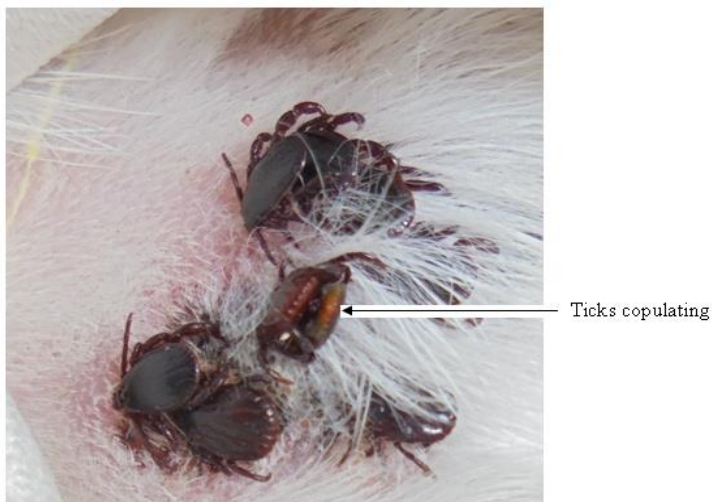
##### 4.1.1 Attachment success of *R. appendiculatus* ticks

The attachment of males in the presence and absence of females was not significantly different from days 1 to 3 as shown in table 4.1. However, on day 4 and 5, significantly more males attached in the presence of feeding females. Tick copulation (evidenced by ventral to ventral co-feeding of male and female ticks as is shown in figure 4.1) coincided with this significant attachment.

**Table 4.1: Mean percentage attachment  $\pm$  SE of *R. appendiculatus* males in the absence and presence of females**

Day	Mean percentage attachment $\pm$ SE		P-value
	Males in absence of females	Males in presence of feeding females	
1	20.00 $\pm$ 10.18 <sup>a</sup>	26.67 $\pm$ 3.85 <sup>a</sup>	0.5734
2	51.11 $\pm$ 8.01 <sup>a</sup>	42.22 $\pm$ 5.88 <sup>a</sup>	0.4215
3	55.56 $\pm$ 5.88 <sup>a</sup>	53.33 $\pm$ 3.85 <sup>a</sup>	0.7675
4	60.00 $\pm$ 6.67 <sup>a</sup>	91.11 $\pm$ 2.22 <sup>b</sup>	0.0114
5	66.67 $\pm$ 7.70 <sup>a</sup>	97.78 $\pm$ 2.22 <sup>b</sup>	0.0178

For a given row, means with the same superscript are not significantly different (Student t-test).



**Figure 4.1:** Adult *R. appendiculatus* ticks feeding on a rabbit, with a pair copulating

Studies by Kuhnert *et al.*, 1995 indicate that female ticks experience a slow feeding phase between day 4 and day 8. During this period, the tick undergoes significant physiological changes that include maturation of the salivary glands, considerable synthesis of the procuticle to accommodate the enormous blood meal during the rapid feeding phase and synthesis and emission of pheromones. Noting that significant pheromone synthesis and emission takes place between the 4<sup>th</sup> and 8<sup>th</sup> day of feeding, it would be reasonable to argue that the significant attachment of the male *R. appendiculatus* ticks in the presence of  $\geq 4$  days fed females and subsequent copulation, was mediated by pheromones emitted by the feeding females.

Female ticks of some *Amblyomma* species e.g. *A. hebraeum* and *A. variegatum* are reluctant to attach on an animal that is not infested with males (Kaufman, 2007). This is clearly not the case with female *R. appendiculatus* ticks. The attachment of female *R. appendiculatus* ticks in the absence and presence of males was not significantly different (table 4.2). By the 2<sup>nd</sup> day almost all females ( $93.33 \pm 3.85\%$ ) had attached.

**Table 4.2: Mean percentage attachment  $\pm$  SE of female *R. appendiculatus* ticks in the absence and presence of males**

Day	Mean percentage attachment $\pm$ SE		P-value
	Females in absence of males	Females in the presence of males	
1	$55.55 \pm 2.22$	$51.11 \pm 8.01$	0.6217
2	$93.33 \pm 3.85$	$93.33 \pm 3.85$	1.0000

For this species, male ticks seem to be reluctant to attach in the absence of females. Results in table 4.3 indicate that significantly higher females attached in the absence of males than males in the absence of females. While almost all females ( $93.33 \pm 3.85\%$ ) had attached by the 2<sup>nd</sup> day, just slightly more than half ( $51.11 \pm 8.01\%$ ) of the males had attached by the 2<sup>nd</sup> day.

**Table 4.3: Mean percentage attachment  $\pm$  SE of *R. appendiculatus* male and female ticks in the absence of ticks of the opposite sex**

Day	Mean percentage attachment $\pm$ SE		P-value
	Males in absence of females	Females in the absence of males	
1	20.00 $\pm$ 10.18	55.55 $\pm$ 2.22	0.0270
2	51.11 $\pm$ 8.01	93.33 $\pm$ 3.85	0.0090

Male ticks in the genus *Amblyomma* attach first and after feeding for  $\geq 4$  days release an attractant-aggregation-attachment pheromone (AAAP) that induces the attachment of conspecific males and females around the pheromone emitting males (Rechav *et al.*, 1997). In *R. appendiculatus*, this role is arguably played by the females. Results in table 4.4 indicate that more male ticks significantly attached in the presence of females that had been feeding for 5 days.

**Table 4.4: Mean percentage attachment of unfed and partially fed males in the presence of unfed and feeding females**

Day	Mean cumulative percentage attachment $\pm$ SE				P-value
	MA	MIF	UMI5FF	5FMI5FF	
1	20.00 $\pm$ 10.18 <sup>a</sup>	26.67 $\pm$ 3.85 <sup>a</sup>	80.00 $\pm$ 3.85 <sup>b</sup>	91.11 $\pm$ 2.22 <sup>b</sup>	<0.0001
2	51.11 $\pm$ 8.01 <sup>a</sup>	42.22 $\pm$ 5.88 <sup>a</sup>	97.78 $\pm$ 2.22 <sup>b</sup>	97.78 $\pm$ 2.22 <sup>b</sup>	<0.0001

**MA**= Males introduced on host in the absence of females; **MIF**= Males introduced on host with females at the same time; **UMI5FF**= Unfed males introduced on host with 5 day fed females; and **5FMI5FF**= 5 day fed males introduced on host with 5 day fed females. For a given row, means with the same superscript are not significantly different (One-way ANOVA,  $\alpha = 0.05$ , SNK test).

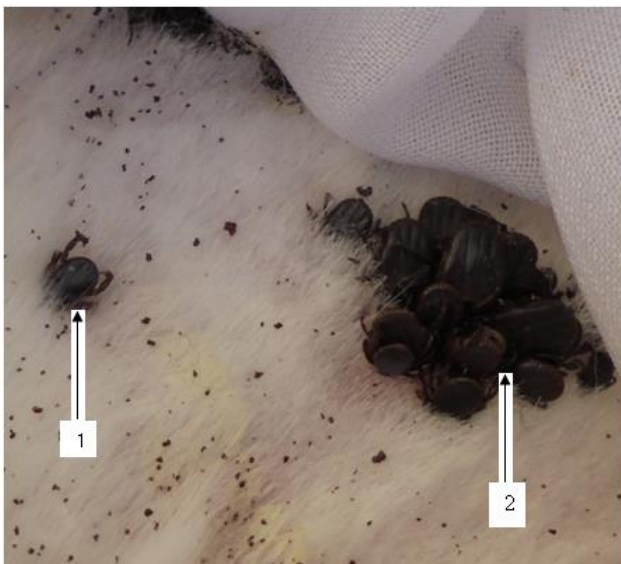
The attachment success of unfed and partially fed males in the presence of feeding females was not significantly different. Both partially fed and unfed males attached in close proximity with the feeding females. On hosts where partially fed males were introduced copulation was observed on the 1<sup>st</sup> day of attachment.

Most metastriate female ticks are known to produce sex pheromones that secure the meeting of partially fed and sexually mature male and female ticks on the host. These female-

produced sex pheromones arguably play no role in the attachment of either sex to the host, but regulate their meeting and consequently mating (Rechav *et al.*, 1997; Sonenshine, 1991). Results in this study, however, indicate that feeding *R. appendiculatus* females produce a pheromone(s) that not only attract partially fed males for copulation but also attract and induce the attachment of unfed males. Since female *R. appendiculatus* ticks readily attach on the host in the absence of males, this attraction and significant enhancement of attachment of unfed males close to the feeding females is possibly an evolutionary strategy that optimizes chances of mating. Arguably, the definition of a sex pheromone should not be therefore narrowed to an attraction between partially fed ticks of the opposite sex.

#### 4.1.2 Attachment and feeding pattern of *R. appendiculatus* ticks

*R. appendiculatus* exhibit both solitary and gregarious kind of attachment and feeding patterns as is shown in figure 4.2. The mean percentage of ticks in gregarious feeding pattern was significantly (Student t-test,  $p < 0.001$ ) higher than those in solitary feeding as indicated in table 4.5.



**Figure 4.2:** The feeding pattern of male and female *R. appendiculatus* ticks on a rabbit. **1=** solitary feeding, **2=** gregarious feeding.

**Table 4.5: Mean percentage per attachment and feeding pattern of male and female *R. appendiculatus* ticks**

<b>Feeding pattern</b>	<b>Mean percentage per pattern <math>\pm</math> SE</b>
Gregarious	84.62 $\pm$ 2.69
Solitary	15.38 $\pm$ 2.69
P-value	< 0.001

While both males and females preferred gregarious kind of feeding, the number of females in this pattern were significantly (ANOVA,  $p = 0.0128$ ) higher than that of males. There was no significant difference between the number of females aggregating and that of males and females combined (table 4.6).

**Table 4.6: Mean percentage aggregation for males, females and combined sexes of *R. appendiculatus* ticks**

<b>Ticks introduced</b>	<b>Mean percentage aggregation <math>\pm</math> SE</b>
Males alone	74.76 $\pm$ 5.02 <sup>a</sup>
Females alone	87.20 $\pm$ 2.78 <sup>b</sup>
Males and females	94.51 $\pm$ 2.21 <sup>b</sup>
P-value	0.0128

Means with the same superscript are not significantly different (One-way ANOVA,  $\alpha = 0.05$ , SNK test).

Tick feeding aggregation is mediated by pheromones (Sonenshine, 1991). Results in this study suggest that adult *R. appendiculatus* ticks synthesise and emit an aggregation pheromone. Synthesis and emission of this pheromone in *R. appendiculatus* cannot however, be assigned to a particular sex of ticks as is the case in some *Amblyomma* species, since both sexes exhibited the aggregation behaviour.

While aggregation behaviour in adult ticks is said to facilitate mating (Sonenshine, 1991), this behaviour may serve additional roles (Norval *et al.*, 1989). Intra-specific gregarious behaviour resulting in group action is beneficial in winning inter-specific competitions (Sirot,

2000; Tullberg *et al.*, 2000). Ticks display between-individual polymorphism of salivary gland proteins (Wang *et al.*, 1999) suggesting that the individual tick saliva may differ in their composition (Wang *et al.*, 2001). Pooling of these individual saliva activities while feeding in aggregation, possibly improves the feeding conditions of all members in the feeding group (Wang *et al.*, 2001).

#### 4.1.3 Role of male ticks on the engorgement mass and repletion time of females

Following mating, female *R. appendiculatus* ticks feed to repletion and detach from the host to search for a suitable oviposition site (figure 4.3).



**Figure 4.3:** Fully engorged female *R. appendiculatus* tick voluntarily detached

The male tick seems to play a role on the engorged mass and repletion time of the females. Table 4.7 shows that the mass of engorged females introduced on the host with males the same day was significantly higher than the engorged mass of those females that were accompanied by 5 days fed males on their 5<sup>th</sup> day of feeding. The repletion time for the two groups of females was however, comparable. While the engorged mass of those females

accompanied by unfed males on their 5<sup>th</sup> day of feeding was comparable to the engorged mass those females that were introduced on the host with males the same day, the repletion time for these two groups of females was significantly different.

**Table 4.7: Mean engorged mass and repletion time of female *R. appendiculatus* ticks**

Group of ticks	N	Mean engorged mass of females $\pm$ SE (mg)	Engorged mass ratio	Repletion time $\pm$ SE (days)
Females introduced with males the same day	15	402.60 $\pm$ 37.30 <sup>b</sup>	170.59	10.47 $\pm$ 0.36 <sup>a</sup>
5 day fed males introduced to 5 day feeding females	18	237.22 $\pm$ 22.28 <sup>a</sup>	100.52	11.17 $\pm$ 0.23 <sup>a</sup>
Unfed males introduced to 5 day feeding females	10	387.90 $\pm$ 32.42 <sup>b</sup>	164.36	13.40 $\pm$ 0.34 <sup>b</sup>
P-value		0.0003		< 0.001

Means with the same superscript in a column are not significantly different (One-way ANOVA,  $\alpha = 0.05$ , SNK test). Mass of unfed female ( $2.36 \pm 0.25$  mg,  $n = 18$ ).

Male ticks certainly, play a critical role on the engorged mass and repletion time of females. Studies have shown that after mating, the female ticks benefit from a ‘male factor’ and an ‘engorgement factor’ i.e. proteins secreted from the male gonad. The male factor hastens the onset of salivary gland degeneration and ovarian development while the engorgement factor stimulates the female to engorge. Full engorgement in female Ixodid ticks is thus attained after mating (Kaufman, 2007). Fed males also secrete specific salivary gland immunoglobulin proteins (IGBP) (Wang & Nuttall, 1995) that help mated females feed (Wang *et al.*, 1998) by modulating the host’s immune system.

Female ticks that were mated and thus benefited from these male derived proteins almost the same time i.e. those females that were introduced with males the same day and those females that were accompanied with 5-days fed (sexually mature) males on their 5<sup>th</sup> day of feeding had comparable repletion times. Females that were mated much later i.e. those females that were accompanied by unfed (sexually immature) males on their 5<sup>th</sup> day of feeding however,

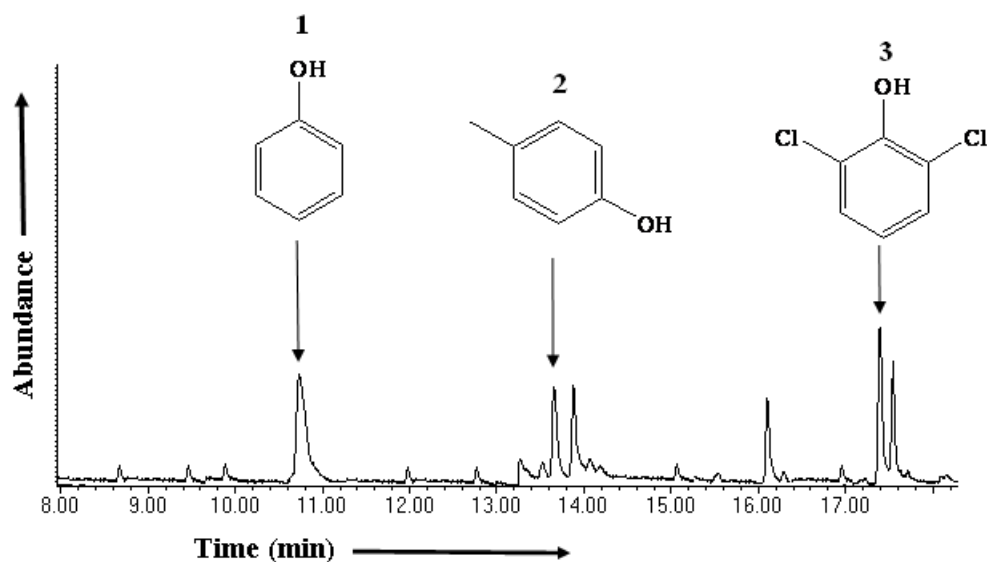
had significantly higher repletion time. These observations underscore the role of the male derived proteins. The duration the female takes on the host is therefore among other factors dependent on the time it was mated.

The differences in the engorged mass of the three groups of females cannot however, be explained in terms of male derived proteins. This can be explained in terms of cuticle extensibility. If the female ticks are not rejected by the host, they will only cease to feed because of a limit to cuticle stretching (Kaufman *et al.*, 2010). The size of the blood meal would thus reflect how developed the cuticle was.

Cuticle secretion occurs during the slow feeding phase (Hackman, 1982; Kaufman *et al.*, 2010). Kaufman, 2007 suggestion that virgin female ticks curtail feeding as they approach the critical weight especially so when feeding in the absence of males makes us hypothesize that they too curtail cuticle development. Sexual maturity is not however, curtailed. If this line of argument is true, we do propose that when such a female is introduced to a sexually mature (fed) male, mating will occur, stimulating engorgement at the expense of cuticle development. The tick will attain repletion faster but have a smaller mass. Introduction of an unfed male to such a female would on the other hand trigger it to resume normal cuticle development. By the time the male completes spermiogenesis and copulates with such a female, its cuticle would have better developed. This would allow the female tick to accommodate a larger blood meal during the rapid feeding phase hence a larger engorged mass. It would be interesting to find out how exactly the attaching of unfed male stimulates the attached female to resume cuticle development.

## 4.2 Gas chromatography-mass spectrometry of tick volatiles

GC-MS analysis of tick volatiles trapped by SPME gave three tick specific compounds. The compounds were identified as phenol (**1**), *p*-cresol (**2**) and 2,6-dichlorophenol (**3**). The gas chromatogram spectrum of the compounds is shown in figure 4.4.



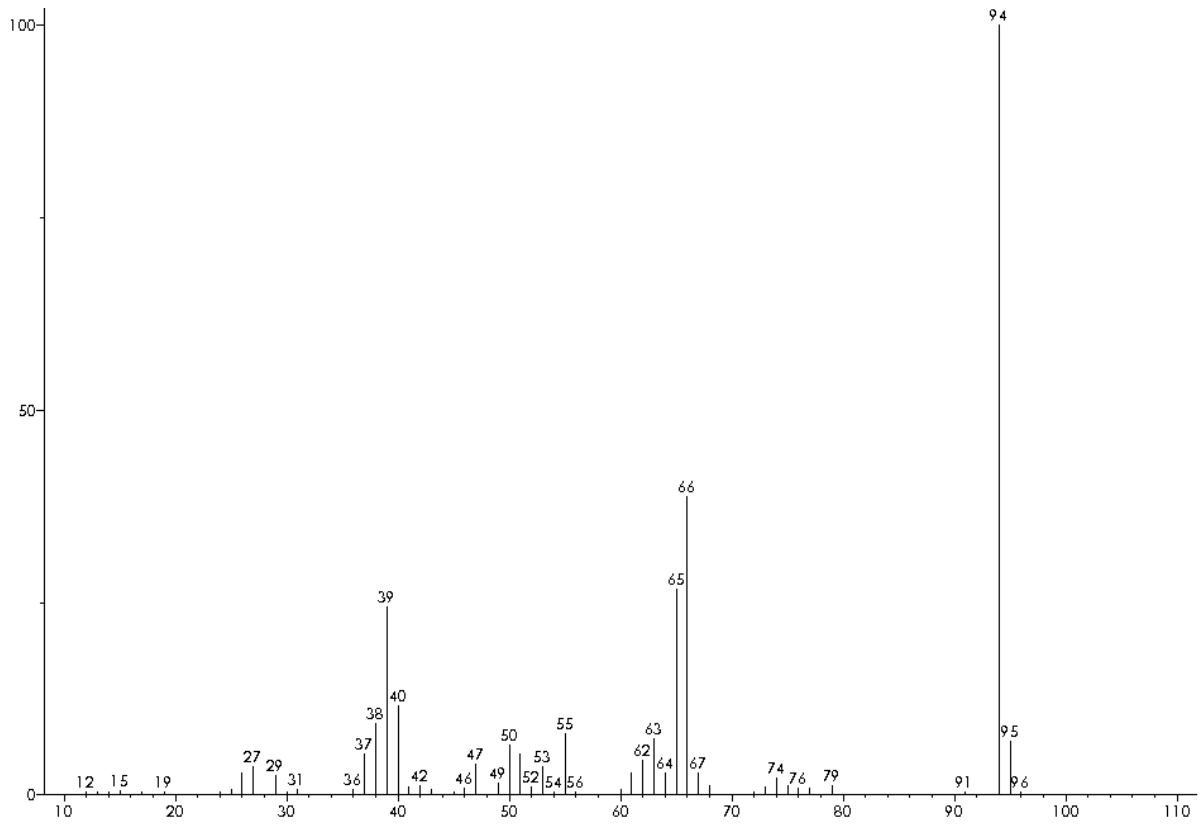
**Figure 4.4:** Gas chromatogram spectrum for the 6 days fed *R. appendiculatus* females' volatiles(1= phenol, 2= *p*-Cresol and 3= 2,6 dichlorophenol)

Table 4.8 shows the retention time and peak areas of the identified compounds. Based on the peak areas the ratio of phenol: *p*-cresol: 2, 6- dichlorophenol = 11:5:6.

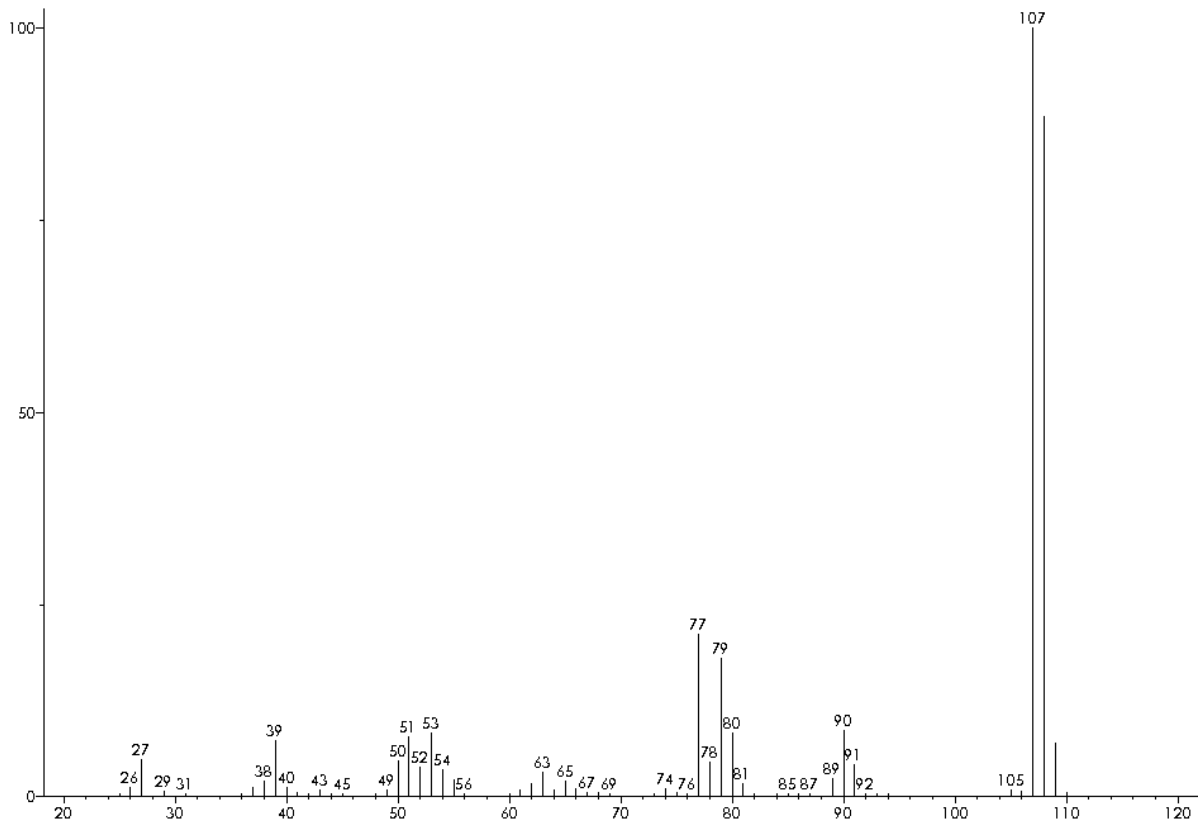
**Table 4.8: Phenolic compounds identified from the SPME traps from 6 days fed virgin females of *R. appendiculatus***

Peak No.	Retention time	Peak area	Compound identity
1	10.7404	8204493	Phenol
2	13.6654	3741463	<i>p</i> -cresol
3	17.3977	4330189	2,6-dichlorophenol

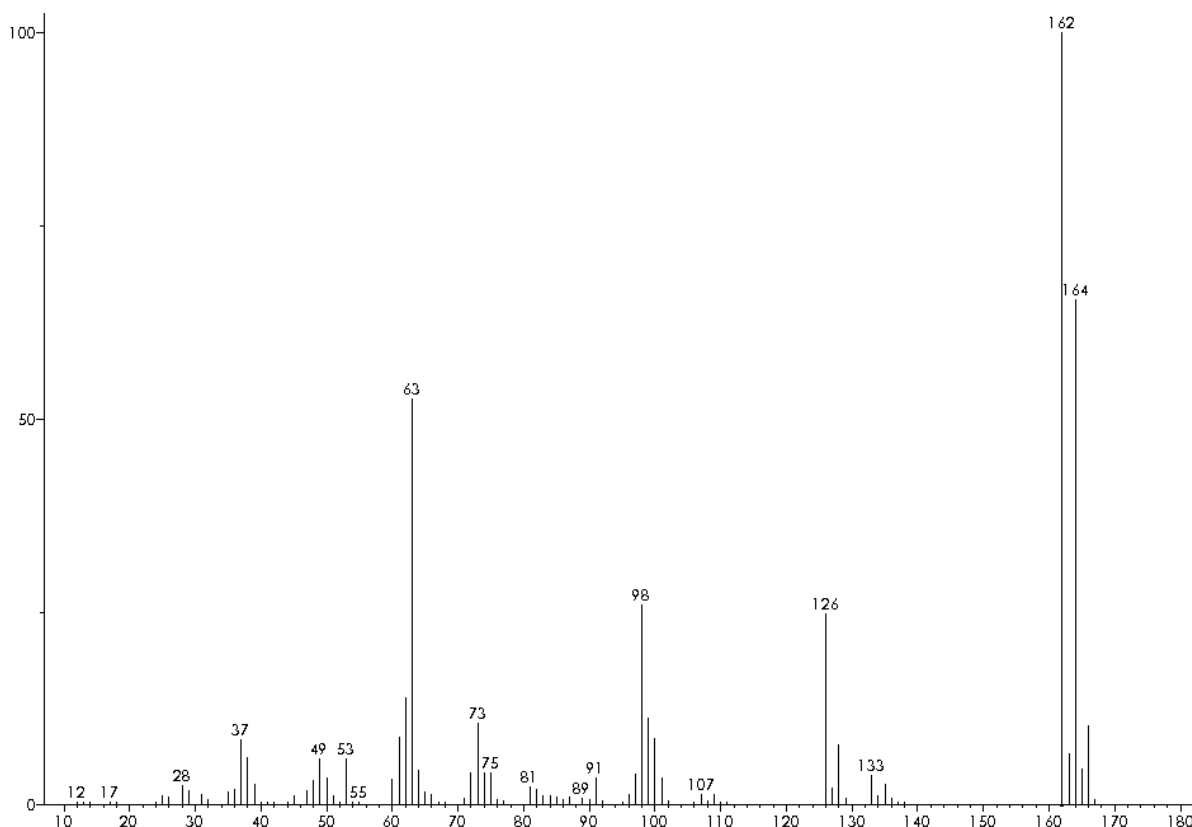
The mass spectra of the three compounds are shown in figures 4.5-4.7.



**Figure 4.5:** Mass spectrum of phenol (1)



**Figure 4.6:** Mass spectrum of *p*-cresol (2)



**Figure 4.7:** Mass spectrum of 2, 6-dichlorophenol (**3**)

Phenol (**1**), *p*-cresol (**2**) and 2, 6-dichlorophenol (**3**) have been reported earlier in fed virgin *R. appendiculatus* females. Compounds (**1**) and (**2**) were first reported by Wood *et al.*, (1975) while compound (**3**) was first reported by McDowell & Waladde (1986). All the three compounds were isolated by solvent extraction. This is however, the first time to report all the three compounds using a relatively new isolation technique (SPME).

The mass spectra in figure 4.5-4.7 of the three phenols exhibit intense molecular ion peaks, a characteristic of aromatic compounds. For compounds (**1**) and (**3**) the molecular ion peak is also the base peak. For compound (**2**) however, M-1 peak is larger than the molecular ion peak as a result of facile benzylic C-H cleavage. Peaks resulting from loss of CO (M-28) and CHO (M-29) are common to the three phenolic compounds.

### 4.3 Percentage attractancy in two choice climbing bioassays

Responses of unfed male *R. appendiculatus* ticks to synthetic phenol, *p*-cresol, 2, 6-dichlorophenol and their blends in a two choice climbing bioassay are shown in table 4.9.

2,6-Dibromophenol, as an analogue of the three phenols was also tested.

**Table 4.9 : Relative percentage attractancy of unfed male *R. appendiculatus* by the GC-MS identified phenols, their blends and 2, 6-dibromophenol**

Test compound/ blend	Dose (ng/ $\mu$ l)	Mean relative % attractancy $\pm$ SE	P-value
Phenol	1.1	-22.99 $\pm$ 2.70 <sup>a</sup>	<0.001
	5.5	37.34 $\pm$ 2.27 <sup>b</sup>	
	11.0	44.10 $\pm$ 1.59 <sup>b</sup>	
<i>p</i> -Cresol	0.5	37.60 $\pm$ 0.47 <sup>a</sup>	<0.001
	2.5	50.41 $\pm$ 1.88 <sup>b</sup>	
	5.0	34.72 $\pm$ 1.39 <sup>a</sup>	
2,6-Dichlorophenol	0.6	3.71 $\pm$ 1.89 <sup>a</sup>	<0.001
	3.0	6.88 $\pm$ 1.59 <sup>a</sup>	
	6.0	48.89 $\pm$ 1.11 <sup>b</sup>	
2,6-Dibromophenol	1.0	-21.32 $\pm$ 0.67 <sup>a</sup>	<0.001
	5.0	37.90 $\pm$ 2.76 <sup>b</sup>	
	10.0	28.38 $\pm$ 1.03 <sup>c</sup>	
Phenol, <i>p</i> -cresol	1.1, 0.5	43.93 $\pm$ 2.14 <sup>a</sup>	<0.001
	5.5, 2.5	50.90 $\pm$ 1.77 <sup>b</sup>	
	11.0, 5.0	39.61 $\pm$ 1.65 <sup>a</sup>	
	5.0		
Phenol, 2,6-dichlorophenol	1.1, 0.6	24.09 $\pm$ 2.15 <sup>a</sup>	<0.001
	5.5, 3.0	30.99 $\pm$ 2.34 <sup>a</sup>	
	11.0, 6.0	6.23 $\pm$ 1.45 <sup>b</sup>	
	6.0		
<i>p</i> -Cresol, 2,6-dichlorophenol	0.5, 0.6	41.40 $\pm$ 3.12 <sup>c</sup>	<0.001
	2.5, 3.0	20.66 $\pm$ 2.87 <sup>b</sup>	
	5.0, 6.0	-17.31 $\pm$ 2.87 <sup>a</sup>	
Phenol, <i>p</i> -cresol, 2,6-dichlorophenol	1.1,0.5, 0.6	10.37 $\pm$ 0.37 <sup>a</sup>	<0.001
	5.5,2.5, 3.0	46.90 $\pm$ 1.84 <sup>b</sup>	
	11.0,5.0 ,6.0	45.77 $\pm$ 2.17 <sup>b</sup>	

Means with the same superscript for each test compound/ blend are not significantly different (One-way ANOVA,  $\alpha = 0.05$ , SNK test).

All the identified phenols and their blends were attractive to the unfed male *R. appendiculatus* ticks. This agrees with earlier studies by Wood *et al.*, (1975). 2,6-Dibromophenol was also attractive to the unfed male ticks. Whether this attraction to 2, 6-dichlorophenol is a result of lack of receptor discrimination in this tick species or because the compound is produced by the feeding female is subject to further investigation

Among the individual compounds tested, *p*-cresol at a dose of 2.5ng/ $\mu$ l ( $50.41 \pm 1.88\%$ ) was the most attractive while the most attractive blend consisted of phenol and *p*-cresol at a dose of (5.5 phenol, 2.5 *p*-cresol) ng/ $\mu$ l ( $50.90 \pm 1.77\%$ ). Phenol at a dose of 1.1 ng/ $\mu$ l, 2,6-dibromophenol at a dose of 1.0 ng/ $\mu$ l, and a blend consisting *p*-cresol and 2,6-dichlorophenol at a dose of (5.0 *p*-cresol, 6.0 2,6-dichlorophenol) ng/ $\mu$ l however, appeared to be avoided by ticks.

For each individual compound or blend, a significant attractancy was observed at one particular dose. This suggests that there is a concentration threshold that enlists an optimal receptor response. These phenols have been reported in other tick species as well. For instance 2, 6-dichlorophenol has been reported in 15 species (Sonenshine, 2004) while phenol and *p*-cresol has been reported in 7 species (Wood *et al.*, 1975). Receptor discrimination based on concentration threshold would therefore be the only way to minimize interspecies interactions especially when different species infest the same host. As a way of maintaining intra-species integrity, a particular species would emit these phenols in a unique ratio. In performing bioassays, this ratio should therefore be taken into consideration.

Phenol, *p*-cresol and 2, 6-dichlorophenol have been suggested as sex pheromones in this tick species (Wood *et al.*, 1975; McDowell & Waladde, 1986). In our on-host studies, we have

shown that both unfed and fed males are comparably attracted by the feeding females as was evidenced by the significant attachment of the males adjacent to the feeding females. Copulation was observed where fed males were involved. We believe that this significant attraction, subsequent attachment and copulation were mediated by these phenols. While copulation demonstrated that they may have acted as attractant sex pheromones, the attraction and attachment of the unfed males adjacent to the pheromone emitting females suggests that these compounds induces attachment and aggregation as well.

## CHAPTER FIVE

### CONCLUSIONS AND RECOMMENDATIONS

#### 5.1 Conclusions

*R. appendiculatus* has been the subject of many scientific studies aimed at its control because of its association with East Coast Fever in cattle. Exclusive use of synthetic acaricides has been the principal way of controlling ticks for decades. More recent studies have however, shown potential of tick semiochemicals such as pheromones in controlling ticks. This later approach demands an understanding of the ticks' behaviour such as feeding and mating. Equally important is the identification of the pheromone(s) mediating such behaviours.

Tick feeding is the main avenue through which ticks transmit disease causing pathogens to the hosts. Ticks seem to have developed attachment and feeding patterns unique to a particular species. In some *Amblyomma* species for instance, the male tick plays a critical role in the attachment of conspecific female ticks. This doesn't seem to be the case in *R. appendiculatus*. Feeding also aids in the physiological development of ticks. Male and female ticks for instance, become sexually mature during feeding. Synthesis and emission of pheromones that mediates on-host behavioural interactions also occurs during feeding for all metastriate ticks. The composition of the pheromones emitted is unique to a particular species. In instances where the composition is the same, the ratio in which such compounds are emitted is however, not the same.

Isolation of pheromones from ticks has over time been done by solvent extraction or effluvial collection. Both methods employ the use of organic solvents. With calls to minimise use of organic solvents, focus is shifting to isolation techniques that would minimise their use. Solid phase micro-extraction technique is such a method. In view of the above discussion, this study makes the following conclusions.

- i. Female *R. appendiculatus* ticks readily attach on the host even in the absence of males.
- ii. After feeding for at least 4 days, female *R. appendiculatus* ticks not only attract partially fed and sexually mature males for copulation but also attract and induce the attachment of conspecific males close to them.
- iii. Female ticks become most attractive to the males on day 5 of feeding. During this period the females emit phenol, *p*-cresol and 2,6-dichlorophenol in the ratio 11:5:6. These phenols are believed to mediate the on-host behavioural interactions between these ticks.
- iv. The role of male *R. appendiculatus* ticks is not limited to mating. Presence of male ticks induces increased feeding of females. The time the male joins the female on the host and the physiological state of the male (whether fed or unfed) by the time it joins the female has an impact on the repletion time and engorged mass of the female.
- v. Solid phase micro-extraction is a rapid technique of isolating volatile pheromones from ticks.
- vi. 2,6-dichlorophenol, an analogue of the identified phenols is also attractive to male *R. appendiculatus* ticks.
- vii. For each individual phenol or blend phenols tested in the climbing assays, a significant attractancy was observed at one particular dose suggesting that there is a concentration threshold that enlists an optimal receptor response.

## 5.2 Recommendations

Based on this study's findings, the following recommendations are made.

- i. On-host studies be carried out using synthetic 2, 6-dichlorophenol, phenol and *p*-cresol to ascertain their specific roles in adult *R. appendiculatus* ticks.
- ii. Further investigations be carried out to establish whether 2, 6-dibromophenol is emitted by *R. appendiculatus* feeding female ticks.
- iii. 2, 6-dichlorophenol, phenol and *p*-cresol be employed in field trials to test their efficacy in semiochemical aided tick control strategies.
- iv. Control of *R. appendiculatus* ticks can target the male ticks.

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