STUDIES ON PHEROMONES AND OTHER SEMIOCHEMICALS OF THE AFRICAN BROWN EAR TICK, *RHIPICEPHALUS APPENDICULATUS* NEUMANN

BY

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A THESIS SUBMITTED IN PARTIAL FULFILMENT FOR THE DEGREE OF MASTER OF SCIENCE OF KENYATTA UNIVERSITY

DEPARTMENT OF ZOOLOGY

1991

DECLARATION

This thesis is a result of my original work, except where acknowledged in the text and has not been submitted for a degree in any other University

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To Cosmas, for his patience and understanding throughout the period of the study, and to our children, Antonette and Linda.

ACKNOWLEDGEMENTS

I am grateful to Kenyatta University for offering me the chance to undertake this study. I thank the Zoology Department and its entire staff for the assistance they gave me while doing the research. I wish to acknowledge with thanks Deutscher Akademischer Austauschdienst (DAAD) for sponsoring this study through Kenyatta University.

I wish to express my gratitude to the Director of the International Centre of Insect Physiology and Ecology (ICIPE), Prof.T.R. Odhiambo, for accepting my request to carry out this research at the centre. I am also grateful to the programme leader, Livestock Ticks Research Programme, Prof.Olusegun O.Dipeolu, for providing the materials which I used in my research work.

I am most grateful to Professors Ochon'g Okelo, Olusegun O.Dipeolu, and Dr.Isaac O.Jondiko for their keen interest in the study, general assistance, and their dedication in supervising the actual research work. My sincere gratitude goes to Professor Ahmed Hassanali for constant advice and suggestions on chromatography and bioassays. I also thank Dr.Suleiman Essuman, Mr.John Chiera, Mr.J.F.Omollo, and Mrs.Esther Mwangi for their encouragements.

It would not have been possible for me to do the work without the technical assistance of Mr.Edward Nyandat who helped with the chromatography; Messrs.Joseph Ndun'gu, Patrick Muchiso and Mark Kimondo who helped with the rabbits; and Mr.Patrick Oluya who fabricated the glassware for bioassays. I thank Mrs.Mary Kichamu and Mrs.Pauline Wangai for their assistance in using word processor and for providing materials for, and typing parts of the thesis; and Messrs.Henry Meena, Onyango Okello and James Netya for their assistance in using Lotus 123.

Last, but not least, I would like to acknowledge support from all my friends and colleagues, particularly Lydia Kutima, Eunice Gai, Patrick Mbugi, Peter Kabii and James Nyarango; and my relatives, especially my husband Cosmas, for his understanding and moral support throughout the study period.

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ABSTRACT

Ticks are of great concern all over the world because of the diseases they transmit in livestock, resulting in a reduction in livestock productivity. Studies of pheromones and other semiochemicals of ticks have shown promise for use in integrated pest control strategies.

Male Rhipicephalus appendiculatus (Neumann), fed for 8 days, and female ticks, fed for two days, were found to attract both unfed male and female conspecific ticks on rabbits. Male tick extracts, when placed on rabbits, were found to attract both male and female ticks. The extracts also attracted male and female ticks in T-tube assays.

Cattle ear swabs attracted males and females in the T-tube assays. However, swabs from the legs, back, perineum and belly did not attract the adults, nymphs or larvae. Swabs from the ears were found to repel the nymphs and larvae. 2,6-dichlorophenol was found to be an attractant for both unfed male and female ticks in a T-tube assay system, and for nymphs and larvae in a Y-tube assay system. Only fed males were attracted to rabbit ears treated with 2,6-dichlorophenol. Chromatographic analyses revealed the presence of 2,6-dichlorophenol in both unfed, and fed male and female ticks. However, fed female ticks contained more of the phenol than fed male ticks.

Mixtures of 2,6-dichlorophenol and ear swabs, and of ear swabs and male tick extracts, produced inhibitory responses from the adult ticks, while responses from nymphs and larvae were inhibited by a mixture of ear swabs and 2,6-dichlorophenol. A mixture of 2,6-dichlorophenol and male tick extract did not affect responses from the adult ticks.

CHAPTER ONE

GENERAL INTRODUCTION

Rhipicephalus appendiculatus is the most important tick species in Kenya, since it is the principal vector of Theileria parva, the protozoon which causes East Coast Fever in cattle. This tick species also infests other domesticated animals, as well as wild animals, causing anaemia, damage to the skin, and secondary infections by bacteria, through the wounds they inflict in the animals. It also transmits the virus which causes Nairobi sheep disease. It is found in warm, humid areas; few cases are reported in dry sub-humid, semi-arid, and arid areas (Walker, 1974).

There is need to control these ticks, because of their economic importance to man and livestock. The commonest method of controlling ticks, at present, is acaricide application, especially by dipping. However, some African tick species have developed resistance to most of the available acaricides and this is a threat to livestock

production (Urquhart et al., 1987). Dipping the domestic stock has to be done regularly, making this method of control costly; for example, 100 ml of Clexon costs K.Shs.1,474.50. Furthermore, the cost of developing new acaricides, in relation to the economic return expected before resistance occurs, has discouraged research leading to new chemicals for tick control (Durand, 1976). Therefore, alternative methods of control need to be developed (Whitehead, 1965, 1973; Drummond, 1970). Concern for environmental safety has caused a shift in control strategies toward the utilization of methods such as biological control, anti-tick vaccines, breeding of tick resistant cattle, sterile hybrid techniques and naturally occurring biological attractants such as pheromones and kairomones (Faustini et al., 1981; Gladney et al., 1974a; Rechav and Whitehead, 1978; Ziv et al., 1981). These methods offer distinct advantages in efficacy and safety as compared to strategies based solely on acaricides.

According to Karlson and Luscher (1959), who first proposed the definition of pheromones (the most widely studied group of semiochemicals), the latter are secreted to the outside by an individual, and received by a second individual of the same species, as a result of which a specific reaction is elicited. Studies, however, have shown

that some pheromones can act across species (Norval and Rechav, 1979; Rechav, 1978; Gladney, 1971; Sonenshine et al., 1982a)

Tick pheromones were first reported in Dermacentor variabilis (Say), Amblyomma americanum (Linnaeus) and A.maculatum (Koch) by Berger et al. (1971). Since then, pheromones have been described in a wide variety of tick species. These include assembly pheromones, sex pheromones and aggregation-attachment pheromones.

Assembly pheromones are insoluble in organic solvents, non-volatile, and environmentally persistent. They arrest the normal ambulatory activity of wandering ticks, contributing to the formation of tight clusters of inactive ticks. They have been reported in both soft and hard ticks (Graf, 1975; Treverrow et al., 1977; Leahy et al., 1973, 1975a,b; Leahy, 1979; Otieno et al., 1985).

Sex pheromones are chemicals which are secreted by animals of one sex and cause behavioural reactions in the opposite sex, thus facilitating mating (Shorey, 1976); they regulate one or more components of the mating process (Sonenshine, 1984). Various workers have reported the presence of sex pheromones in a number of tick species

(Sonenshine et al., 1976; Sonenshine et al., 1984; Chow et al., 1975; Berger, 1972; Berger, 1974; Leahy and Booth, 1978; McDowell and Wallade, 1986; Andrew and Bull, 1982; Obenchain, 1984; Schlein and Gundess, 1981; Silverstein et al., 1983; Sonenshine et al., 1974; Sonenshine et al., 1982a; Sonenshine et al., 1985).

Aggregation-attachment pheromones induce ticks to aggregate and attach around other, previously attached, feeding individuals (Sonenshine et al, 1982b). This ensures that ticks attach preferrentially to hosts on which they are likely to feed successfully (Norval et al., 1988). This pheromone has been described in a number of Amblyomma species. (Rechav et al., 1977a, b; Gladney et al., 1974a, b; (Norval and Rechav, 1979; Rechav, 1978; Obenchain et al., 1977; Obenchain and Ojowa, 1978; Obenchain et al., 1979).

Apart from the pheromones produced by ticks, other semiochemicals may also play an important role in host recognition, selection of feeding sites, and subsequent attachment of ticks to hosts. One such group of semiochemicals are the kairomones which are of adaptive benefit to the organisms which receive them. It has been suggested that kairomones may have been pheromones that evolutionarily backfired (Blum, 1978).

In view of the advantages and promise of using semiochemicals in control of ticks, this study was undertaken to establish the roles, if any, of certain semiochemicals in R.appendiculatus.

CHAPTER TWO

THE ROLE OF 2,6-DICHLOROPHENOL IN THE TICK, R.APPENDICULATUS

INTRODUCTION

2,6-dichlorophenol (2,6-DCP) was first identified as a stimulant of sexual behaviour in the lone star tick, A.americanum (Berger, 1972). Berger (1974) showed that the compound is biological in origin and is synthesised by the ticks. It has since been found in several other species of hard ticks. Wood et al. (1975) showed that adult R.appendiculatus males were attracted to 2,6-dichlorophenol in a T-tube assay system. They did not test female ticks in their investigation. Although these investigators did not detect 2,6-dichlorophenol in fed female R.appendiculatus tick washes, later, McDowell and Wallade (1986) reported the presence of this phenolic compound in the ticks. The latter workers did not quantify this compound in the ticks by age, feeding duration, or mating. It is necessary to determine the trend of 2,6-dichlorophenol concentration with age and physiological state, since changes in the concentration of this compound can serve as indicators of important physiological events in the life of this tick. McDowell and Wallade (1986) postulated that 2,6-dichlorophenol may act as a sex pheromone in this tick species, as in other species of ticks. Production of a chemical in significant quantities

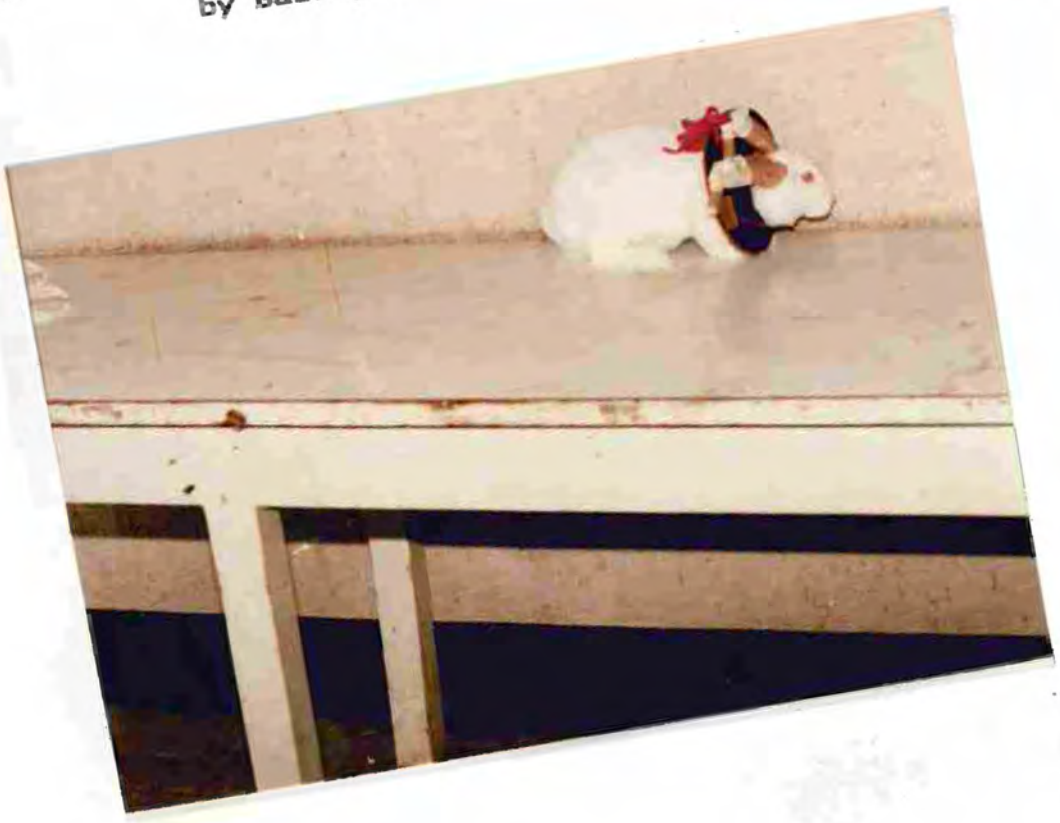
by members of only one sex and its mediation in mating behaviour of the other sex, lends credence to the conclusion that the compound serves as a sex pheromone in that species. In R.appendiculatus, however, the compound is found in all the developmental stages, except eggs (McDowell and Wallade, 1986). This observation does not support the definition of a sex pheromone. This study was, therefore, set up to examine the changes in 2,6-dichlorophenol content in the adult ticks by age, different feeding periods, and in mated females. Another objective was to carry out behavioural bioassays on the adults, nymphs and larvae, using 2,6-dichlorophenol,in order to determine its role in the ticks.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Laboratory colonies of R. appendiculatus were maintained at the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi. The colonies had been maintained for approximately twenty years without the introduction of outside stock. Ticks were allowed to feed on the ears of New Zealand white rabbits, according to the method described by Bailey (1960) (Figure 1). Engorged ticks were collected and transferred to a dark incubator at 28°C and 85% relative humidity (R.H), for them to moult. Humidity was maintained using supersaturated potassium chloride solution. Ticks which had moulted were kept in a dark incubator at 18°C and 85% R.H. White rabbits, weighing approximately 2kg, were used for in vivo bioassays and for feeding the ticks. The rabbits were confined in standard cages, and fed on rabbit pellets, carrots and cabbages.

Figure 1 Method of feeding ticks on rabbits, as described by Bailey (1960)



EXPERIMENTAL PROCEDURE

QUANTITATION OF 2,6-DICHLOROPHENOL FROM THE TICKS

Quantitation of 2,6-dichlorophenol from the males and females of this tick species was done by days post-moulting, different feeding durations, and for mated female ticks. Engorged nymphs were collected and transferred to a dark incubator at 28^{o}C and 85% R.H. The ticks were checked daily and the moulted ticks were collected, sexed, and kept separately. Extraction of 2,6-dichlorophenol was carried out on ticks which were 2, 16, 30, 60, and 90 days post-moulting. Some one month-old ticks from the same group were fed on rabbits for 5 and 8 days; extracts were obtained from them and the extracts were used for chromatographic analysis. Three samples of 10 adults of each sex, in each age group or feeding category, were collected in 5 ml vials. The vials were sealed tightly with aluminium foil-lined caps, and the extraction carried out as described below.

Extraction of 2,6-Dichlorophenol

Extraction of 2,6-dichlorophenol was done by immersing ten ticks in 4 ml of hexane, at 4^{o}C for 48 hours, after which the extract was filtered, and kept at 4^{o}C. This procedure was repeated using a minimum volume of hexane (2 ml) for the same period of time.

The extracts were combined and partitioned with 25 ml of 1N NaOH solution into a lipid and an aqueous phase. The aqueous phase was acidified with 50 ml of 2N hydrochloric acid upto pH 1, and then extracted with 100 ml of hexane to recover the phenols and acids. This fraction was further separated by extraction with 25 ml of 5% aqueous sodium bicarbonate, to remove strong acids. Each of the steps above was carried out twice. The lipid and strong acids fractions were then discarded. The phenolic fraction (80 ml) was collected and dried using 3g of anhydrous sodium sulphate. This fraction was then evaporated under vacuum to a small volume, in a Buchi RE 111 Rotavapor at 45mm Hg and a Buchi 461 Water Bath (Switzerland), operated at 40°C. It was then transferred to a 5 ml vial, evaporated to dryness under a stream of nitrogen, weighed, and later dissolved in 2 ml of hexane.

Analysis of Tick Extracts

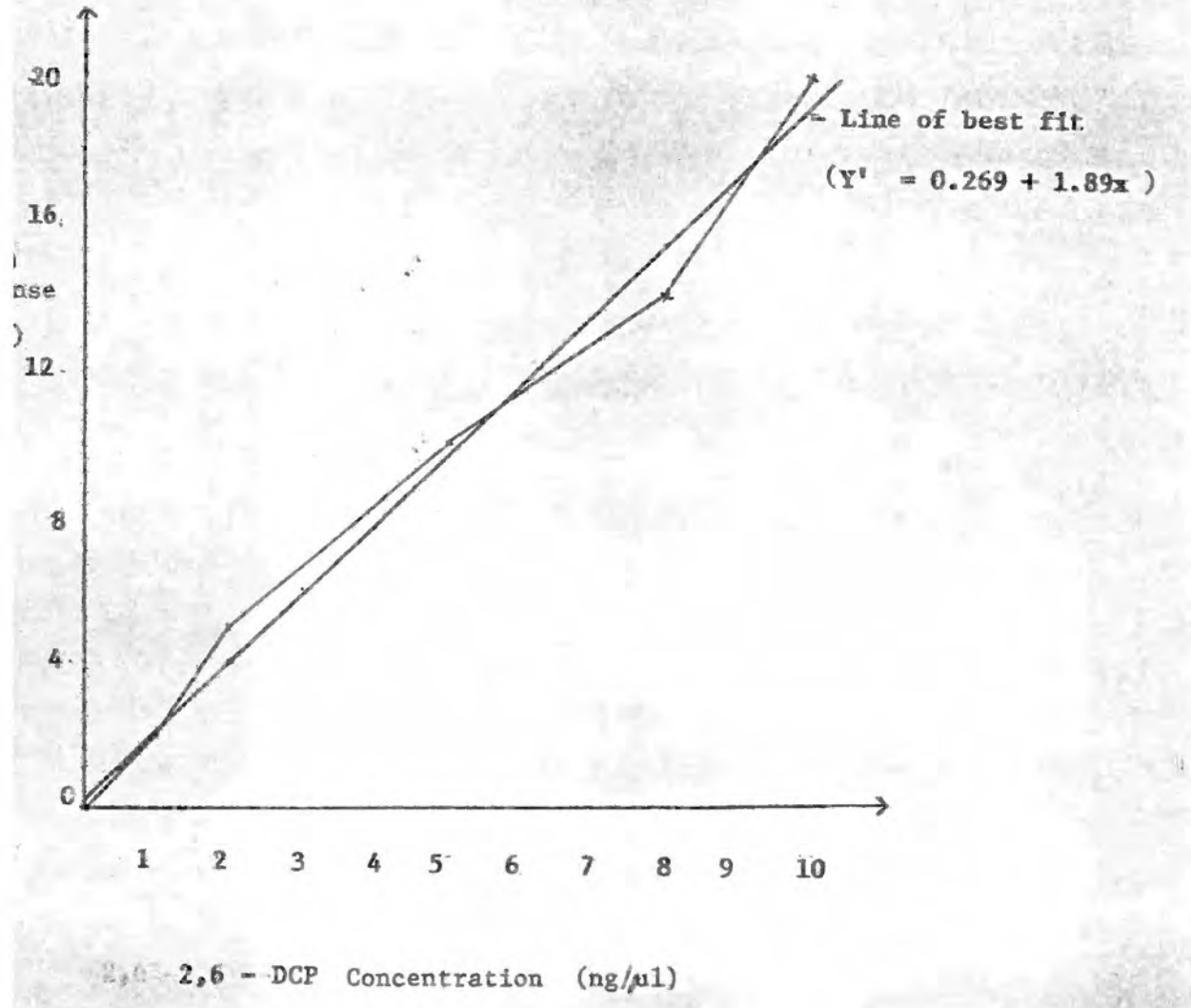
Aliquots of the tick extracts were injected in a Packard model 428 column gas chromatograph (GC), equipped with a Flame Ionization detector (FID). The column employed was a 25-mm x 0.32-mm x 0.52 um Cross Linked Methyl Silicone Gum (Ultra 1), operated at an initial temperature of 50°C, with a rise of 5°C/min and a final temperature of 150°C.

The carrier gas used was nitrogen. The detector and injector temperatures were 240 and 250^{o}C, respectively. Co-injection of known amounts of 2,6-dichlorophenol were done to confirm the identification, and to quantitate the amounts of this compound in the ticks. Quantities of 2,6-dichlorophenol in the extracts were calculated by use of a calibration curve (Figure 2), drawn after injecting known concentrations of pure 2,6-dichlorophenol into the GC.

PERCENTAGE RECOVERY OF PHENOLS

The efficiency of the isolation (extraction) procedure described above was determined by following the same procedure, using 10 ml of 10ng/ul 2,6-dichlorophenol dissolved in hexane. A similar quantity of HPLC grade hexane was also run through the procedure. These chemicals were then injected into the GC for analysis as above. The control used was 10ng/ul 2,6-dichlorophenol which was not partitioned as described in the procedure. The quantity of phenols recovered was then calculated as a percentage of the total before extraction (isolation).

FIG. 2: CALIBRATION CURVE OF FLAME IONIZATION DETECTOR (FID) FOR THE DETERMINATION OF THE CONCENTRATION OF 2,6 - DCP IN TICK EXTRACTS



BIOASSAY OF 2,6-DICHLOROPHENOL

Various concentrations of commercial 2,6-dichlorophenol (99%, Aldrich Chemical Co.Ltd, Gillingham, Dorset-England) were made by dissolving 1mg of the compound in 20ml hexane, and making serial dilutions. The concentrations used were 0.005, 0.01, 0.05, 0.1, 0.5, and 1.0, and 10 ng/ul. These solutions were tested against males, females, nymphs, and larvae of this tick species using the T-tube, Y-tube, and on rabbits, as described below. For the larvae, additional concentrations of 0.001, 25, 50 and 500 ng/ul were also used.

Bioassays Using a T-tube

This was used for the adults only. It was carried out essentially as described by Wood *et al.* (1975), using 10 ticks in each replicate. In brief, a glass T-tube (2 cm stem, 6 cm top, and 8 mm ID) was used (Figure 3). 100 ul of 2,6-dichlorophenol, dissolved in hexane, was dropped on a filter paper disc (Whatman No.1, 3 cm diameter) and the solvent allowed to evaporate (ca.3 minutes). The disc was made into a plug and inserted into one arm of the T-tube. The other arm (control) was plugged with a similar paper disc treated with 100 ul of solvent. The T-tube was then placed over a single unfed male or female tick, which was used at each trial. The tick climbed up the stem and, on

Figure 3 The T-tube which was used for *in vitro* bioassays of 2,6-DCP, swabs from cattle, and male tick extracts, on adults.

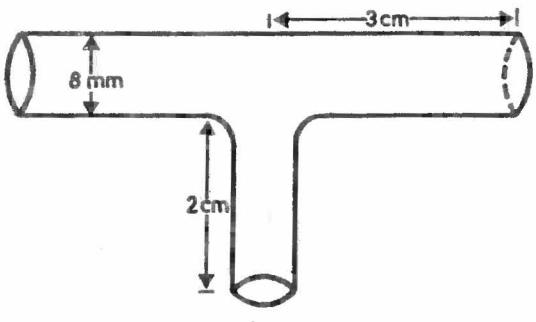


Fig 3: T– tube

reaching the horizontal portion, made a choice between either of the arms. The first choice of the tick was recorded, to give the attractant activity of the extract. The test was replicated 10 times, and the mean number of ticks that chose either arm of the T-tube was calculated. The mean number of ticks in the experimental and control arms of the T-tube were then compared using student's t-test.

Bioassays Using a Y-tube

The Y-tube was used for the nymphs and larvae, because of the ease of introducing these immature ticks into the tube through an introduction pot (A) (Figure 4). The length of each of the anterior arms (B) measured 3 cm while the posterior arm (C) was 4 cm long. The angle between the anterior arms was 35^{o}. The internal diameter of the glass tube was 8 mm. An outlet (D) at the junction of the three arms permitted air to be drawn out of the olfactometer by means of a water pump. This ensured that odours from either side of the arms did not get into the lower arm. One anterior arm of the olfactometer was plugged with filter paper treated with 100 ul of 2,6-dichlorophenol, while the other arm was plugged with filter paper treated with 100 ul of hexane. Approximately 100 larvae, or 20 nymphs, were put into the olfactometer through an introduction pot at the posterior arm. The ticks moved upto the junction of the

Figure 4 The Y-tube which was used for *in vitro* bioassays of 2,6-DCP, swabs from cattle, and male tick extracts, on nymphs and larvae.

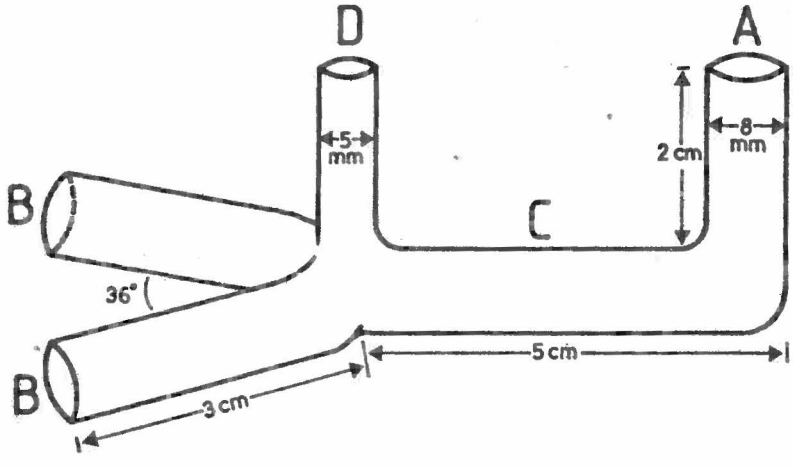


Fig. 4: Y- tube

olfactometer arms and made a choice between the experimental and control arms. The test was replicated 10 times. For each replicate, a time limit of 60 seconds was allowed before the ticks in the two arms were counted. The mean percentage of ticks that chose either of the two arms was then calculated, and compared using student's t-test.

Bioassay of 2,6-DCP on Rabbits

Twenty-five male or female ticks were introduced to the right ears of 4 rabbits, under ear bags. After 24 hours, the single ear bags were removed and both ears enclosed in a larger ear bag. 100 ul of 0.5 ng\ul 2,6-dichlorophenol was then applied to the left ear of the rabbits everyday, for 8 days. On control rabbits, only 100 ul of solvent was applied. The ears were observed every day and the number of ticks attached on the left ears recorded. Fifty nymphs, or 100 larvae were also applied separately to the right ears of other rabbits. After attachment (1 day), the single ear bags were removed and both ears enclosed in a larger ear bag. The concentration of 2,6-dichlorophenol used for the nymphs and larvae was 0.05 and 0.001 ng\ul, respectively. The rest of the experiment was carried out as described above for the adults. The percentage of ticks in the

experimental and control ears of the rabbits was calculated, and compared using X^2 test.

RESULTS AND DISCUSSION

TABLE 1

2,6-DCP CONTENT IN DIFFERENT AGES / STATES OF R.APPENDICULATUS

Days post-emergence / post-fed / mated	Amount of 2,6-DCP (ng/tick + S.E.)		
	Males	Females	P Value
0	0	0	
2	0.40 ± 0.00	0.25 ± 0.15	0.2
16	0.47 ± 0.29	0.53 ± 0.29	0.2
30	1.43 ± 0.17	1.53 ± 0.26	0.05
60	1.17 ± 0.43	0.63 ± 0.27	0.2
90	0.67 ± 0.44	0.60 ± 0.20	0.2
30 (fed 5 days)	0.28 ± 0.12	1.35 ± 0.17	0.01
30 (fed 8 days)	0.00 ± 0.00	2.80 ± 0.80	0.01
Mated females (fed 5 days)		1.05 ± 0.28	

2,6-dichlorophenol was found in both male and female R.appendiculatus adults (Table 1). The amount of this compound increased significantly in both males and females, from two day-old ticks to thirty day-old individuals. In

the unfed ticks, the highest concentrations were found in ticks 30 days post-emergence. Although the amount of 2,6-dichlorophenol in both males and females decreased gradually after 30 days, the difference between the concentration at 30 days and each of the age groups extracted was not significant.

Feeding resulted in a significant decrease ($p < 0.001$) in the concentration of 2,6-dichlorophenol in 30 days-old male ticks, but not in females of the same age. Female ticks fed for five days, however, contained significantly ($p<0.001$) more 2,6-dichlorophenol than male ticks of the same age and feeding category. Eight days-fed male ticks had no detectable 2,6-dichlorophenol. Mating did not affect the amount of this phenolic compound in five days- fed females.

It is apparent that in R.appendiculatus, newly moulted male and female ticks already synthesize 2,6-dichlorophenol. The release of this compound increases significantly until the ticks are 30 days old, after which it gradually declines. The decline in the concentration of this compound in the unfed male and female ticks was not significant and could be due to partial degradation of the stored compound.

The quantities of 2,6-dichlorophenol found in this study correlate with the findings by Joyner and Purnell (1968), who observed that ticks are more inclined to attach to host skin when they are about five weeks old. After attachment, the ticks start to feed, and mating commences 5-6 days later. Thus the highest concentration of 2,6-dichlorophenol appears to be associated with the period when the ticks are more likely to feed, and hence attain sexual maturity.

In the females, no significant reduction in the concentration of this phenolic compound occurs following feeding. In female D.variabilis and D.andersoni, feeding appears to cause release of the stored compound (Sonenshine et al., 1974; Sonenshine et al., 1982b; Sonenshine et al., 1984). The observations from this study suggest that female R.appendiculatus more or less maintain the level of 2,6-dichlorophenol released. According to the scheme of synthesis, storage and release of female-produced sex pheromones proposed by Sonenshine et al. (1979), release of the compound in response to feeding stimulus may cause renewed synthesis.

In unfed male R.appendiculatus, concentrations of 2,6-dichlorophenol that parallel those found in the females were recorded. In most cases, for a compound to be classified as a sex attractant, it should be present in one sex, but not

in the opposite sex of that particular species. This phenol also occurs in males of Amblyomma maculatum in amounts similar to those found in females of the same species (Kellum and Berger, 1977). Foveal glands (sex pheromone glands) have been found in male D.variabilis and H.dromedarii (Sonenshine et al., 1977; Sonenshine et al., 1983). Evidently, the glandular system needed to secrete and store 2,6-dichlorophenol is present in males of at least several ixodid ticks. Feeding, however, seems to cause release of the compound but no synthesis occurs to renew the compound. This is reasonable since at sexual maturity, only one sex would be expected to contain a compound considered as a sex attractant. The foveal glands appear much smaller in the males than in the females of D.variabilis (Sonenshine et al., 1977). This could also be the case in R.appendiculatus; the glands have not been identified in this tick species. The differing concentrations of 2,6-dichlorophenol in the 5 days-fed male and female ticks may help the males in locating their mates: In H.dromedarii, 2,6-dichlorophenol occurs at a higher concentration than in H.analoticum excavatum, and males of the two species respond only to the right concentration of pheromone produced by its conspecific female (Silverstein et al., 1983).

The reduction in the concentration of 2,6-dichlorophenol in the fed male ticks may also be an attempt to avoid the confusion that might ensue as the male ticks

become sexually mature. In ticks, sexual maturity commences following feeding. While these observations appear to support the view that 2,6-dichlorophenol may be a sex pheromone of R.appendiculatus, its presence in the unfed and fed male ticks, and even in the immature stages which never mate, suggest an additional role for the compound. The compound could be a by-product of metabolic reactions in the ticks, and its use as a pheromone may represent a specialised adaptation which evolved following the development of mechanisms for their release and adaptation of receptor mechanisms for their perception (Galun, 1974).

The quantities of 2,6-dichlorophenol found in this study were lower than those recorded by McDowell and Wallade (1986). It is not clear from their work, the age of ticks on which they carried out their analysis, and this could be a contributory factor to the differences in levels of 2,6-dichlorophenol recorded. These workers used the Electron Capture Detector (ECD) in their investigation while in the present study, the Flame Ionization Detector (FID) was used. However, it is important to note that the trend in the concentration of 2,6-dichlorophenol released by the ticks during their feeding paralleled that found by these workers.

In this study, the percentage recovery of phenolic extracts from the isolation procedure was 50%.

T-TUBE BIOASSAY OF 2,6-DICHLOROPHENOL (2,6-DCP)

TABLE 2

PERCENT RESPONSE (X ± S.E) OF *RHIPICEPHALUS APPENDICULATUS* MALES TO 2,6-DCP IN A T-TUBE

Conc.of 2,6-DCP(ng/ul)	2,6-DCP	Control	P Value
0.5	50.00 ± 5.375	50.00 ± 5.375	0.1
1.0	51.11 ± 3.514	48.89 ± 3.514	0.1
5.0	53.89 ± 3.889	46.11 ± 3.889	0.1
10.0	62.00 ± 5.121	38.00 ± 5.121	0.01
50.0	64.00 ± 2.211	36.00 ± 2.211	0.01
100.0	68.00 ± 5.538	32.00 ± 5.538	0.01
1000	11.80 ± 3.441	88.20 ± 3.441	0.01

The bioassay of 2,6-DCP on males showed response at concentrations of 10 ng/ul and above. Concentrations of 0.5 ng/ul, 1.0 ng/ul and 5.0 ng/ul did not elicit significant responses from the male ticks (Table 2). The highest mean percentage response for the males was observed at a concentration of 100 ng/ul. At a concentration of 1000 ng/ul, the ticks were repelled and did not move up the stem of the T-tube.

TABLE 3

PERCENT RESPONSE (X ± S.E) OF *RHIPICEPHALUS APPENDICULATUS* FEMALES TO 2,6-DCP IN A T-TUBE

Conc.of 2,6-DCP (ng/ul)	2,6-DCP	Control	P Value
0.5	52.45 ± 4.899	47.55 ± 4.899	0.1
1.0	53.00 ± 6.155	47.00 ± 6.155	0.1
5.0	57.78 ± 3.239	42.22 ± 3.239	0.01
10.0	59.00 ± 7.491	41.00 ± 7.491	0.1
50.0	73.00 ± 3.667	27.00 ± 3.667	0.01
100.0	71.00 ± 6.227	29.00 ± 6.227	0.01
1000	13.90 ± 5.242	86.10 ± 5.242	0.01

Females started showing significant ($P < 0.01$) responses at 5 ng/ul. A concentration of 50 ng/ul elicited the highest mean percentage response. The ticks showed repulsion from the chemical at a concentration of 1000 ng/ul (Table 3).

TABLE 4

PERCENT RESPONSE (X ± S.E) OF *RHIPICEPHALUS APPENDICULATUS* NYMPHS TO 2,6-DCP IN A Y-TUBE

Conc.of 2,6-DCP (ng/ul)	2,6-DCP	Control	P Value
0.5	72.43 ± 0.463	27.57 ± 0.463	0.01
1 0	73.63 ± 0.415	26.37 ± 0.415	0.01
5.0	78.62 ± 0.551	21.38 ± 0.551	0.01
10.0	76.50 ± 0.431	23.50 ± 0.431	0.01
50.0	75.71 ± 0.241	24.29 ± 0.241	0.01
100.0	52.72 ± 0.471	47.28 ± 0.471	0.2
1000	26.3 ± 0.840	72.7 ± 0.840	0.01

Nymphs showed significant responses ($P<0.01$) at all the concetrations tested. However, 100 ng/ul elicited significantly ($P< 0.01$) reduced responses, compared to that elicited by a concentration of 50 ng/ul (Table 4). As Figure 5 indicates, nymphs showed the highest mean percentage response, at 5 ng/ul, while for the adults, a higher concentration was required to elicit maximal response.

Figure 5 Attraction of R.appendiculatus to 2,6 -dichlorophenol in T-tube and Y-tube assays

TICKS ATTRACTED TO 2,6—DCP (%)

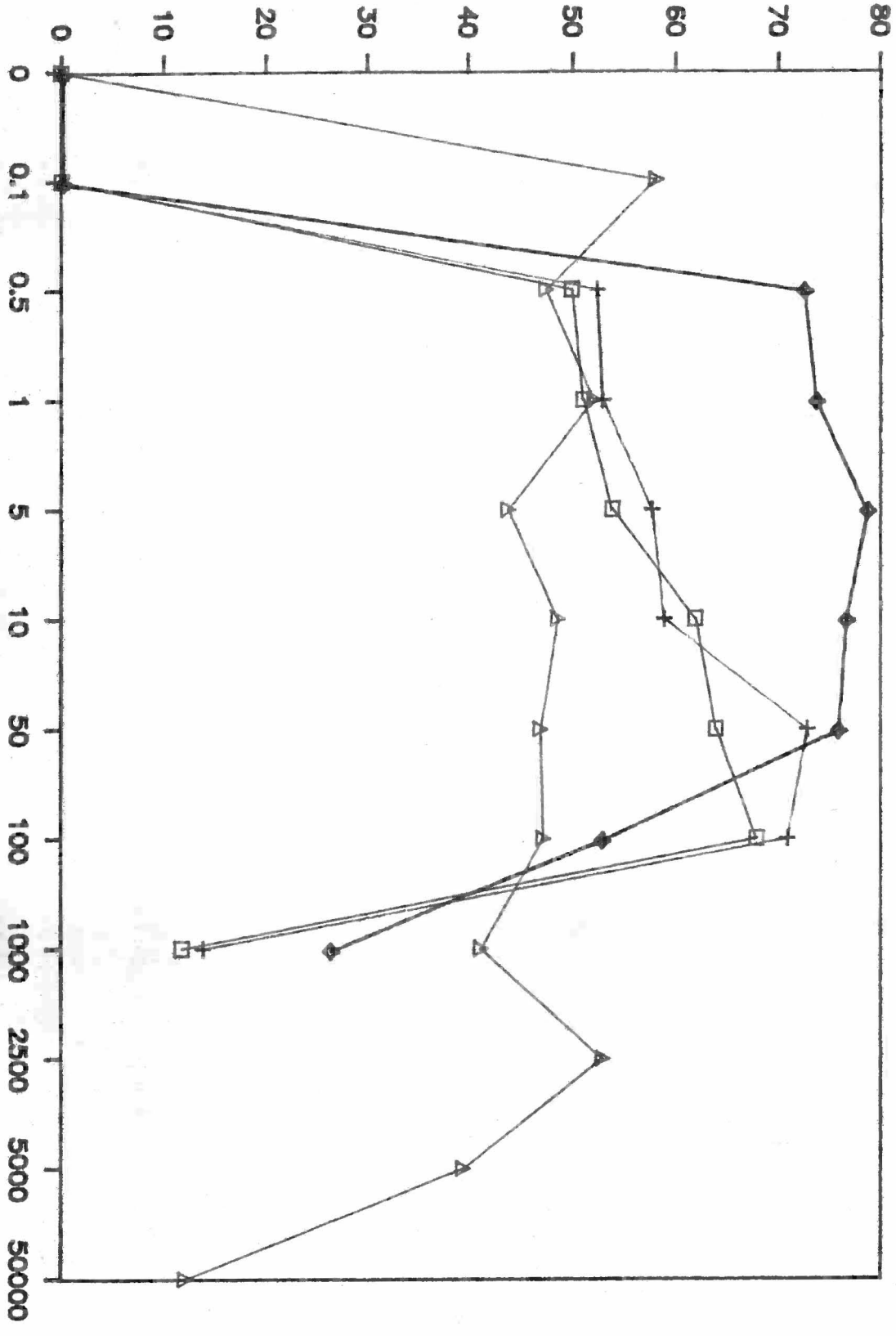


TABLE 5

PERCENT RESPONSE (X + S.E.) OF R.APPENDICULATUS LARVAE TO 2,6-DCP IN A Y-TUBE

Conc.of 2,6-DCP (ng/ul)	2,6-DCP	Control	P Value
0.1	58.1 ± 2.18	41.9 ± 2.12	0.05
0.5	45.7 ± 2.80	54.3 ± 5.31	0.2
1.0	52.2 ± 2.02	47.8 ± 2.44	0.2
5.0	43.9 ± 5.15	56.2 ± 6.60	0.2
10.0	48.7 ± 3.34	51.3 ± 2.81	0.2
50.0	47.0 ± 3.06	53.0 ± 2.21	0.2
100.0	47.3 ± 3.67	52.7 ± 2.49	0.2
1000	41.3 ± 3.41	58.7 ± 5.73	0.2
2500	53.0 ± 2.60	47.0 ± 2.41	0.2
5000	39.6 ± 5.88	60.4 ± 3.26	0.01
50000	12.2 ± 0.94	87.8 ± 1.55	0.01

Larvae showed significant attraction ($P < 0.05$) to 2,6-DCP only at 0.1 ng/ul. Significant repulsion ($P < 0.01$) was observed at 5000 and 50000 ng/ul. A lot of variation in the responses was observed at the other concentrations tested.

TABLE 6

PERCENT RESPONSE OF MALES (BY DAYS) TO 2,6-DCP ON RABBIT EARS

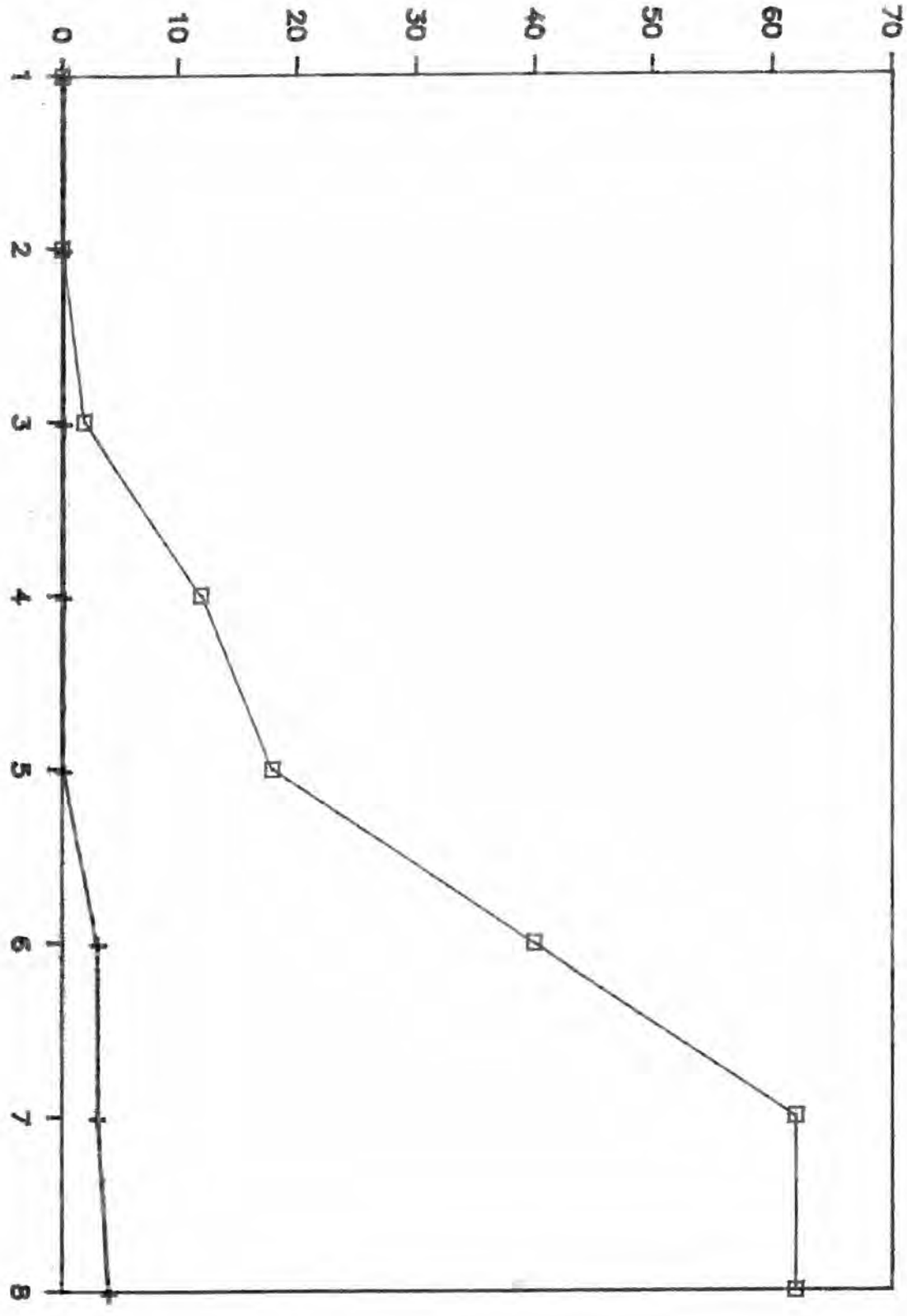
Days after introduction of 2,6-DCP	Number of ticks on left ear (%) Experimental	Control	Ticks Expected
1	0	0	0
2	0	0	0
3	2	0	1
4	12	0	6
5	18	3	10.5
6	40	3	21.5
7	62	5	33.5
8	62	4	33

Table 6 shows the response of male ticks to 2,6-DCP applied on rabbit ears. In this study, rabbits were used as hosts because these are commonly used for the experimental maintainance of R.appendiculatus, and they offer the easiest means of standardizing conditions. In the experiments carried out here, the ticks moulted and were reared in the laboratory under constant conditions of temperature and humidity, while their environment within the ear bags, in close proximity to the rabbit's body, was assumed constant.

In the males, response to the chemical became evident on day 3 and maximal response was observed on day 7, by which time, 62% of the males were attached on the ear treated with 2,6-DCP (Figure 6). This attraction was highly significant (X^2 = 73.04, degrees of freedom = 1). It was observed that ticks detached and later reattached to the ear with the chemical. Similar experiments with females, nymphs and larvae showed that these were not attracted to 2,6-dichlorophenol-treated ear of the rabbits.

2,6-dichlorophenol elicited responses from unfed males, females, nymphs and larvae of R. appendiculatus, in the T-tube and Y-tube assay systems. This shows that 2,6-dichlorophenol acts as a general attractant for this tick species. This observation supports electrophysiological studies using 2,6-dichlorophenol, which indicated that male R.appendiculatus responded to the chemical, with no relationship to the state of feeding (Wallade, 1982). Unfed ticks showed significantly more response to the chemical than fed ticks. This investigator did not test female ticks using this technique. Sonenshine et al. (1974) and Homsher and Sonenshine (1976) reported that in the presence of feeding females, male D. andersoni and D. variabilis ticks detach earlier than when females were absent. Some of the detaching males, which reattached beside the females, were sexually immature. Dissection of the males detaching in environments where 12 or 25 females were present revealed

Figure 6 Attraction of R.appendiculatus males to 2,6 -dichlorophenol on rabbit ears


MALES ATTRACTED TO 2,6—DCP (%)
0
10
20
30
40
50
60
70
1
2
3
4
5
6
7
8

that as many as 39.4% or 80% respectively, of these males were immature. Thus in the metastriate ixodidae, sexually immature males perceive, and are sometimes attracted by chemicals produced by feeding females. The detachment of males coincides with the release of 2,6-dichlorophenol by females (McDowell and Wallade, 1986).

In the unfed male and female ticks, nymphs and larvae, 2,6-dichlorophenol could function in bringing the ticks together, in natural situations. The bioassays carried out here show that each of the developmental stages of this tick respond optimally to different concentrations of the compound, a finding which may correlate with periods when each of these stages occur in the field. In Kenya, the work of Yeoman (1966) has shown that R.appendiculatus are active all the year round in some parts of the country. The adult peak is closely followed by the larval peak and the nymphal peak occurs later.

In the bioassay on rabbits, 62% of the males migrated to the ear with 2,6-dichlorophenol, while no females, nymphs, or larvae were attracted to the chemical. These findings seem to suggest that 2,6-dichlorophenol is a sex pheromone. In the Gulf Coast tick Amblyomma maculatum, both males and females were found to contain 2,6-dichlorophenol. However, males responded to spot applications of this compound on rabbits, but no evidence was seen that females

were induced to migrate to the chemical (Kellum and Berger, 1977). These workers concluded that the compound acts as a sex pheromone in that species. Sonenshine et al. (1982b) suggested that 2,6-dichlorophenol functions in these situations as a generalized excitant, stimulating males to detach. It is worth noting here that in Metastriate Ixodidae, males become motile after initial feeding, and become stationary again upon contacting a female (Oliver, 1974). Nymphs and larvae of this tick species do not detach once they have attached on a host, until after engorgement. Probably, the response of ticks already attached on a host, to the chemical, is different from that of ticks not attached, as was observed in the bioassays using the T-tube, Y-tube, and the assays on rabbits. It was also observed in the experiments that newly introduced male ticks located feeding females within hours, moved, and attached with them, whereas it took 3 days for the males attached on the right ears to detach, and re-attach on the left ears of the rabbits. This can partly be explained by the physiological changes that take place in the ticks after commencement of feeding. In ticks, sexual maturity commences following feeding.

CHAPTER THREE

SELECTION OF FEEDING SITES BY THE TICK, R.APPENDICULATUS

INTRODUCTION

Gregson (1973) stated that once a tick is on the host, it encounters stimuli which, with its own predisposition, may either favour or discourage subsequent attachment. The stimuli concerned may be highly specific for the tick species concerned (Wallade and Rice, 1982). A tick species may need only non-specific stimuli; for example, I.ricinus will attach to almost any part of the body of a wide variety of animals. Alternatively, a tick may require very specific stimuli; for example, R.evertsi larvae and nymphs display strong predilection for the inside of the ears of bovids, whereas adult R.evertsi attach around the anus; R.appendiculatus adults prefer the inner surface of the ears of cattle, the upper edge being especially favoured, whereas the larvae and the nymphs attach to any part of the body, although relatively more attach to other parts of the head (Yeoman and Walker, 1967; Baker and Ducasse, 1967). One of the objectives of this study was to determine whether there is a kairomonal cue, which leads to the selection of

attachment and feeding sites, on the hosts of this tick species.

MATERIALS AND METHODS

Tick-naive Friesian calves, kept at ICIPE, were used in experiments on selection of feeding sites by R.appendiculatus.

The ears, belly, perineum, legs, and back of the calves were swabbed with hexane, using a pair of sterile forceps and cotton wool. Preliminary tests, using various solvents, indicated that ethanol and hexane swabs produced positive responses from the ticks. However, hexane was preferred because of its ease of evaporation. The swabs were concentrated to a volume of about 2 ml by vacuum evaporation. The concentrates were then transferred to 5 ml vials, evaporated to dryness, and weighed. They were later dissolved in 2 ml of hexane and used for bioassays.

Bioassay of Cattle Swabs

Assays were carried out on the ticks using the T-tube and the Y-tube, as described in Chapter Two, under the section on bioassay of 2,6-dichlorophenol.

RESULTS AND DISCUSSION

TABLE 7

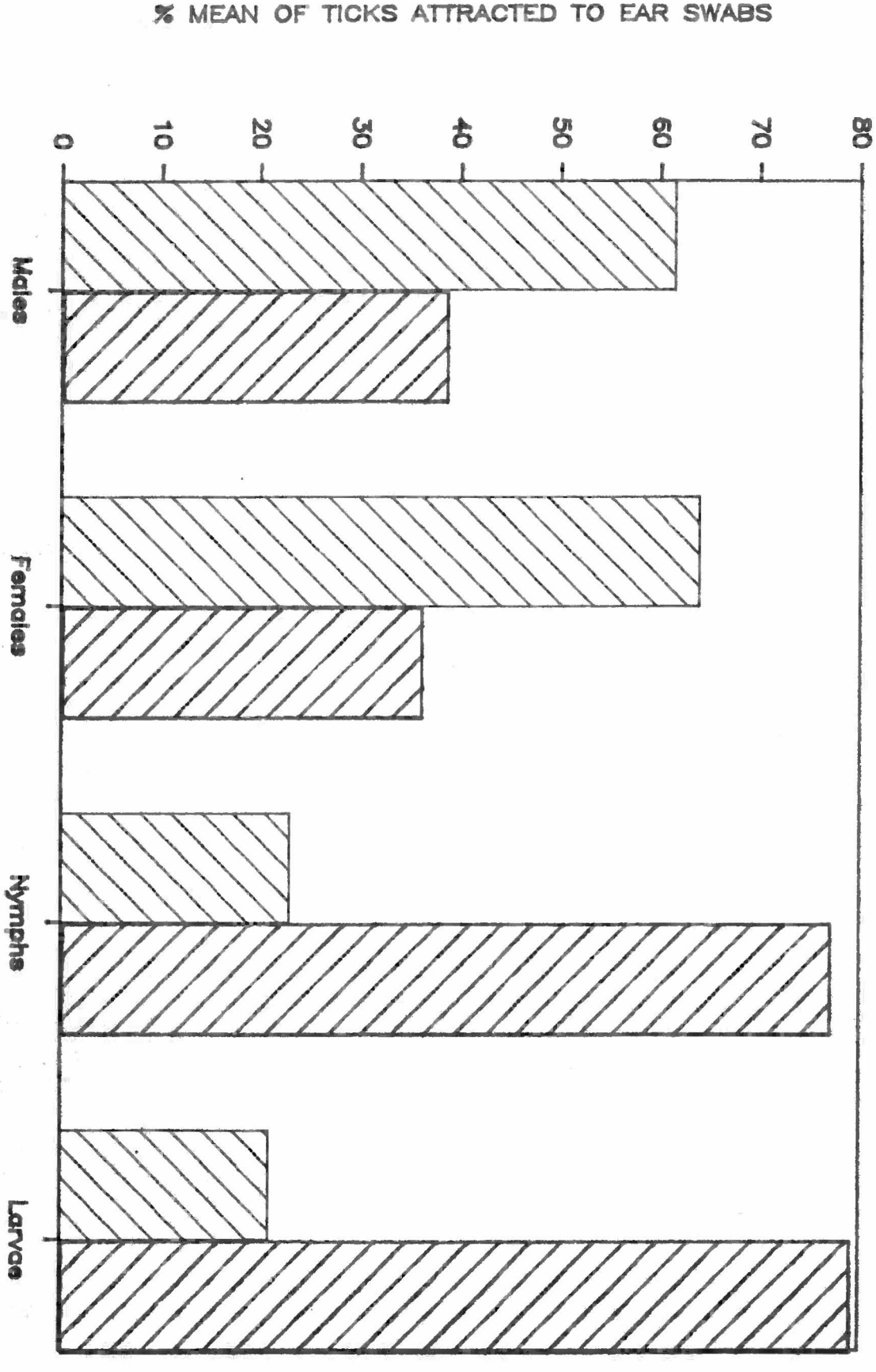
PERCENT RESPONSE (X + S.E.) OF R.APPENDICULATUS TO SWABS FROM VARIOUS PARTS OF CATTLE BODY

Part of Body swabbed	Ticks Tested	Exptal	Control	P Value
EAR	Females	64.0 ± 3.232	36.0 ± 3.232	0.01
	Males	61.5 ± 3.253	38.5 ± 3.253	0.01
	Nymphs	23.2 ± 0.471	76.8 ± 1.495	0.01
	Larvae	21.1 ± 0.842	78.9 ± 1.082	0.01
LEGS	Females	55.0 ± 7.032	45.0 ± 7.032	0.2
	Males	47.0 ± 6.333	53.0 ± 6.333	0.2
	Nymphs	45.3 ± 1.163	54.7 ± 0.914	0.2
	Larvae	30.3 ± 1.690	69.7 ± 1.943	0.01
BACK	Females	44.0 ± 7.774	56.0 ± 7.774	0.2
	Males	48.0 ± 5.333	52.0 ± 5.333	0.2
	Nymphs	51.3 ± 1.322	48.7 ± 0.934	0.2
	Larvae	46.5 ± 2.254	53.5 ± 2.065	0.2

Part of Body swabbed	Ticks tested	Exptal	Control	P Value
PERINEUM	Females	50.0 ± 5.375	50.0 ± 5.375	0.2
	Males	44.0 ± 4.000	56.0 ± 4.000	0.1
	Nymphs	54.7 ± 0.491	45.3 ± 0.554	0.2
	Larvae	47.1 ± 1.404	52.9 ± 1.572	0.2
BELLY	Females	47.0 ± 7.461	53.0 ± 7.461	0.2
	Males	53.0 ± 5.972	47.0 ± 5.972	0.2
	Nymphs	50.7 ± 1.026	49.3 ± 0.561	0.2
	Larvae	50.4 ± 9.035	49.6 ± 9.923	0.2

As shown in Table 7, male and female R.appendiculatus respond to cattle ear swabs, but not to swabs from other body areas. A mean percentage of 61.5 of the males, and 64% of the females responded significantly ($P<0.001$) to the ear swabs in the T-tube assay system, as compared to 38.5% for the males, and 36% for the females, in the hexane arm of the T-tube (Figure 7). In the tests with swabs from the perineum, belly, legs, and back, there was no consistent attraction, and the difference between the experimentals and controls were not significant. Nymphs and larvae showed significant ($P< 0.01$) repulsion from the ear swabs. The

Figure 7 Attraction of R.appendiculatus to cattle ear swabs in T-tube and Y-tube assays


% MEAN OF TICKS ATTRACTED TO EAR SWABS
0
10
20
30
40
50
60
70
80
Males
Females
Nymphs
Larvae

bioassay also indicated that larvae were repelled by the leg swabs ($P < 0.01$). Since legs come into contact with dung, among other materials where cattle are kept, an extract of cow dung was tested against the larvae. No significant response was observed. Swabs from the back, perineum, and belly did not elicit significant attraction from the nymphs and larvae.

The attraction of unfed male and female ticks to cattle ear swabs indicates the presence of compounds which are perceived by this tick species. Cattle ear swabs have been shown to enhance attachment of R. appendiculatus ticks on membranes during in vitro feeding experiments (Waladde et al., unpublished). Probably, the ticks are attracted by chemicals produced from the ears, the combinations of which may be specific for this particular species. Carbon dioxide and ammonia are known host-originated stimuli to which ticks respond (Wilson et al. 1972; Haggart and Davis, 1980, 1981), and butyric acid is reputed to be an excitant (Waladde and Rice, 1982). From the data shown in the experiments, it seems that the compounds that attract adults to the ears act as repellants to the immature stages. Surveys carried out by Yeoman and Walker (1967) and Baker and Ducasse (1967) showed that relatively few larvae (10%) and nymphae (24%) attached to ears of cattle , while 83% of the adults were found on the ears. The ticks never occurred on the back, ventral parts of the body, perianally, or on the legs,

except in cases of very heavy infestations (Walker, 1974). From the experiments carried out here, the adult ticks seem to perceive chemicals which make them prefer the ears to other parts of the body, although they can attach to the unpreferred areas in cases of high competition for feeding sites. An additional experiment here would have been to take the ear secretion (cerumen) and analyse it for the presence of attractants such as ammonia and butyric acid. However, this was not done because of the pressure of time allocated for this investigation.

Many other factors could also influence the selection of feeding sites for the ticks, such as carbon dioxide concentration and organic compounds in cattle breath, and the location, and the physical characters of the ears. These could act synergistically with the chemical cues from the ears, to make the adults prefer the ungulate ears. This study, however, dealt with chemical cues only, and these other factors were not tested.

CHAPTER FOUR

AGGREGATION BEHAVIOUR OF R.APPENDICULATUS

INTRODUCTION

In Amblyomma species, unfed female ticks do not attach readily, if males are absent (Gladney, 1971; Lounsbury, 1899, Norval and Rechav, 1979). In R.appendiculatus, unfed female ticks attach to a host, irrespective of the presence of feeding males. However, the unfed ticks prefer to attach to areas where other ticks are attached and already feeding, suggesting the involvement of a chemical cue. Experiments were therefore designed to find out whether ticks of this species show aggregation response to other conspecific ticks, and tick extracts.

MATERIALS AND METHODS

TEST FOR AGGREGATION OF TICKS ON RABBITS

General Methods

Twenty-five male or female ticks were enclosed in cotton ear bags on the right ears of seven rabbits. The left ears were similarly enclosed in ear bags , but with no ticks introduced. Female ticks were allowed to feed for two days, while males were fed for eight days. Female ticks were fed for two days only, since reports indicate that after two days, females start producing sex pheromones, and these were not being investigated in this study. It has also been reported that in *Amblyomma* species, maximum production of aggregation pheromones occurs after 7-8 days of feeding (Gladney, 1971; Norval and Rechav, 1979; Rechav *et al.*, 1976; Rechav *et al.*, 1977a). Both ear bags were then removed, and fifty unfed male or female ticks were released from the back of the rabbit, 7 cm from the ears. The number of ticks that attached with the preattached ticks was then scored after every hour, for five hours. The mean percentage of ticks in the experimental and control ears was then calculated, and compared using student's t-test.

Specific Methods

Movement of Unfed Males to Preattached Females

This test was carried out as described above. Females ticks were introduced into the ear bags, and were allowed to feed for two days. Unfed male ticks were then released on the rabbits.

Movement of Unfed Females to Preattached Females

This test was conducted as described above, except that unfed female ticks were released on the rabbits after two days.

Movement of Unfed Females to Preattached Males

This test was carried out as described above, except that male ticks were introduced into the ear bags, and were allowed to feed for eight days. After removal of the ear bags, unfed female ticks were released on the rabbits.

Movement of Unfed Males to Preattached Males

This test was conducted as described above. Males were introduced into the rabbit ears, and unfed males released on the rabbits eight days later.

PREPARATION OF MALE TICK EXTRACT

One month-old male ticks were introduced under cotton ear bags to the ears of rabbits. Previous reports indicate that pheromone production is age-related, and maximum production occurs 2-4 weeks after moulting (Graf, 1975). After feeding for 8 days, the ticks were forcibly detached from the rabbits using a pair of forceps and brought to the laboratory for extraction. 10,000 ticks were extracted by packing them in a glass column and adding sufficient hexane (150 ml) to cover the ticks. The extraction procedure was then carried out as already described in Chapter Two, under the section on extraction of 2,6-dichlorophenol.

BIOASSAY OF THE MALE TICK EXTRACTS

This test was carried out using the T-tube, Y-tube and on rabbits, as described in Chapter Two, under the section on bioassay of 2,6-dichlorophenol.

RESULTS AND DISCUSSION

BIOASSAY ON RABBITS

TABLE 8

MOVEMENT OF MALES TO PREATTACHED FEMALES

Time after release (hrs)	Percentage (Mean ± S.E.) of males that were observed attached With Females	On control ear	P Value
1	0.86 ± 0.297	0.29 ± 0.184	0.2
2	4.31 ± 0.261	0.43 + 0.202	0.01
3	7.40 ± 0.286	0.85 ± 0.261	0.01
4	10.32 ± 0.670	1.43 ± 0.297	0.01
5	14.34 ± 0.857	2.00 ± 0.488	0.01

Table 8 shows the attractiveness of fed female ticks for unfed male ticks. The unfed male ticks were significantly ($P < 0.01$) attracted by the female ticks, from two hours on. Some of the unfed males were found attached in mating position with the preattached female ticks.

Figure 8 Movement of unfed male ticks to preattached females on rabbit ears

MEAN NO. OF MALES ATTACHED (%)

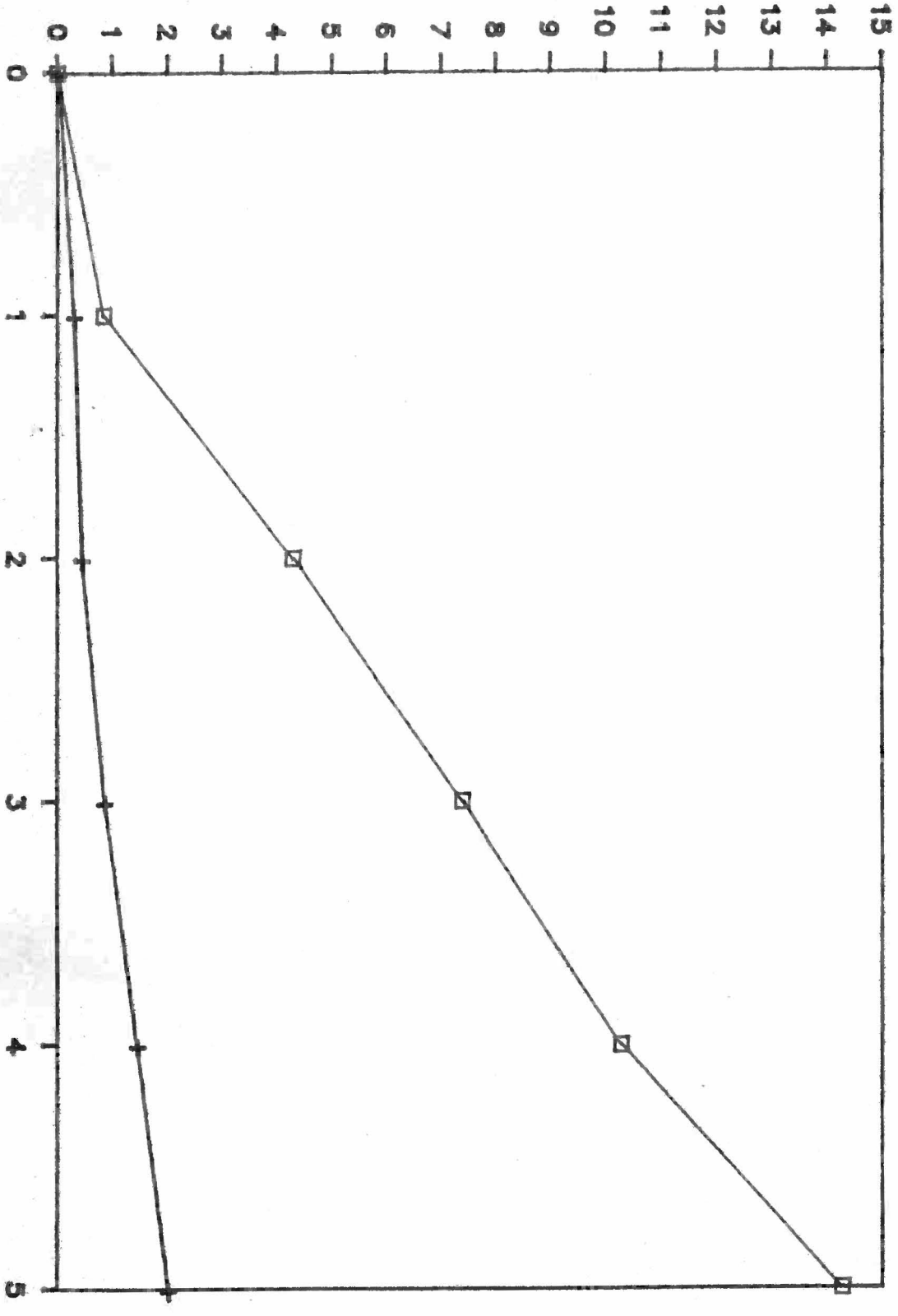


TABLE 9

MOVEMENT OF FEMALES TO PREATTACHED FEMALES

Time after release (hrs)	Percentage (X ± S.E) of females that were observed attached		
	With females	On control ear	P Value
1	4.00 ± 1.574	1.71 ± 0.808	0.1
2	6.86 ± 2.502	2.86 ± 0.962	0.1
3	9.14 ± 3.622	3.14 ± 1.438	0.1
4	10.00 ± 3.651	4.00 ± 1.690	0.1
5	11.14 ± 1.876	4.29 ± 1.658	0.05

There was significant ($P < 0.05$) attraction of fed females for unfed females. The number of females that went to either ear of the rabbits gradually increased with time (Figure 9).

Figure 9 Movement of unfed female ticks to preattached females on rabbit ears

MEAN NO. OF FEMALES ATTACHED (%)

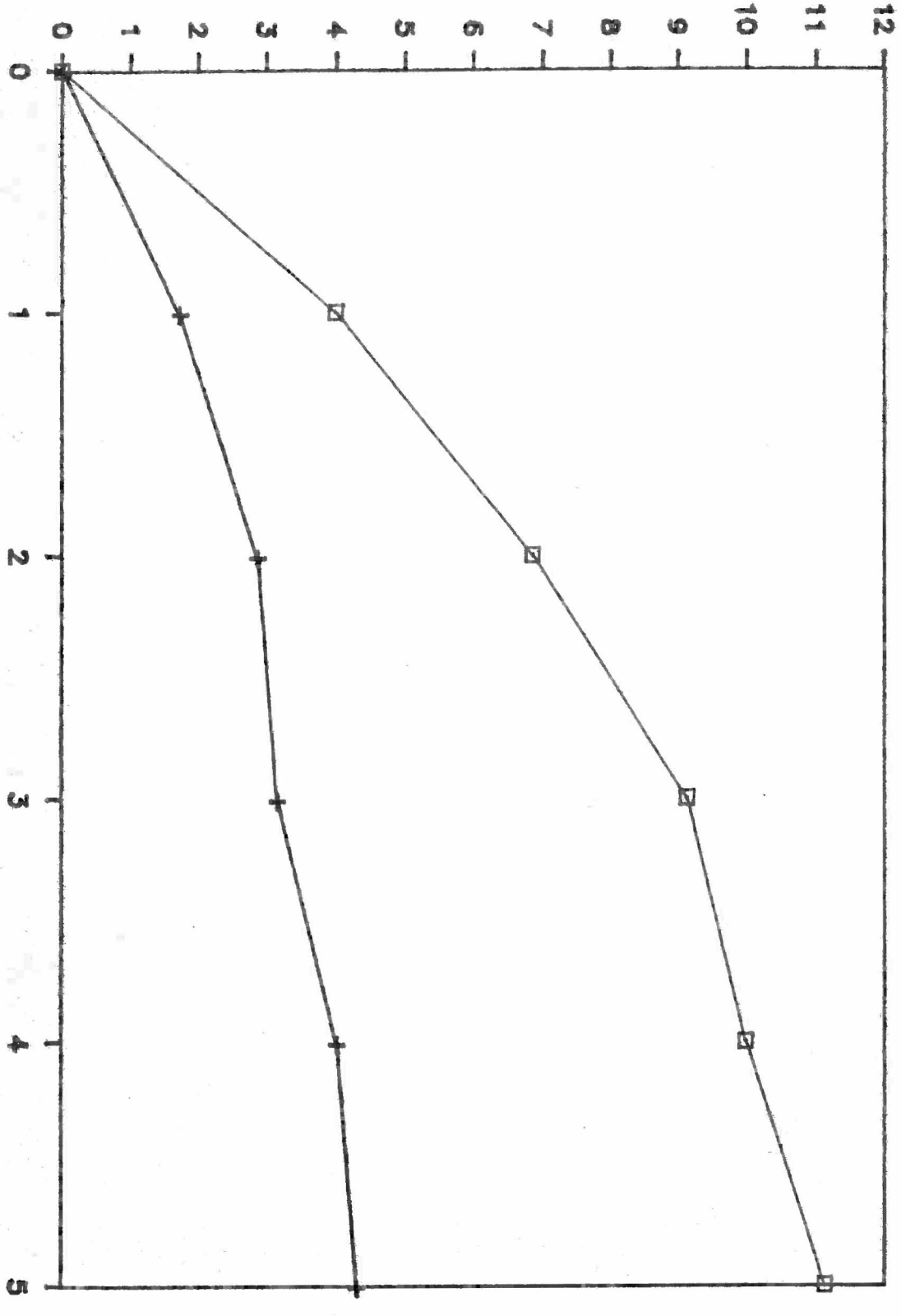


TABLE 10

MOVEMENT OF FEMALES TO PREATTACHED MALES

Time after release (hrs)	Percentage (X ± S.E) of females that were observed attached		
	With males	On control ear	P Value
1	11.14 ± 4.973	3.14 ± 1.143	0.1
2	14.86 ± 6.216	4.86 ± 1.299	0.1
3	17.43 ± 6.043	5.43 ± 1.494	0.1
4	23.14 ± 6.077	7.43 ± 1.986	0.05
5	25.71 ± 5.959	8.57 ± 1.986	0.05

Table 10 shows that significantly ($P < 0.05$) more females attached with the preattached males after 4 and 5 hours, compared to those that attached on the control ear. The mean percentage of female ticks that were attracted to the males (25.71%) was much more than the percentage of males (9%) that were attracted to fed males (Table 11, Figure 10).

Figure 10 Movement of unfed female ticks to preattached males on rabbit ears

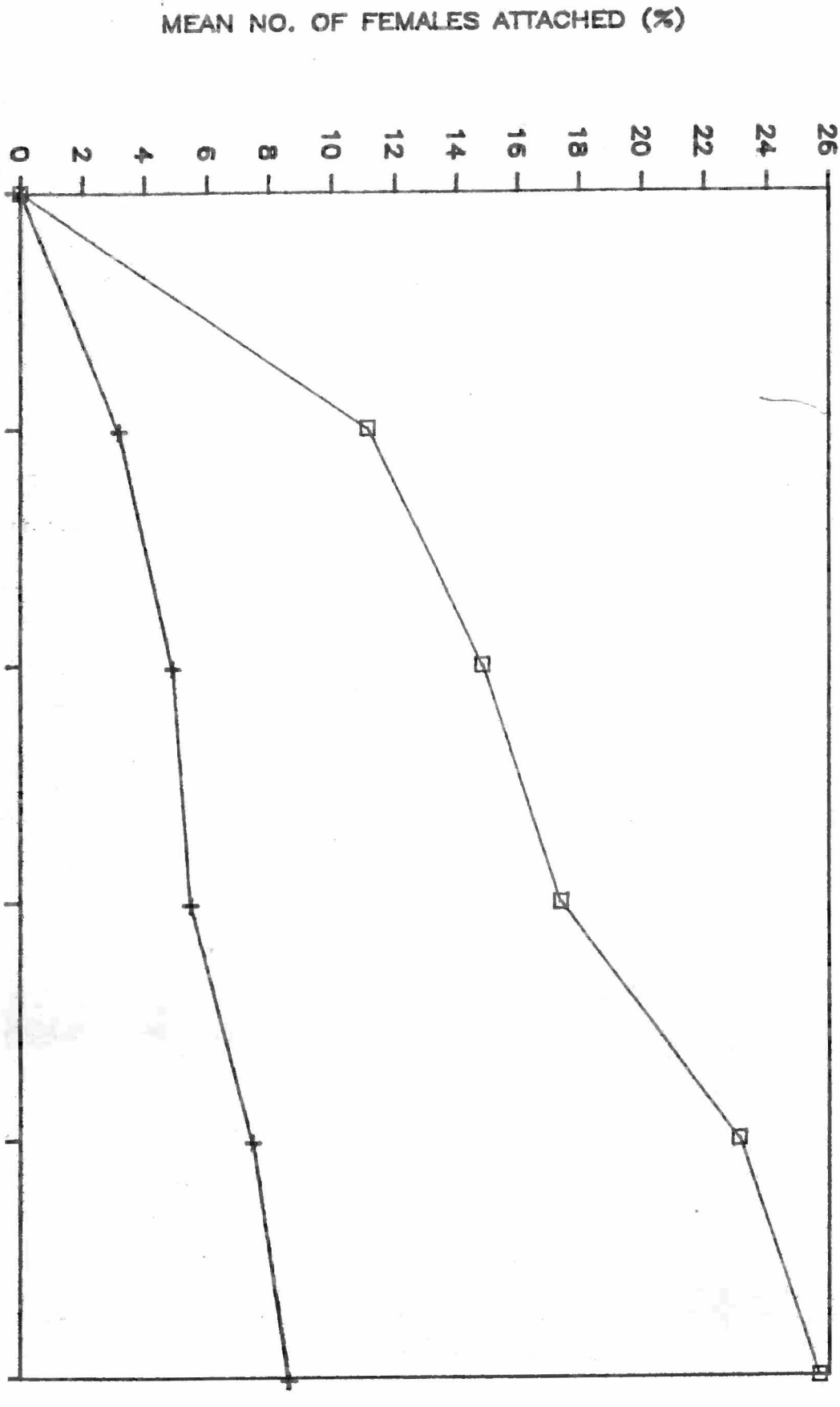

MEAN NO. OF FEMALES ATTACHED (%)
0
2
4
6
8
10
12
14
16
18
20
22
24
26

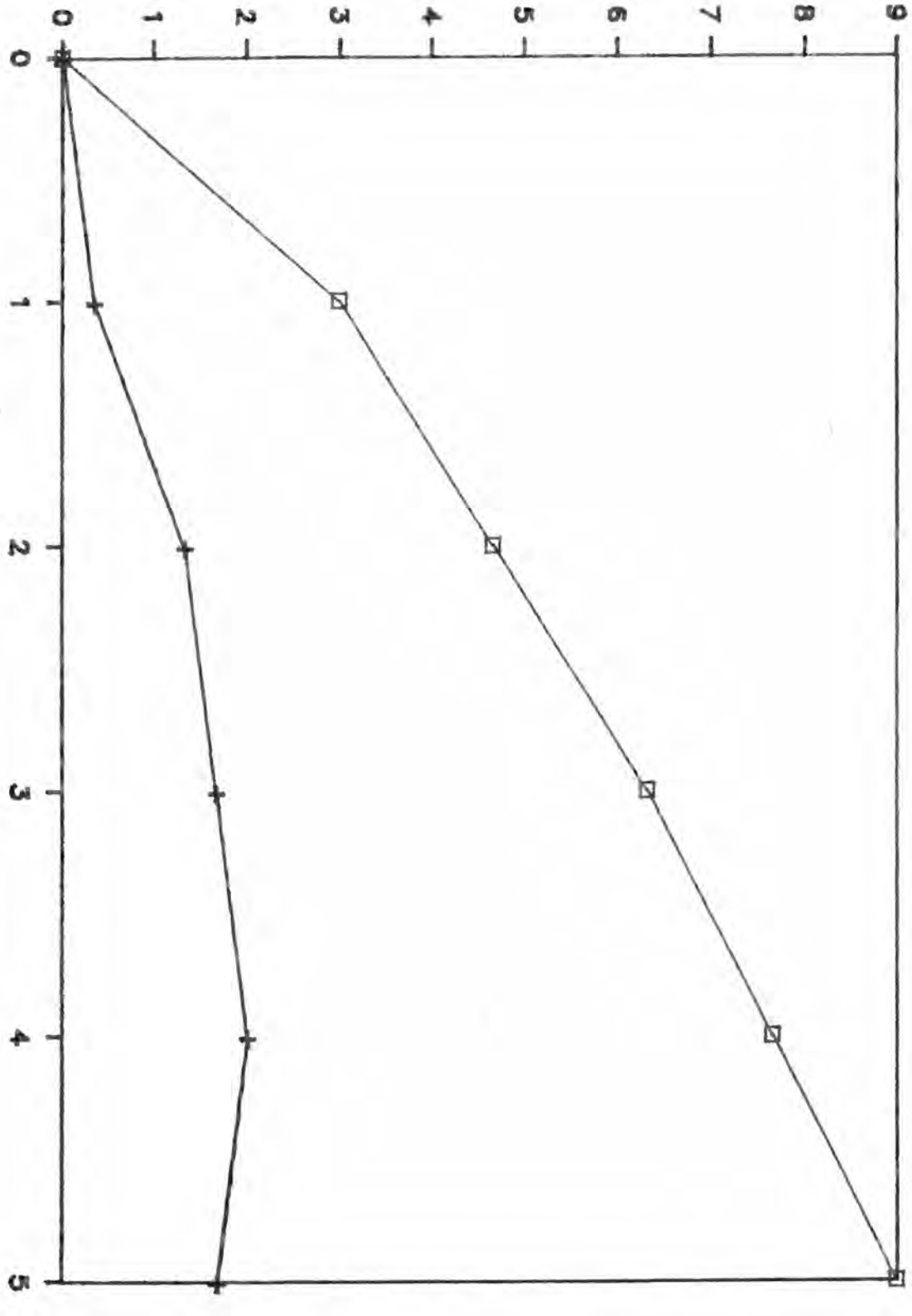
TABLE 11

MOVEMENT OF MALES TO PREATTACHED MALES

Time after release (hrs)	Percentage (Mean ± S.E) of males that were observed attached		
	With males	On control ear	P Value
1	3.00 ± 1.125	0.34 ± 0.333	0.1
2	4.67 ± 1.687	1.33 ± 0.667	0.1
3	6.33 ± 1.085	1.67 ± 0.615	0.01
4	7.67 ± 1.308	2.00 ± 0.894	0.01
5	9.00 ± 1.693	1.67 ± 0.615	0.01

Males were also attracted by 8 days-fed males (Table 11). The mean percentage number of males that attached with the preattached males, and on the control ear, was comparatively less than in all the other tests. This suggests that males are less responsive than females to the presence of preattached males and whatever chemicals may emanate from them. In all the tests, the number of ticks that attached on the ears increased with time (Figures 8, 9 10 and 11).

Figure 11 Movement of unfed male ticks to preattached males on rabbit ears


MEAN NO. OF MALES ATTACHED (%)
0
1
2
3
4
5
6
7
8
9
0
1
2
3
4
5

T-TUBE ASSAY OF TICK EXTRACTS

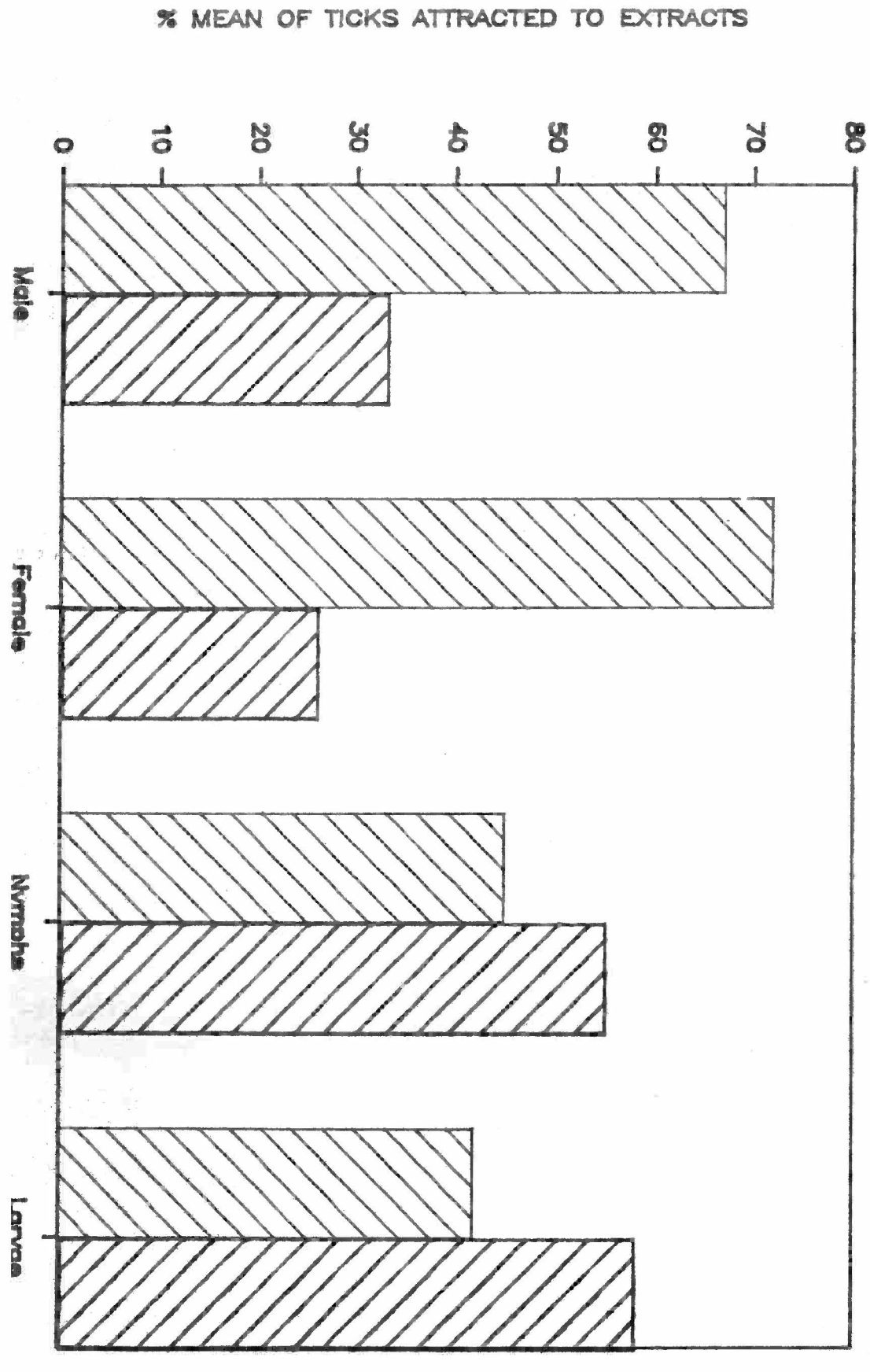
TABLE 12

PERCENT RESPONSE (X ± S.E) OF *RHIPICEPHALUS APPENDICULATUS* TO TICK EXTRACTS IN A T-TUBE

	Experimental	Control	P Value
Male	67.0 ± 4.485	33.0 ± 4.485	0.01
Female	72.0 ± 5.121	26.0 ± 5.121	0.01
Nymphs	45.1 ± 0.780	54.9 ± 1.128	0.2
Larvae	42.2 ± 1.496	57.8 ± 2.121	0.1

The T-tube assay indicates significant attraction of both males and females to the tick extracts ($P < 0.001$) (Table 12). As Figure 12 shows, 67% of the males were attracted to the extracts, compared to 72% for the females, indicating again more responsiveness of the females to the extracts. Nymphs and larvae, however, did not show significant attraction to the extracts.

Figure 12 Attraction of R.appendiculatus to tick extracts in T-tube and Y-tube assays


% MEAN OF TICKS ATTRACTED TO EXTRACTS
0
10
20
30
40
50
60
70
80
Male
Female
Nymphs
Larvae

BIOASSAY OF THE TICK EXTRACTS ON RABBITS

TABLE 13

PERCENT RESPONSE OF FEMALES TO TICK EXTRACTS ON RABBIT EARS

Days after introduction of extract	Number of ticks on left ear (%) Experimental	Control	Ticks Expected
1	0	0	0
2	0	0	0
3	0	0	0
4	1	0	0.5
5	2	0	1
6	5	0	2.5
7	5	0	2.5
8	5	0	2.5

TABLE 14

PERCENT RESPONSE OF MALES TO TICK EXTRACTS ON RABBIT EARS

Days after introduction of extract	Number of ticks on left ear (%) Experimental	Control	Ticks Expected
1	0	0	0
2	1	0	0.5
3	4	0	2
4	7	1	4
5	18	2	10
6	17	3	10
7	19	3	11
8	25	4	14.5

The *in vivo* bioassay of the extracts on rabbits showed that both males (X^2 = 19.44, degrees of freedom = 1) and females (X^2 = 5.05, degrees of freedom = 1) were significantly attracted to the extracts. In the case of females, 5% were found attached on the extract-treated rabbit ears (Table 13). In contrast to the control experiments, in which a percentage of 4 males were observed attached on the left ears of the rabbits after 8 days, 25% of the males were found on the extract-treated rabbit ears (Table 14). 4% of the males were found detached. In the

control experiments with females, none was found attached on the left ear of any of the rabbits. Nymphs and larvae did not show any movements.

In this study,the attraction of males and females, for unfed conspecific ticks, was seen after the males had fed for 8 days. Female ticks, fed for two days, attracted other conspecific ticks, probably due to release of 2,6-dichlorophenol.

The data from the experiments reported in this Chapter show that R. appendiculatus actually exhibits aggregation behaviour, similar to that observed with Amblyomma species. R. appendiculatus prefer to attach to those areas on the hosts where other conspecific ticks are already feeding. This response must involve chemical cues, released by the feeding ticks and perceived by the unfed ticks. Female ticks were found to be relatively more responsive to the extracts than male ticks. Other workers have reported that males are more attractive to unfed females than to unfed males (Gladney et al., 1974b; Rechav et al., 1977b).

R. appendiculatus females attach to hosts irrespective of the presence of feeding males, indicating that the chemical cue from other ticks is not a prerequisite to attachment. Generally, once females have attached, they do not detach until after engorgement. Hence the observation that only 5% of the females released on the right ears of

rabbits were found attached on the left ears, while a higher percentage of the males were found on the left ears and others were still detached, probably prior to re-attachment on the ears with the male tick extract. Nymphs and larvae also, once attached on a host, feed until they engorge and drop off (3 to 4 days for larvae, and 5 to 6 days for nymphs). Apart from the work of Rechav et al. (1976), who reported that nymphs aggregate to areas on cattle with aggregation pheromone extracts, there have been no other reports on aggregation behaviour of these immature ticks. These workers also reported that larvae were not attracted to the aggregation pheromone extracts, to which nymphs were attracted. Probably, in R.appendiculatus, the immature stages do not have well developed receptors to detect the pheromone and so are not guided by the pheromone to a feeding site.

CHAPTER FIVE

EFFECT OF VARIOUS MIXTURES OF EAR SWABS, TICK EXTRACTS AND 2,6-DICHLOROPHENOL ON R.APPENDICULATUS

INTRODUCTION

In certain situations, mixing various behaviourally significant compounds enhances the attraction of ticks to the compounds involved (Wood *et al.*, 1975). Wallade (1982), using electrophysiological techniques, observed that stimulating male *R.appendiculatus* with various combinations of p-cresol, salicylaldehyde, and 2,6-dichlorophenol did not produce any synergistic effects

Enhanced attraction would be expected when mixtures of ear swabs, male tick extracts, and 2,6-dichlorophenol, are presented to the ticks, since after the ticks have been attracted by chemicals from the ears and have attached on a bovine host, the ticks produce 2,6-dichlorophenol which, together with other odours, may cause attraction and attachment of more ticks.

This study was set up in order to determine whether various combinations of male tick extract, 2,6-dichlorophenol, and cattle ear swabs would enhance responses from the ticks.

MATERIALS AND METHODS

BIOASSAY OF A MIXTURE OF EAR SWABS AND TICK EXTRACTS

This test was carried out on adults only, since nymphs and larvae did not show response to either of the extracts. The ear swabs and tick extracts were obtained as already described in Chapters Three and Four of this thesis. 0.5 ml of the tick extract was mixed with 0.5 ml of the ear swabs, and bioassayed against the ticks using the T-tube, as described in Chapter Two, under the section on bioassay of 2,6-dichlorophenol.

BIOASSAY OF A MIXTURE OF EAR SWABS AND 2,6-DCP

This test was done by mixing 0.5 ml of 50ng/ul 2,6-DCP (the concentration that was most attractive to the adults), with 0.5 ml of the ear swabs. For the larvae and nymphs, 0.5 ml of 0.1ng/ul and 5ng/ul 2,6-DCP respectively, were mixed with 0.5 ml of the ear swabs. This test was carried out using the T-tube and the Y-tube, as described in Chapter Two, under the section on bioassay of 2,6-dichlorophenol.

BIOASSAY OF A MIXTURE OF TICK EXTRACTS AND 2,6-DCP

This assay was carried out on adults only, since nymphs and larvae did not show attraction to tick extracts. It was conducted using the T-tube, as described in Chapter Two, under the section on bioassay of 2,6-dichlorophenol.

To test for the effect of the various mixtures, bioassayed against the ticks, expected responses were calculated using the formula, $E = O_a + O_b - O_aO_b$,

where O_a is the observed proportional response caused by one test material

O_b is that caused by the other test material in the mixture.

X^2 values were then calculated as $(O_c - E)^2/E$, where O_c is the percent response caused by the combined treatment (Salama et al., 1984).

RESULTS AND DISCUSSION

TABLE 15

PERCENT RESPONSE (X + S.E.) OF R.APPENDICULATUS ADULTS TO A MIXTURE OF TICK EXTRACTS AND EAR SWABS

	Experimental	Control	P Value
Males	65.0 ± 4.282	35.0 ± 4.282	0.01
Females	54.0 ± 5.617	46.0 ± 5.617	0.2

Males responded significantly ($p < 0.01$) to the mixture of ear swabs and tick extracts. However, females were not significantly attracted by the mixture.

TABLE 16

PERCENT RESPONSE (X + S.E.) OF R.APPENDICULATUS ADULTS TO A MIXTURE OF TICK EXTRACTS AND 2,6-DCP

	Experimental	Control	P Value
Males	72.0 ± 3.590	28.0 ± 3.590	0.01
Females	72.0 ± 3.887	28.0 ± 3.887	0.01

Significant attraction was observed with both males ($p<0.01$) and females ($P< 0.01$).

TABLE 17

PERCENT RESPONSE (X + S.E.) OF R.APPENDICULATUS ADULTS TO A MIXTURE OF EAR SWABS AND 2,6-DCP

	Experimental	Control	P Value
Males	63.0 ± 3.958	37.0 ± 3.958	0.01
Females	59.0 ± 5.044	41.0 ± 5.0440	0.05

Significant attraction was observed with both males ($p<0.01$) and females ($p<0.05$).

TABLE 18

EFFECT OF VARIOUS MIXTURES 2,6-DICHLOROPHENOL, EAR SWABS, AND TICK EXTRACTS ON FEMALE R.APPENDICULATUS

Treatment	Observed response (%)	Expected response (%)	X^2 value	Conclusion
Tick extract + 2,6-DCP	72	89.92	3.57	No effect
Ear swabs + 2,6-DCP	59	90.28	10.84**	Depressive
Ear swabs + Tick extract	54	92.44	15.98**	Depressive

TABLE 19

EFFECT OF VARIOUS MIXTURES OF 2,6-DICHLOROPHENOL, EAR SWABS, AND TICK EXTRACTS ON MALE R.APPENDICULATUS

Treatment	Observed response (%)	Expected response (%)	x^2 value	Conclusions
Tick extract + 2,6-DCP	72	88.12	2.95	No effect
Ear swab + 2,6-DCP	63	86.14	6.76*	Depressive
Ear swab + Tick extract	65	87.29	5.69*	Depressive

TABLE 20

EFFFECT OF A MIXTURE OF EAR SWAB AND 2,6-DICHLOROPHENOL ON NYMPHS AND LARVAE OF R.APPENDICULATUS

Stage of ticks	Observed response (%)	Expected response (%)	X^2 value	Conclusions
Nymphs	51.03	88.39	15.79**	Depressive
Larvae	54.3	75.9	6.15*	Depressive

$X^2_{0.05, 1} = 3.84$; $X^2_{0.01, 1,} = 6.64$

* - significant at 5% level of probability

** - significant at 1% level of probability

The study on the effect of the various mixtures of tick extracts, ear swabs, and 2,6-dichlorophenol, on the responses produced by each of the materials acting singly indicate that the mixtures inhibit the responses from the ticks, apart from the experiment on a mixture of tick extracts and 2,6-dichlorophenol, in which responses from the males and females were not affected. It appears that presenting a mixture of ear swab and 2,6-dichlorophenol, or ear swab and tick extract simultaneously, inhibits responses

from the ticks, while a mixture of tick extract and 2,6-dichlorophenol does not affect responses produced by either material acting alone. This is in agreement with findings of Wallade (1982), that stimulating male R.appendiculatus with various combinations of 2,6-dichlorophenol, p-cresol, and salicylaldehyde did not produce any synergistic effects. O'Connell (1986), working with the moth Trichoplusia ni, observed that responses were significantly reduced when three behaviourally significant compounds from female pheromone glands, were presented as a mixture, to male moths.

SUMMARY

From this study, it is evident that chemical cues, acting as aggregation pheromones are present in R.appendiculatus ticks. The fact that unfed ticks of this species prefer to attach where other conspecific ticks are already attached and feeding, together with the T-tube assays, lends support to the conclusion that R.appendiculatus also exhibits aggregation response, similar to that reported in Amblyomma species.

The chemical, 2,6-dichlorophenol, was found to attract unfed larvae, nymphs, males, and females of this tick species, in a T-tube assay system. These findings suggest a general role of the compound. The in vivo assays on the rabbits, however, suggest that the compound may act as a sex pheromone. It is worth noting here that while males are mobile, females of this tick species do not generally detach once they have attached, until at the time of engorgement. The experiment on quantitation of 2,6-dichlorophenol suggests that the compound may act as a sex pheromone, since higher concentrations were found in feeding females than in feeding male ticks. This is the period when sexual maturity commences. It is, therefore, apparent that the compound has a dual role, depending on whether the ticks are unfed or

fed: In the unfed ticks, it acts as a general attractant, probably aiding the ticks in clustering together. When the adult ticks feed, it may act as a sex attractant, stimulating the males to detach, orient, and move to attached, feeding female ticks.

Hexane swabs from the legs, back, belly and perineum of tick-naive calves did not produce significant responses from the ticks. However, it was shown that swabs from the ears actually repelled the nymphs and larvae, probably due to the nature of odours emanating from the ears. Only the adult ticks were attracted to ear swabs.

Mixtures of 2,6-dichlorophenol and ear swab, and of ear swabs and tick extract, produced inhibitory responses from the ticks, compared to responses produced by each of the materials acting singly. A mixture of 2,6-dichlorophenol and tick extract has no effect on the responses produced by each of these materials acting on the males and females. This is explained by the inhibition of responses from the ticks, when these materials are presented simulteneously to the ticks.

The results from this study can be used in control of this tick species, using the methods of Gladney et al., (1974a) and Ziv et al., (1981). The chemical composition of the aggregation pheromone of this tick species is not known.

It does not contain 2,6-dichlorophenol, since the chromatographic analyses indicated that the chemical is below detectable amounts in male ticks fed for 8 days.

Future research is needed to elicidate the chemical composition of the extract from R.appendiculatus males and cattle ear swabs, in order to identify the attractants in these extracts.

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