Mate choice in *Prostephanus truncatus* (Horn) (Coleoptera:Bostrichidae): The role of male-produced aggregation pheromone

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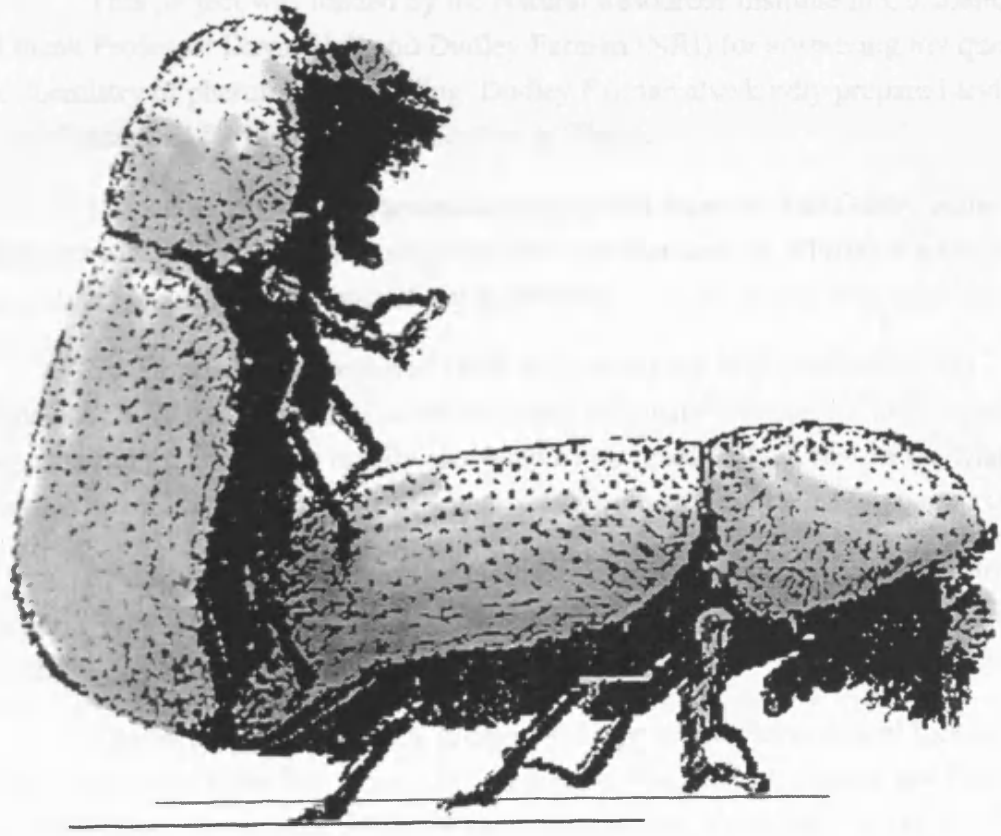

Thesis submitted for the degree of
Doctor of Philosophy

JANUARY 1998

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ACKNOWLEDGMENTS

I thank my supervisor, Professor Robert 'thrasher' Smith, for a great three years of constructive criticism, discussion, editing, and taking my ankles out at unihoc. Similarly, I thank my second supervisor, Dr. Rick Hodges. Without Rick's support I know the work performed in Ghana would not have got past Rob's initial reaction of, 'No you can't go to Africa!'.

The field work would also not have been possible without the support of the Post Harvest Management Division, Ministry of Food and Agriculture in Ho. I thank Mr. Kwaku Nicol, Mr. Sam Addo and the staff at the ministry for their help. We thank the Forestry Department in Ho for permission to work on their land.

This project was funded by the Natural Resources Institute in Chatham, Kent, UK. I thank Professor David Hall and Dudley Farman (NRI) for answering my questions on the chemistry of pheromone signalling. Dudley Farman also kindly prepared and analysed filters used for pheromone collection in Ghana.

I thank Mick Ward for technical support in Leicester. Ted Gaten, Stans and Ewen taught me all I know about computers. Bev Sherbon and Dr. Muriel Walker helped me take, develop and print pictures of my dissections.

Sterilization of insects was made possible by the kind permission and assistance of Dr. David Bonnett at Leicester Royal Infirmary (chapter 7). Initial questions about sperm competition were readily answered by Dr. Paul Eady. Later on Dr. Matt Gage took on the role of 'advisor on sperm' and also taught me the subtle art of sperm counting.

I must thank those who have recently gone before me, Dr. Manuel Vazquez-Arista, Dr. Henry Fadamiro and Dr. Dagmar Scholz. All of these LGB Doctors have readily given advice/ reprints and best wishes.

I thank my aged ancestors, damned average genetic inheritance! Life in Leicester has always been fun. Cheers in particular to Chris, Matt, Sharon and Boz; Jens and my mate Vivi; Stans, Ems, Mike, Noosie, Pips, Helen, Karen and David. My final word is, 'beetles are NOT just bird food!'.

ABSTRACT

Lucy A. Birkinshaw 1998: **Mate choice in *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae): The role of male-produced aggregation pheromone.**

Prostephanus truncatus (Horn) (Coleoptera: Bostrichidae) is a destructive pest of stored maize and cassava that has recently been accidentally introduced into tropical Africa. Males produce an aggregation pheromone when on food, that attracts dispersing males and females. *P. truncatus* aggregation pheromone is being used to monitor the spread of *P.truncatus* (Larger Grain Borer) across Africa. The biological function of this pheromone is controversial. This thesis investigates the role of aggregation pheromone in mate choice in *P. truncatus*.

The literature on Coleopteran aggregation pheromones was reviewed, with particular reference to the possible adaptive functions of aggregation pheromones.

Variation in *Prostephanus truncatus* aggregation-pheromone signalling was detected. Conspecifics can detect these differences and are preferentially attracted to some males more than others. Both males and females 'agree' which males are most attractive (shown in a laboratory bioassay and in trapping experiments in the field). Females also discriminate between potential mates on contact by a stylised pushing behaviour. Some males consistently secure more matings than others when two males are presented at once to a female. Discrimination between males mediated on contact through pushing is not influenced by the male's aggregation pheromone signal (both natural variation and manipulation of the pheromone signal were studied).

Observation of adult beetles in an artificial host sandwiched between two glass plates revealed that males and females pair up, and cohabit within the same tunnel system. Pairs mate multiply (up to 20 times per 12 hours) and dissection of recently mated females revealed that males deliver an oversized ejaculate (approx. 50 000 sperm) as an oval spermatophore. Male investment in ejaculate was not found to be influenced by male crowding or the presence of Female Factor (an involatile pheromone produced by females, which can trigger aggregation pheromone shut down in males).

These results are discussed in the context of sexual-selection theory and also with reference to the Integrated Pest Management of this insect.

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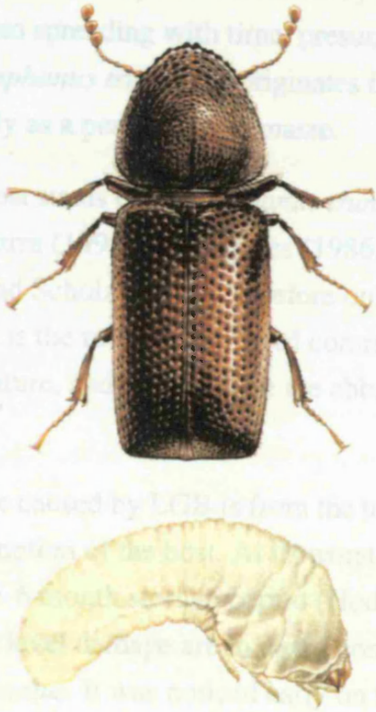


Fig. 1: Adult *Prostephanus truncatus* (length 2.5-4.5mm), and larva (reproduced from GASGA/ CTA technical leaflet No.1 (1993)).

CHAPTER 1: INTRODUCTION

1.1 THE INSECT

1.1.1 Pest status in Africa

Prostephanus truncatus (Horn) (Coleoptera: Bostrichidae) is a destructive pest of stored maize and cassava that has recently been accidentally introduced into tropical Africa. Its presence was first officially confirmed in the Tabora region of Tanzania in the early 1980's (Cross, 1985). Since this time it has spread across six countries in the east of Africa and what is thought to be a second introduction (Vazquez-Arista, 1997), now covering at least seven West African countries has radiated from Togo (Scholz, 1997). *P. truncatus* has most likely arrived in totally new locations by hitching a ride in grain shipments. Local ranges are also spreading with time, presumably via insect dispersal by flight (Fadamiro, 1995). *Prostephanus truncatus* originates from Mexico and Central America where it is known only as a pest of stored maize.

The biology and pest status of *Prostephanus truncatus* are reviewed in detail in Markham, Wright and Rios Ibarra (1991) and Hodges (1986; 1994). Recent reviews are included in Fadamiro (1995) and Scholz (1997), therefore only the most relevant details are given here. Larger Grain Borer is the most widely used common name for *Prostephanus truncatus* in the scientific literature, and LGB will be the abbreviated form used throughout this thesis.

Most of the damage caused by LGB is from the tunnelling activity of adults and larvae rather than actual consumption of the host. At its worst *P.truncatus* reduced yields by up to 34% weight loss in a 3-6 month storage period (Hodges *et al.*, 1983, quoted in Hodges, 1986). Patches of high level damage are in part a consequence of the aggregation behaviour of *Prostephanus truncatus*. It was noticed early on that, although one store may become seriously infested, neighbouring stores, presumably equally suitable as hosts, would remain untouched. This phenomenon has since been attributed to a system of aggregation pheromone communication in LGB. Males that locate a suitable host produce a volatile chemical signal that is attractive to both sexes. Aggregation on food hosts has most certainly been key to the creation of conflict between the interests of man and insect and will be the focus of this study.

1.1.2 Life history

LGB is a fairly small beetle, typical of many stored product pests (approx. adult length= 3-5mm, width= 1-1.5mm, fresh weight= 2.5-4.5mg). Stored-product pests commonly fall into one of two life-history strategies: short adult life where many small eggs are produced in a short period of time; long adult life with an extended reproductive period,

where a few fairly large offspring are produced at a constant rate (Burkholder and Ma, 1985). LGB falls into the second category (Li, 1988).

If a suitable host is available a female can consistently lay an average of four eggs per day pausing only to construct new tunnels (Nyakunga, 1982, quoted in Hodges, 1986). Mean lifetime-fecundity estimates vary but a figure of 430 eggs is given by Bell and Watters (1982) (quoted in Fadamiro, 1995 for LGB cultured on maize). Egg production peaks at about three weeks after emergence and declines slowly from then on until death (see Bell and Watters, 1982, quoted in Fadamiro, 1995). Both males and females will mate multiply in the laboratory (pers. obs.) and females require at least three matings spaced approximately one month apart to maximise the number of viable offspring she can produce (Li, 1988).

When LGB is reared on maize under laboratory conditions of 32°C and 80% r.h., small clutches of about eight eggs develop at the end of blind ending tunnels bored into the host and hatch into cream coloured larvae after about five days (Li, 1988). Larvae pass through three instars (possibly five instars see Ramirez, 1990, quoted in Markham *et al.*, 1991) and metamorphose into pupae after about three weeks. Pupae are often surrounded by a thin-walled, closed cylindrical case in part constructed of flour. Beetles remain as pupae for about four to five days, then hatch into lightly sclerotised adults. These newly hatched adults are relatively inactive for their first few days, reach sexual maturity after 4-5 days and are reproductively active for the remainder of their life span. LGB adults live for about three to four months under ideal laboratory conditions.

1.1.3 Host range

Not surprisingly, diet greatly influences many life-history characters of this beetle. This is an important consideration since LGB is not constrained to one host and has been found breeding in plant species of different plant families. The life history described above is based on studies of LGB on maize, currently its main host in the stored-product environment. It is likely that LGB's ancestors were woodborers as Bostrichids are generally wood boring in habit (Fisher, 1950). Indeed, LGB has been reared in the laboratory on the wood taken from trees of the Anacardiaceae, Burseraceae, Euphorbiaceae and Leguminosae (Helbig and Schulz, 1994; Nang'ayo *et al.*, 1993, quoted in Fadamiro, 1995).

One mystery is the difficulty in locating LGB in wood in the field. LGB has yet to be found living and breeding in wood in the wild except when it tunnels into grain-store structures. A considerable population away from cultivation has been demonstrated (Rees *et al.*, 1990, in woodland areas of Mexico; and Nang'ayo *et al* ., 1993, in Tsavo national park, Kenya - all quoted in Fadamiro, 1995). Traps baited with artificially produced aggregation pheromone were found to attract LGB just minutes after they were set up in

areas many kilometres away from the nearest known source of maize (Hodges pers. comm. in a bush area in Kenya.). Large aggregations of beetles have never been found naturally occurring in a wood host despite considerable effort to identify such populations (Hodges pers. comm.). This contrasts with the large aggregations of beetles that may be found in food stores. It may be the case that large aggregations are a feature only of the stored-product environment where food only becomes limiting only for very large populations, or perhaps the location of aggregations in wood remains to be discovered. Ramirez *et al* ., (1991), quoted in Markham *et al.,* (1991), suggested that LGB inhabits the drying out ends of dead branches and twigs as a host, possibly those recently killed by the action of Cerambycid beetles. This is compatible with the theory that LGB is particularly well suited to outcompeting other species in relatively dry conditions (Howard, 1983; Markham *et al.,* 1991).

1.1.4 Research emphasis to date

Much research effort has addressed the aim of damage limitation of this pest. The main challenge has been to devise solutions that are practical and acceptable to small scale farmers, and to provide this information through a network of extension workers. It is unlikely that *Prostephanus truncatus* will be eliminated from Africa, but the devastation of its initial introduction need not be repeated. A truly Integrated Pest Management approach has been employed against LGB. Maize variety, storage practices, use of pesticides, use of inert admixes, and biological control, are all factors that have been considered in an effort to limit LGB damage. For example, *Teretriosoma nigrescens* (Histeridae) is a predatory beetle found naturally in the American continent, which feeds on LGB larvae. Since it was first isolated in infested maize at Reading University in the late 1970's (Howard, 1983), it has been studied as a potential biocontrol agent. *Teretriosoma nigrescens* is attracted to the aggregation pheromone of LGB and uses this signal to locate its prey. *T. nigrescens* has now been released in both East and West Africa as part of a biocontrol program against LGB (Boeye *et al.,* 1994).

Two components of LGB aggregation pheromone have been identified and given the trivial names of Trunc-call 1 and Trunc-call 2, (hereafter referred to as T1 and T2). T1 is 1-methylethyl(E)-2-methyl-2-pentenoate and T2 is 1-methylethyl(E,E)-2,4-dimethyl-2,4-heptadienoate. These components can be manufactured by man and are used to bait pheromone traps that have been successfully used to study LGB biology and to monitor the spread of LGB (Hodges, 1986). Initially pheromone-baited traps were employed in store to provide an early warning of LGB infestation. Unfortunately, once LGB tunnel into a suitable host, they become relatively unresponsive to aggregation pheromone. Regrettably these traps tended to attract the dispersing population around the store instead of the population already within store, and thus they probably promoted infestation of the store (Pike, 1993). It is now recommended that pheromone traps for

monitoring be placed at least 100m away from any stores (Hodges and Pike, 1995). The occurrence and spread of *T.nigrescens* has been monitored using these pheromone traps (Markham *et al.*, 1994).

Host-plant volatiles are often used to increase the efficiency of aggregation-pheromone traps for other pest species, as they can also be attractive and indeed act synergistically with pheromone lures (see chapter 2). So far this has not been found to be the case in LGB. As yet, no strong directional response to plant volatiles from a distance has been observed (Fadamiro, 1995; Scholz *et al.*, 1997b) although some short range arrestant activity has been demonstrated for maize and cassava (Pike *et al.*, 1994). For general reviews of the use of pheromones as tools in stored product pest management see Phillips (1994), and Chambers (1990).

Semiochemicals have so far fulfilled only part of the initial vision for their application as a tool in pest management (Silverstein, 1990). It is fair to say that the main successes have been realised in monitoring and the other main potential application, mating disruption, has been relatively ineffective. Lack of understanding of the natural functioning of semiochemicals has been highlighted as the main shortcoming of programmes utilising these biological signals. The work presented here is a study of the reproductive biology of *Prostephanus truncatus* with particular reference to the possible evolutionary advantages of aggregation pheromone signalling. Aggregation pheromone signals (those that attract both sexes) are a common feature of pest species so insights gained in this study are potentially applicable to other systems of economic importance.

1.2 STARTING HYPOTHESIS

This thesis starts with the hypothesis that males signal to attract mates. A corollary is that other males attracted are exploiting the signaller and represent a cost of signalling through increased competition for mates. The finding that only males signal using this pheromone was influential in the formulation of the hypothesis that *Prostephanus truncatus* aggregation pheromone serves a sexual function, first formally proposed in print by Hodges (1994). This idea was aired at a meeting in 1984 (Group for Assistance on Systems relating to Grain After harvest (GASGA), 1984) (R.H. Smith, pers. comm.), and its development was in part inspired by the discovery that males decrease their pheromone signal in response to an involatile chemical produced by females but not males (Smith, 1996).

The main critic of this theory so far has been Dr.H.Y.Fadamiro (see Fadamiro, 1995). Fadamiro suggests that the pheromone shut down in response to females is a general response to overall population density and not necessarily a response specifically to the presence of a potential mate. Fadamiro's work has focused mainly on the flight behaviour of LGB. He consistently found no sex-specific differences in response to

aggregation-pheromone signals and concluded that aggregation pheromone was more likely to function as, 'a suitable resource location and colonisation signal', (Fadamiro, 1995); this may be a fair description of part of the motivation that leads beetles to respond to the signal, however surely any functional explanation for the existence of a signal needs to address the question, why do males signal in the first place?

1.3 SEXUAL SELECTION

Sexual selection is defined by Darwin as, "The advantage which certain individuals have over others of the same sex and species solely in respect of reproduction", (Darwin, 1871). It is quite common for one of the sexes of a species to be more ornamented (either visually, chemically, structurally, or acoustically) than the other and such ornamentation is often attributed to sexual selection (Cronin, 1991; Andersson, 1994; chapter 8 this thesis). Classic examples include the elaborate Peacock's tail and the large antlers of male deer. Perhaps aggregation pheromone signalling in male LGB owes its origins and/or current maintenance to similar processes that have produced these other ornaments?

Two of the earliest mechanisms proposed to result in sexual selection were female choice (inter-sexual selection) and male contests (intra-sexual selection). More recently scramble competition (favouring the first males to locate mates) and endurance rivalry (favouring males who are able to remain reproductively active for an extended period of time) have been added to the list (Andersson, 1994). The 'battlefield' on which relative male reproductive success through sexual selection is determined has extended to competition between ejaculates within the female reproductive tract (Parker, 1970). Historically, females tended to be viewed as passive commodities that males strive to obtain. Females are now recognised to be far more pro-active determinants of relative male reproductive success (Eberhard, 1996; Wirtz, 1997). Determining parts of the complex whole of sexual selection in any one species is a daunting, yet rewarding task. See Cronin (1991) for a comprehensive review of sexual-selection theory.

1.3.1 Low sexual dimorphism of LGB external morphology

Current morphology can hint at past selection pressures. Externally, adult *Prostephanus truncatus* are not strikingly dimorphic in form. The relative difficulty in sexing this species pays testament to the lack of sex-specific structures, although means of continuously variable features are different (sex specific body size and size and shape of a pair of bumps on the head for instance (Shires and McCarthy, 1976)). The horns of male stag beetles, and the longer antennae and bristles on the legs of males of some *Monochamous* species of the Cerambycidae are two examples of features that warrant more investigation for their possible role in sexual selection (see Hughes, 1981 for study of a *Monochamous* species). Females are often larger than males in invertebrates (Andersson,

1994). Higher fecundity with increased size of female and better manouvrability used in mate acquisition with decreasing size for males are cited as two possible explanations for this difference. Shires and McCarthy's method for sexing LGB is based on the fact that females generally have more prominent clypeal tubercules (bumps on the head), which are spaced further apart in females than males (Shires and McCarthy, 1976). The function of these bumps is unknown. Perhaps these protrusions are useful during aggressive encounters. It will be noted in chapter 6 that females are generally more aggressive than males and appear to use pushing behaviour to choose between mates. Such 'rejection' hypothesis for traits that are more elaborate in females than males where no reversed sex role is inferred are discussed briefly in Andersson (chapter 13, 1994). Boring insects such as LGB may be particularly constrained against possessing elaborate external features since they could impede tunnelling.

In this study I will look across the spectrum of possible determinants of male-fertilization success in LGB to determine the consequences of male aggregation pheromone signalling. The response of both males and females to different aggregation pheromone signals is perhaps the first sieve in the behavioural sequence that determines mating success. The question of whether males can bypass this sieve and cheat has been addressed by considering the next possible sieves, behaviour on contact (both between the sexes and between males) and processes within the female tract. Practically nothing was known about the reproductive biology of this insect, so initially a huge range of determinants of mating success were possible. This study presents new findings on all levels of the reproductive biology of this species, from which it is hoped that more accurate hypotheses can be derived in the future.

1.4 SUMMARY OF CHAPTERS

The idea that aggregation pheromone signals are exploited sex pheromone signals was first investigated by a review of the available literature on Coleopteran aggregation pheromones in chapter 2, where the main rival theory, that of a food conditioning benefit, is also discussed. Chapters 3-7 deal more specifically with *Prostephanus truncatus*. Basic patterns of signalling and response are presented in chapter 3, a mandatory prerequisite to a more in-depth study. Chapters 4 and 5 demonstrate that considerable variation exists between male aggregation pheromone signals both in a laboratory bioassay and in trapping trials in the field. This variation is perceived by conspecifics who 'agree' which signals are the most attractive, therefore females may mate non-randomly on the basis of this pheromone signal. The courtship behaviour of *Prostephanus truncatus* is described in chapter 6. On contact, females continue to discriminate between males by aggressively pushing potential suitors away. In common with the preferences shown to different pheromone signals, different males were ranked in the same order by different females. The relationship between success in attracting females

by signalling and success in physical courtship was then tested. Natural variation in both traits revealed no consistent relationship between these two possible components of mate choice. The pheromone signal was then manipulated to show conclusively that this signal is not a determinant of courtship preferences. Behaviour of adults within an artificial plant host, recorded using time-lapse photography is also reported in chapter 6. The potential for sperm competition in LGB is evaluated in chapter 7 along with some assessment of male ejaculate investment in differing sociosexual environments. Lastly (in chapter 8), the results are discussed in the context of sexual selection theory and also with reference to the continuing pest management of LGB.

1.5 GENERAL MATERIALS AND METHODS

1.5.1 Source of insects

All insects used were originally collected from Tanzania in the 1980's unless otherwise stated. These insects were provided by the Natural Resources Institute (NRI) and were maintained on maize for 2-3 years at Reading University and then a further 3 years at Leicester University, prior to the start of this study in February 1995.

1.5.2 Source of plant material used as insect hosts

Whole maize was supplied by Spillers, Bristol, UK. Maize meal used was 'Natco medium corn meal' supplied by T. Cholthram and sons Ltd., Middlesex, UK. Cassava (unfermented) was obtained from Dr. Rick Hodges at NRI and came originally from Ghana.

1.5.3 Insect cultures

Insects were maintained on whole maize in glass jars (capacity=550ml). All food sources were sterilized by freezing and stored at -20°C. Each new culture consisted of approximately 200 adult *Prostephanus truncatus* placed on half a jar of maize. Jars were sealed with a plastic screw lid with most of the top replaced by a filter paper held in place with metal gauze to ensure no insects escaped. All jars were placed on an oil coated surface to prevent the spread of any insect or mite infestations. No cultures ever became infested with mites during this study. All cultures were kept in a constant temperature humidity (CTH) room set to 30 °C and 70% relative humidity. There were occasional problems with the humidifier and although for approximately 80% of the time conditions were as stated above, the range recorded in the CTH room was 25-33°C and 40-85% r.h. Cultures will be affected much less by short-term fluctuations than the sensors in the CTH room. The life cycle from egg to adult took approximately four weeks under these conditions.

Insects were removed from culture by sieving through brass sieves of mesh sizes 850μm and 3.35mm (Endecotts Ltd., London, UK). If the protocol required insects

to be reared separately for a time prior to an experiment, then insects were usually reared in small glass pots with perforated plastic lids (height=5cm, diameter=2.3cm). Unless stated otherwise, approximately $5cm^3$ of coarse ground maize meal was used to feed these insects. Demianyk and Sinha (1988) found that a single whole grain of maize was sufficient to feed one adult for an average life span so the amount of food used in this study was never limiting.

1.5.4 Experimental conditions

Unless stated, all behavioural observations were carried out in the same CTH room where the cultures were kept (generally 28-31 °C and 55-75% r.h.). Initially care was taken to rear some test insects in a separate 'pheromone free' incubator, but it became apparent that this was not necessary for the trials conducted.

1.5.5 Insect handling

Insects were generally moved using a small piece of paper towel. It was found that if the towel was brushed lightly against the insect's legs, LGB will cling on and can easily be moved without the need for forceps that can on occasion damage limbs. 'Featherlite' forceps were, however used to transfer insects during weighing as this required an accurate placement of the beetles on the small weighing platform. During examination of genitalia required for sexing (see section 1.5.6), a short length of thread was taped across the tip of the forceps. This thread was pinned across the ventral side of the upper abdomen of the adult and was used for holding the insect on its back.

1.5.6 Sexing *Prostephanus truncatus*

Clypeal tubercle size and shape were initially used to sex adults as suggested by Shires and McCarthy (1976). In this method the sexes are distinguished by looking at two bumps on the head (clypeal tubercles) that are larger and spaced further apart in females relative to males. This method is not 100% accurate since variation in this character is continuous and the female and male range overlaps. It is possible to reduce the rate of error by only selecting individuals at the extreme ends of the spectrum, but since the extent of variation between individuals was the subject of some of this work, this selection would be inappropriate. I decided to sex beetles by looking at a more definitive indicator of their gender, their genitalia. Females possess a bright yellow, sac-like structure that is revealed when the last abdominal segment is lifted in response to slight pressure applied to the abdomen. Likewise, males will extrude their three pronged intromittent organ in response to this pressure (see section 7.4.1 for description of the intromittent organ). The main disadvantage of this method compared to that of Shires and McCarthy (1976) is that insects are more likely to be damaged whilst the pressure is applied. Scholz independently decided to use genitalia to sex LGB (Scholz, 1997). Scholz specifically investigated the risk of damage from sexing by everting genitalia and found no significant effect on either mortality

or fecundity arising from this procedure (Scholz, 1997). After practice, beetles can easily be sexed at a rate of approximately 80 per hour if live undamaged adults are needed, or at about twice this rate if the sex of freshly killed insects is required.

1.5.7 Video equipment

All video recordings were taken using a Vista (Japan) NCL1100 CCTV camera with a Vista (Japan) CCTV lens, 3.5mm, F1.4. An Hitachi time-lapse video recorder (model VT-L2000E) was also used.

1.5.8 Statistical analysis

All error bars are standard errors unless otherwise stated. Likewise, all statistical analysis were performed in SYSTAT 5 for the Macintosh unless otherwise indicated.

CHAPTER 2: LITERATURE REVIEW OF COLEOPTERAN AGGREGATION PHEROMONES

2.1: DEFINITION

Communication between organisms takes many forms. From a human perspective watching and listening to others are perhaps the most obvious ways in which we receive signals. For insects, the subject of this review, the world of smells/tastes has heightened importance and can relay surprisingly precise information (Butler, 1967). Karlson and Butenandt proposed the term 'pheromone' in 1959 to describe, 'substances which are secreted to the outside by an individual and received by a second individual of the same species in which they release a specific reaction'. This definition serves to distinguish pheromones from hormones, which are chemicals conveying information within the body of a single organism, and also from kairomones (attracting exploiters) and allomones (attracting organisms of benefit), which are chemical messages, intentional or otherwise between individuals of different species. These definitions are in no way mutually exclusive, however, and the same chemical may be acting in more than one capacity at any one time depending on whom you are considering. Semiochemicals is a more general term encompassing pheromones, kairomones and allomones. Bossert and Wilson classified pheromones further in 1963 into 'releaser' substances and 'primer' substances. Releaser substances are those which induce an immediate and reversible change in the recipient acting more or less directly on the central nervous system, and primer substances are those which trigger a more permanent physical change in the recipient, for example retarding sexual maturity. In this review I will be dealing with releaser pheromones, more specifically, "substances produced by members of either or both sexes that induce members of both sexes to aggregate", termed aggregation pheromones (Borden quoted in Kerkut and Gilbert, 1985). These are superficially distinct from sex pheromones, which attract just the opposite sex from that of the signaller.

2.2: OCCURRENCE

Aggregation pheromones as defined by Borden, are fairly widespread in insects. They are found in cockroaches, social Hymenoptera and many beetles (Coleoptera). The literature on Coleopteran aggregation pheromones is composed mainly of various phytophagous insect-pest species (see Burkholder and Ma, 1985). The impact created by such aggregations has been the inspiration behind much of the funding given over to this subject. For this reason, it is these insects, the weevils (Curculionidae), the grain borers (Bostrichidae), the flour beetles (Tenebrionidae), the sap beetles (Nitidulidae),

the Chrysomelidae, the flat bark beetles (Cucujidae), and the well studied bark beetles (Scolytidae) that this review concentrates on.

2.3: CHEMICAL NATURE

Pheromones are organic molecules ranging in size from simple benzene derivatives to longer carbon chains and more complex multiple ring structures. Pheromones are often a blend of components whose ratio and chirality can be crucial in determining the response elicited (Vanderwel and Oehlschlager, 1987; Seybold, 1993). Coleopteran aggregation pheromones are either chemicals sequestered from the host which are often slightly modified; or are effectively built up from scratch from simpler precursors (said to be synthesised *de novo*).

The relatively well studied bark-beetle aggregation pheromones consist mainly of bicyclic ketals and secondary alcohols. The biosynthetic pathways leading to the construction of bicyclic ketals are unresolved but the secondary alcohols are thought to be mostly derived from host monoterpenes (see Vanderwel and Oehlschlager, 1987 for a summary). These monoterpenes are often toxic to the beetles that feed on them and it has been proposed that the biochemical pathways that produce these pheromones may have initially evolved as part of a detoxification process. The detoxification strategy is generally geared to converting hydrophobic structures into hydrophilic ones, which are easier to eliminate. Various isomers of verbenol and verbenone and myrtenol were thought to be derived from the tree oleoresin component alpha-pinene in bark beetles. More recent evidence, however, indicates a greater role of *de novo* synthesis of Scolytid pheromones. Ivarsson *et al.*, (1993) and Seybold *et al.*, (1995) have both studied biosynthesis of pheromone in *Ips* species and found that Ipsenol, Ipsdienol and E-myrcenol can be produced *de novo*. Myrtenol, another derivative of alpha-pinene, is known to be produced by *Ips paraconfusus* , *Dendroctonus frontalis, Dendroctonus ponderosae,* and *Dryocoetes confusus.* Although myrtenol has so far no known pheromonal function in *Ips* species, it is a multifunctional pheromone of *Dendroctonus frontalis* and an aggregation pheromone of *Dryocoetes confusus* (Discussed in Prestwich and Blomquist, 1987).

Bostrichid aggregation pheromones are relatively simple compounds containing 9-12 carbons possibly constructed *de novo* and not obviously sequestered from the host (Prof. D. Hall pers comm.). So far the aggregation pheromones of Cucujid grain beetles have been found to be macrolides of either terpenoid or fatty acid origin or possibly by *de novo* synthesis (see Vanderwel and Oehlschlager, 1987; and Vanderwel *et al.*, 1992). Both *Tribolium castaneum and T. confusum* (Tenebrionidae) use 12 carbon alkanals as aggregation pheromones. *Rhynchophorus* species of weevil have been found to use various alcohols as pheromones (Jaffe *et al.*, 1993; Weissling *et al.*, 1994). Maize and rice weevils (*Sitophilus zeamais and S. oryzae*) both use the same compound which has the

typical 1,3-oxygenation pattern characteristic of polyketides (see Vanderwel and Oehlschlager, 1987). Boll weevils were shown by Mitlin and Hedin (1974) (quoted in Prestwich and Blomquist, 1987) to incorporate radio-labelled acetate, mevalonate and D-glucose into all four components of grandlure thus showing that this pheromone could be constructed *de novo*. Similarly, *Cryptolestes ferrugineus* (Rust red grain beetle) was shown to use both acetate and mevalonate for the construction of a terpenoid pheromone (quoted in Prestwich and Blomquist, 1987). However, both these species can also derive these pheromones more directly from dietary components.

Micro-organisms residing in the guts of insects have sometimes been shown to convert dietary chemicals into semiochemicals. For example, a *Bacillus cereus* strain has been isolated from *Ips paraconfusus*. Experiments using externally-sterilised eggs that were then grown in axenic conditions, and adults treated with antibiotics have indicated that both insect and microbes are involved in ipsdienol production by male *Ips paraconfusus*. No such microbial influence was shown for *Dendroctonus ponderosae* (work quoted in Prestwich and Blomquist, 1987). The micro-organisms do not necessarily always add to pheromone output, indeed axenically-reared male *Ips paraconfusus* and female *Dendroctonus ponderosae* contain significantly more alpha-pinene metabolites than wild beetles. Increased pheromone production in microbe-suppressed insects might simply be due to healthier insects or competition for pheromone substrates between microbe and insect. It has also been recognised that microbes may convert attractive compounds made by the insect into anti-aggregation compounds, which could play a significant part in terminating the attack on a host.

To summarise, so far pheromone origins have been proposed from fatty acids, polyketides, and terpenoids. Many unknowns still exist particularly for low molecular-weight pheromones. Much of the work performed so far has been on bark beetles, but results of studies in a few other beetle groups can be found.

2.4: BIOLOGICAL FEATURES

In this section, biological attributes of aggregation pheromones are given as generalisations (in italics) followed by a justification and expansion.

Pheromone produced only when signaller is on the host:

Aggregations of insects often form away from an appropriate food source, usually for the purpose of mating. Alternative localities include potential adult emergence sites, potential oviposition sites and conspicuous features of the landscape like hill tops (Thornhill and Alcock, 1983). All the aggregation pheromones reviewed here are usually produced in the presence of a suitable food source. All the food sources reviewed also double as sites of oviposition, perhaps their more important role since often more of the host is consumed by the developing larvae than by the mature adults.

The necessity of a host as a stimulus for pheromone production has been specifically demonstrated in Chrysomelidae (Peng and Weiss, 1992), Nitidulidae (Bartelt and James, 1994), Bostrichidae (Mayhew and Phillips, 1994), and Scolytidae (Vite and Pitman, 1968). With the exception of Peng and Weiss (1992) these studies all assess pheromone production directly by collection of volatiles and not via the response of conspecifics. This response may be influenced by the presence of food directly as well as its effect on pheromone production (see next sub-heading). Peng and Weiss (1992) controlled for this effect by separating potential producers from the food source with a screen. Faustini *et al.,* (1982) and Rochat *et al.,* (1991a) have obtained similar results in their studies in Curculionidae but without such a control being included.

Even the distinction insinuated between host odours and insect-produced pheromones is far from obvious since many pheromones are derived more or less directly from host chemicals ingested by a feeding insect (see previous discussion). Pheromones may be assembled from many basic building blocks by the signaller. Where the pheromone is derived more directly from the host-plant chemistry, presence of the host is obviously required for pheromone production. Interestingly, the host plant also seems to be required for production of aggregation pheromone even when the pheromone is truly being synthesised *de novo* by the insect. It is easy to imagine that some pheromone systems could have evolved from signallers enhancing cues given out by host plants for their own gains, perhaps initially by simple physical damage, and later by synthesis of these chemicals by the insect itself. Such, 'sensory trap', ideas are discussed by Christy (1995). Christy notes that in Lepidoptera, 'unlike female odours, male odours usually are similar to or the same as the odours of adult or larval food plants'. Thus, for these species at least, sensory traps are a tactic more often employed by males during mate attraction than females.

There is evidence that some *Dendroctonus* species of Scolytids do not require feeding as a cue for aggregation-pheromone production (see Wood, 1982). In these cases pheromone release from females can be stimulated by a sonic signal from the male.

Response to pheromone is often increased by confirmation of the presence of food via perception of food volatiles:

Potential responders to a pheromone signal may assess the existence of a food source before responding, by using host volatiles (for a review see Visser, 1986). The importance of the pheromone signal to be in the context of such volatiles varies from very strong synergism such as that found in Nitidulid beetles (Bartelt *et al.,* 1993a), to as yet no detectable influence (Pierce *et al.,* 1995 for Conophthorus species of Scolytidae, but see enhancing effect noted by Birgersson *et al.,* 1995). Bartelt *et al.,* (1993a) found that pheromone alone attracts only 1-29% as many beetles as the combinations with dough (source of food volatile), and dough alone only 0-2.9% as many as in combination. Here it is volatiles produced by the action of yeast on the host rather than volatiles emitted by a host

alone that seem to be most influential. Other synergistic influences of pheromone/food volatile combinations have been described in the Curculionidae (Walgenbach and Burkholder, 1986; Weissling *et al.,* 1994; Giblindavis *et al.,* 1994; Gries *et al.,* 1994b; Rochat *et al.,* 1993; and Phillips *et al.,* 1993) and Scolytidae (Pitman, 1969; Byers *et al.,* 1990). Other patterns of influence include additive effects (Renwick and Vite, 1969) and density-dependent but ill-defined effects (Dowdy *et al.,* 1993), and Jaffe *et al.,* (1993) proposes an interaction that is distance-dependent. Petroski and Vaz (1995) proposed that the pheromone and co-attractant bind in close proximity on the receptor surface. This evidence comes from molecular modelling of 26 host-type volatiles with pheromone from Nitidulidae. A recent review of the influence of host chemistry on the biology of attracting pheromones is given by Landolt and Phillips (1997).

Host volatiles are used as a tool in pest management. The use of host volatiles in combination with pheromone components in traps is reviewed in Faustini *et al.,* (1990). Host volatiles could also be used to confuse beetles. Inappropriate host volatiles have been shown to interrupt responses to true host volatiles and to an aggregation signal in bark beetles (Dickens *et al.,* 1992).

Produced by species with a relatively long lived adult stage:

According to Burkholder and Ma (1985), "two general types of communication and reproductive strategies characterise stored product insects". They propose that insects are either short lived, do not feed as adults, and produce sex pheromones; or they are long lived, have to feed as adults, and produce aggregation pheromones.

The food source is capable of supporting many individuals:

Here the data available are, unfortunately biased towards studies on insect-pest species. Therefore food sources are often artificially large, a good example being a grain store. I have found no studies on beetle species that generally feed on very small patches of food and produce aggregation pheromones. Such situations, however, could not result in the large groups of insects which tend to stimulate research funding, so the possibility of this occurring cannot be dismissed.

The identity of the signaller is sex specific:

Both males and females have been found to produce aggregation pheromones. Male-produced aggregation pheromones have been described in Bostrichidae (Dendy *et al.,* 1991; Khorramshahi and Burkholder, 1981); Cucujidae (White *et al.,* 1989; references in Phillips, 1994); Curculionidae (Budenberg *et al.,* 1993; Faustini *et al.,* 1982; Hibbard and Webster, 1993; Jaffe *et al.,* 1993; Nielsen and Jensen, 1993; Patrock *et al.,* 1992; Perez *et al.,* 1994; Rochat *et al.,* 1991b and 1993; Walgenbach *et al.,* 1983; Weissling *et al.,* 1994); Nitidulidae (Bartelt *et al.,* 1993b; Petroski *et al.,* 1994); Tenebrionidae (Obeng-Ofori and Coaker, 1990); Scarabae (Gries *et al.,* 1994a), and some genus of Scolytidae including Ips

(Akers *et al.*, 1993; Borden, 1967; Miller *et al.*, 1991; Seybold *et al.*, 1995; Byers *et al.*, 1990; Teale *et al.*, 1991a; Wood, 1982; Zuber *et al.*, 1993); Polygraphus (Bowers and Borden, 1990) Dryocetes (Camacho *et al.*, 1994), and Pitogenes (Byers, 1993).

Females have been described as the initial signallers in several genera of Scolytidae including *Dendroctonus* spp. (Agosta, 1992; Renwick and Vite, 1969; Wood, 1982), *Scolytus* spp. (Wood, 1982) and also *Trypodendron lineatum* (Raffa *et al.*, 1993). Usually female weevils produce sex pheromones but an aggregation pheromone has been described from female cabbage seed weevils, *Ceutorhynchus assimilis* (Evans and Bergeron, 1994).

Mostly only one sex produces aggregation pheromones. Where both sexes signal it is mostly with different chemicals. Cases where both sexes signal with the same chemicals are rare and in the case of the Cucujid beetle, *Ahasverus advena* the rate of production differs with males producing at least four times as much as females (Pierce *et al.*, 1991).

The pattern of response is sex specific:

Aggregation pheromones by definition attract both males and females. There are, however, many sex-specific differences in response to aggregation pheromones (review of Scolytidae, Raffa *et al.*, 1993). Sex specific differences can be complex to detail since the behaviour these pheromones elicit appears to be very variable both between individuals and for any one individual at different times. Thus any comparison between the sexes in terms of their response can give widely varying results very much dependent on the design of the experiment. Experiments that record the number and categories of beetles attracted to a pheromone source in the field can give insight into which conspecifics a signaller may expect to attract. Attraction depends on many factors including differential sensitivity of the responders to pheromone and which portion of the population is dispersing.

Zuber and Benz (1992) found a higher proportion of males in traps at the beginning of a flight period in *Ips typographus* thought to arise from sex specific patterns of dispersal. A similar situation was found by Chapman (1966) for *Trypodendron lineatum* , and by Rudinsky (1963) for *Dendroctonus pseudosuage* where additionally female numbers peaked late in the season in synchrony with re-emergence for "second brood" formation. *Polygraphus rufipennis* showed no sex-specific catch differences in the Spring and Summer, however more of the opposite sex to the signaller were caught in Winter (Bowers and Borden, 1990). Teale *et al.*, (1991a) found a seasonal variation in response to ipsdienol by *Ips pini. Ips typographus* and *Ips pini* response to pheromone was also found to be affected by nutritional state (Nemec *et al.*, 1993 and Gast *et al.*, 1993 respectively). Mating status can also affect response (see next sub-heading). Laboratory-based studies can

be designed to look specifically for any patterns of differing sensitivity to pheromone, which may give us further insight into the motivation behind the behaviour of responders.

Some studies have found no difference in response between the sexes (Field studies: Patrock *et al.*, 1992; Peng and Weiss, 1992. Laboratory studies: Dowdy *et al.*, 1993; Obeng-Ofori and Coaker, 1990; and Walgenbach *et al.* 1983). The opposite sex to the signaller usually shows the greater response where there is a difference in response between the sexes: Field data: Weissling *et al.*, (1994); Krausseopatz *et al.*, (1995); Evans and Bergeron (1994); Renwick and Vite (1969). Laboratory data: Borden (1967); Weissling *et al.*, (1994); Evans and Bergeron (1994); Faustini *et al.*, (1982); Gast *et al.*, (1993, Bowers and Borden 1990. The results obtained by Obeng-Ofori and Coaker (1990) for *Tribolium castaneum* are the expection where male pheromone attracted more males than females in a laboratory bioassay.

Schlyter *et al.*, (1987) suggested that males responding to male produced pheromone may avoid high concentrations of the signal and land a short distance from the source to avoid inter-male competition. Some sex-specific patterns of response recorded from trap-catch data may therefore arise from sex specificity in landing behaviour rather than from differences in the numbers of insects orientating towards the signal.

Sex-specific differences have also been found at the level of pheromone perception. Chambers *et al.*, (1990) found females of the Cucujid beetles, *Cryptolestes ferrugineus* and *C. pusillus* had larger electroantennogram (EAG) responses than males when exposed to the male-produced pheromone components. Faucheux (1994) has even found structural differences between the sexes with respect to their sensilla cells. EAG results are just one level of influence on behaviour, and studies by White and Chambers (1989) show marked sex-specific behavioural differences where no differences in EAG patterns were found.

Discrepancies between the response of the sexes can be further complicated when different components of pheromone blends skew the sex ratio in different directions. As we find out more about pheromone systems it is likely that more components will be revealed and an ever more complex pattern of response will become apparent. *Ips pini* and *Ips typographus* are good examples where complicated patterns of response are specific to the sex of the responders (Gast *et al.*, 1993; Schlyter *et al.*, 1987; Teale 1991). In another Scolytid, *Dendroctonus brevicomis*, at least one attractive pheromone component is produced by each sex that preferentially attracts the opposite sex; the combined blend results in an approximately equal sex ratio (Agosta, 1992). The Saw toothed grain borers, *Oryzaephilus surinamensis* and *Oryzaephilus mercator* have a multi-component pheromone whose blend also determines the degree of sexual dimorphism of response (White and Chambers, 1989 and refs. therein). The Tenebrionid, *Tribolium castaneum* shows sex

specific differences of response than can be altered by changing the ratio of the components (Rangaswamy and Sasikala, 1991). The enantiomeric ratio of single components can also have a sex-specific effect on response (Salom *et al.*, 1992). The ratio of pheromone to host volatiles may also influence the sex ratio of beetles attracted. In *Dendroctonus ponderosae* low trans-verbenol:resin ratio attracts mainly females where a high ratio results in a male bias (Quoted in Raffa *et al.*, 1993).

Production and/or response to pheromone often decreases with mating experience:

Arrival of the opposite sex and/or mating experience have often been found to decrease pheromone production. This is not always the case, for example mating was not found to affect pheromone output by males of the maize weevil, *Sitophilus zeamais* (Walgenbach *et al.*, 1983) although boll-weevil males decrease aggregation-pheromone production in response to a single mating with females (Dickens and Wigul, 1987). As yet, no effect has been demonstrated in the Bostrichid, *Rhyzopertha dominica*, (Dowdy *et al.*, 1993; Mayhew and Phillips, 1994), however in a related species, *Prostephanus truncatus*, pheromone production by the male can be decreased dramatically by exposing the signaller to females, even if indirectly in the form of grain previously infested by females (Smith *et al.*, 1996). Both *Polygraphus rufipennis* and *Ips confusus* males showed decreased pheromone production in the presence of females (Bowers *et al.*, 1991; and Borden, 1967 respectively). Production decreases in direct correlation with the number of females per male in *Ips confusus* (Borden, 1967). Decline in female attractivity on addition of males has been demonstrated in Chapman's studies on *Trypodendron lineatum* and Rudinsky's study of *Dendroctonus pseudotsuage* (Chapman, 1966; Rudinsky, 1963).

Some sex-pheromone systems also exhibit pheromone shut down in response to mating of the signaller. Pheromone titre produced by the female dropped dramatically within just two hours of mating in the moth, *Heliothis virescens* (Raina, 1990), and was associated with the female becoming unreceptive to further matings. In *H. virescens* receptivity and pheromone signalling can resume after a one-day refractory period, although in some moths females only mate once and females never resume signalling (discussed in Raina and Stadelbacher, 1990).

Mating status can also be correlated to the level of response to pheromone. Quoting from Borden (1967) when discussing *Ips confusus*, "reproducing females show a greatly reduced response to male attractant". Walgenbach *et al.*, (1983) found that only the female's response to pheromone decreased with mating and the male's response remained high whatever the mating status. No differences have been found between the responses of virgins versus non-virgins in Bostrichidae (Obeng-Ofori and Coaker, 1990; Dowdy *et al.*, 1993), or Tenebrionidae (Obeng-Ofori and Coaker, 1990), and Rochat *et al.*, (1991a) found no obvious effect for the American palm weevil, *Rhynchophorus palmarum*. Gast *et al.*, (1993) noted a general pattern where gonad development in adults was significantly

correlated to response to pheromone and so, "proportionately more responders were sexually mature than in the total population".

2.5: ANTI-AGGREGATION PHEROMONES

The growth of aggregations is often limited at least to some degree by repellent pheromone signals and/or the cessation of signalling. Repellents can be distinct chemicals from the original pheromone or different blends, or concentrations of chemicals that in another format, are attractive (see discussion in Schlyter *et al.*, 1987). For example the blend of endo- and exo-brevicommin determines whether it is an attractant or repellent to responders of *Dryocetes confusus* (Camacho *et al.*, 1994).

The trigger for anti-aggregants is thought to be a function of population density (Agosta, 1992). It is also often found that the rate of production of attractive pheromone per beetle decreases with the number of signallers in an aggregation. For instance, Australian sap beetle, *Carpophilus davidsoni* males in groups of 50-60 emitted a peak level of 0.09μg per beetle per day compared with a single male emission rate of over 3μg per beetle per day (Bartelt and James, 1994). Amount of pheromone per male decreased when population density was artificially manipulated in the Bostrichid *Rhyzopertha dominica* in an experiment using mixed cultures (Dowdy *et al.*, 1993; and Mayhew and Phillips, 1994). Pierce *et al.*, (1991) found that pheromone production of the Cucujidae, *Ahasverus advena* was, 'barely detectable', in the highest population densities of mixed sex cultures tested. Unfortunately it would be very difficult, if not impossible to monitor the breakdown of pheromone production across many co-habiting individuals. It is not known whether such decreases in pheromone production arise from some individuals completely ceasing production or a more general lowering of production by all individuals. In common with attractants, response to repellents can vary with their release rate (Bertram and Paine, 1994; Miller *et al.*, 1992), the season (Devlin and Borden, 1994), and many other influences.

Unlike the attracting pheromones, aggregation inhibitors are usually produced by both sexes. For example both males and females of *Dendroctonus brevicomis* (Bertram and Paine, 1994) and *D. frontalis* (Payne, 1992) emit verbenone, a common aggregation inhibitor in Scolytidae. 3,2-MCH has inhibitory effects for *D. pseudotsuage* and is produced by both sexes (Wood, 1982; Ross, 1994). Both males and females of *Ips paraconfusus* produce cis-verbenol (Wood, 1982), which acts as an inhibitor of male produced aggregation pheromone components (Akers *et al.*, 1993). Anti-aggregation pheromones are generally much less species specific than their corresponding attractants. As already mentioned verbenone is common amongst the Scolytidae, as is Ipsdienol (references above and; Safranyik, 1992; Shore *et al.*, 1992; Paine and Hamilton, 1991; Devlin, 1994; Miller and Borden, 1992; and Byers, 1993). It is proposed that the higher generality of these compounds between species allows them to function to limit both inter as

well as intra-specific competition. Indeed the pheromone of one species often has an inhibitory effect on other sympatric species (Schlyter and Anderbrandt, 1993; Miller and Borden, 1992; Byers, 1993).

Anti-aggregation compounds have obvious potential to be applied to pest management. They can be used to disrupt colonisation (Payne *et al.*, 1992) and they are often used in conjunction with attractants in a 'push-pull' strategy that can concentrate the pest away from high value commodities (Ross and Daterman, 1994; Smart *et al.*, 1994).

2.6: NON ADAPTIVE EXPLANATIONS

The problem to be discussed is, 'why do insects send these signals?'. Both adaptive and non-adaptive explanations might explain signallers' behaviour. We might expect the behaviour of *responders* to be adaptive, if not at present then surely at some point in the past. I suggest this because orientating towards pheromone sources may be expected to be costly in terms of both time and energy, particularly if flight is involved. In contrast, signalling could be of little cost, unavoidable or in fact costly to prevent. Many pheromones, as already mentioned are derived from host compounds and may be waste products of metabolism, in particular digestion. To *eliminate* the emission of such compounds following feeding may represent a significant cost to the signaller (Renwick and Hughes, 1975 quoted in Alcock, 1982). Here the signaller may actually suffer from advertising its position, and so no benefit from attracting conspecifics is necessarily inferred. The finding that generally only one sex signals implies that pheromone production is at least avoidable in these cases. If the pheromone is synthesised *de novo* as is indicated to be the case in some beetles studied (Seybold *et al.*, 1995; Ivarsson *et al.*, 1993), then production is likely to represent an energetic cost of some description, and indeed Schlyter and Birgersson (quoted in Schlyter and Birgersson, 1989) estimates that male *Ips typographus* expends the equivalent of 2% of its body weight per day when emitting pheromone.

2.7: ADAPTIVE EXPLANATIONS

It is tempting to look on the scale of the group when looking for adaptive explanations for the formation of aggregations, after all, it is a phenomenon involving many individuals (see Shorey, 1973). Explanations like, "...because the food resource may be either ephemeral or not available until the tree dies, these beetles have evolved a pheromone that elicits behaviour resulting in aggregation of the population on the new host.." are put forward (Wood, 1982). Here I stress that potential explanations should be described in terms of individuals to avoid wrongly categorising such explanations as group selectionist. Therefore I would start rephrasing Wood's proposal to read, "...a beetle that locates a host might benefit from advertising its location because...", followed by, "...beetles may move towards/away from this signal because...". Notice how the signaller cannot necessarily

dictate the response of the receivers of the signal and consequently may expose themselves to exploitation. Alcock (1982) has led the way in demanding explanations phrased in terms of individuals for the existence of bark beetle pheromone systems. Here I would like to extend this method to encompass other Coleoptera.

2.8: COSTS OF SIGNALLING

Costs of signalling are not limited to energetic costs since the signal may also create costs from its influence on other species. Potential competitors and/or predators can be attracted. Predators from a wide range of taxa have been shown to follow aggregation signals (Prestwich and Blomquist, 1987; Wood *et al.*, 1968; and refs. in Wood, 1982) and can be a selection pressure capable of changing pheromone structure/ composition. *Ips pini* varies the enantiomeric blend of ipsdienol from place to place and from year to year, in concordance with changes in response of its predator *Thanasimus dubius,* this possibly represents predator/prey co-evolution in action (Herms *et al.*, 1991; Raffa and Dahlsten, 1995). The possibility of such a system leading to polymorphism in respect to pheromone-production characteristics has been modelled for the European corn borer moth (Guldbrandtsen, 1995). Interestingly in this case just one-locus seems to determine pheromone blend in females, which can be traced using simple mendelian genetics.

Increased intraspecific competition for host resources is a potential cost incurred by signallers. As discussed above, however, hosts of these species are typically large and able to support many individuals. Where attracted conspecifics are the same sex as the signaller, a cost of increased competition for mates may be incurred. Mate availability is usually most limiting for males for whom this cost may be most significant.

2.9: BENEFITS OF SIGNALLING

The outcome of aggregation pheromone signalling systems is the formation of a cluster of individuals of both sexes on a food source. Potential benefits received by the individuals who invest in signalling could be better defending capacity against predators, overcoming the resistance of a potential host, and increased opportunity to mate and leave offspring (Thornhill, 1983).

Defence against predators has been proposed as an adaptive advantage of aggregating in the aposematically coloured beetle, *Lycus loripes* (Eisner and Kafatos, 1962, quoted in Thornhill, 1983).

Collective overcoming of host defences is a convincing function of aggregation in some species of bark beetles, particularly those that attack healthy trees (Wood, 1982). When a pioneering beetle has located a potential host it is proposed that it will either not be able to feed and/or oviposit efficiently until the host is transformed or 'conditioned' by the combined action of many beetles. Such a conditioning benefit is often inferred, but rarely

has it been demonstrated directly (with the exception of Raffa and Berryman's study of *Dendroctonus ponderosae* quoted in Alcock, 1982). An early study of *Dendroctonus ponderosae* demonstrated a possible food conditioning motivation for signalling, since a higher rate of pheromone production was observed when females were introduced to logs with higher oleoresin content in exudent (a method of host defence) (Vite and Pitman, 1968).

Conditioning may involve the inoculation of the host with fungal pathogens as is the case in some Scolytidae including *Dendroctonus pseudotsuage. Scolytus multistriatus* is also thought to inoculate the tree with pathogens e.g. Dutch Elm disease (Wood, 1982). Conditioning can simply involve the mechanical action of many beetles. 'Mass attack', the situation where an aggregation forms very rapidly, could be a method of killing a tree. Alternatively, signalling may be an incidental product of feeding, and the 'mass attack' often observed may simply arise as individuals race to take advantage of a comparatively short window where the host is relatively safe in terms of lowered defences and yet still not totally used up by those beetles already present (see Alcock, 1982). Although some Scolytid beetles are capable of attacking healthy trees, these are usually only colonised when stressed or dying trees are unavailable, and the majority of species will only colonise weakened or even dead hosts. *Dendroctonus ponderosa* and *D. brevicomis* can attack healthy trees but beetles have been found to be preferentially attracted to stressed trees (Wood, 1982). The Palmetto weevil, *Rhynchophorus cruentatus* is specifically attracted to volatiles from dying palms, its host (Giblindavis *et al.*, 1994).

In the stored product environment where the host resource is commonly made up of much smaller units of food, grain for instance, conditioning could take the form of changed humidity and substrate consistency from the action of many beetles. Beetles could potentially share the cost of burrowing through hard seed coats and use common entrances into a host. However, the benefit of this must be limited by the size of many grains inhabited by these beetles. A single ovipositing female may utilise more than one, and not just part of one grain. For example in the grain borer, *Prostephanus truncatus* a female will distribute her eggs on average in 12.5 grains (SE = 4.17, n=30) when ovipositing alone (Li, 1988). However, Fadamiro (1995) still proposes that the individual cost of boring can be decreased by feeding with more conspecifics. Walgenbach *et al.*, (1983) proposed that chewing through the seed coat of a grain kernel required much energy in *Sitophilus zeamais* aggregations since females require some 30 minutes or more to make a hole in a wheat kernel large enough to deposit an egg. They observed that when a weevil was feeding on a fresh kernel of wheat one or more companions were often present and all the grain was consumed before a new grain was burrowed into. I have yet to find a study that looks specifically at the division of cost of burrowing between individuals and obviously if a

beetle does not defend the food resource it has created access to there is potential here for exploitation by conspecifics.

Another potential benefit of high adult numbers could be increased offspring survival from a general increase in the amount of food found as dust/flour outside the grains. The survival of juveniles may be largely determined by the environment which they find outside the grain. Offspring of species who oviposit within grains like *Prostephanus truncatus* may show particularly high larval mortality if they happen to leave the grain whilst tunnelling for food. For this species risk of death may be expected to be highest when the adults are ovipositing on smaller grains since the larvae are more likely to fall out (suggested by Li, 1988). Li has found no evidence for such a collective-conditioning benefit in this context for the Larger Grain Borer. For initial adult densities ranging from 6-26 pairs per 60g of maize the average number of offspring per female emerging was never found to be higher for an increase in adult density (Li, 1988).

Aggregation pheromones may be used as sex attractants and the benefit incurred to signallers may not be one associated with a general increase in population size, more an increase in the availability of mates. At least some studies assume that the main benefit of aggregation pheromones is increased mating opportunities for the signaller (Lewis and Austad, 1994; Landolt and Phillips, 1997). Sex pheromones, i.e. those that attract *only* the opposite sex have been well studied (see a review for moths by Svensson, 1996). Initially signallers may benefit from attracting members of the *same* sex through increase in the efficiency of signalling, but these beetles may be more accurately described as a cost of signalling since they are also potential competitors for mates. This is thought to be the situation found in some field crickets described in Alcock (1982) who signal by chirping.

Where males are the signallers it is feasible to imagine that females could choose between mates on the basis of these signals if they are variable, and therefore some pattern of assortative mating could be governed by these pheromones. Where different pheromones are used for short and long range signalling it may be predicted that the shorter range components will be most influenced by sexual selection since it is at this point that a female is most likely to encounter more than one signal and has most opportunity to make comparisons. It has now been shown that female preference in *Ips pini* is actually correlated to the blend of enantiomers of ipsdienol produced by individual males which leads to this feature being a main determinant of the pattern of assortative mating in this species (Teale *et al.*, 1994). Alternatively the signaller may be exercising a form of mate choice by selecting only those insects that have survived the dispersal phase and have successfully orientated to the pheromone signal (discussed in Raffa *et al.*, 1993).

2.10: COSTS AND BENEFITS OF RESPONDING TO PHEROMONE

Beetles may respond to a pheromone signal as it indicates the location of a host usually used for both feeding and oviposition, and potential mates. Host distribution and defences as well as any pattern of mate choice determined by pheromone characteristics may be predicted to influence the propensity of beetles to follow pheromone plumes instead of locating an unused host. Also crucial to this choice may be the nutritional and reproductive state of the insect. A dispersing male low on food reserves that feeds on a rare host would surely gain most from orientating to pheromone in contrast to a well fed, impregnated female who utilises a very common host.

2.11: HYPOTHESES DISCUSSED IN TERMS OF THE BIOLOGICAL FEATURES OF AGGREGATION PHEROMONES

In this section, I return to the generalisations made in section 2.4 and consider the specific questions that arise in relation to adaptive functions of aggregation pheromones.

Pheromone produced only when signaller is on the host:

Collective conditioning of a host is a benefit which will obviously only be realised if the signaller is close to such a host. Mate attraction need not necessarily occur at a host (Thornhill and Alcock, 1983). However, as discussed above, the chemical nature of the pheromone may constrain would be signallers to signal only when they have recently been feeding, and/or the importance of the pheromone signal to be in the context of host volatiles may constrain signallers to signal only when on a host. In the species considered here the host is a food source for both adults and developing offspring. Any delay between mating and oviposition while a female locates such a host will potentially increase the risk of females mating again before fertilizing her eggs and so promoting sperm competition and possible loss of paternity for the signaller. See chapter 3 (this thesis) for an assessment of whether LGB males continue to signal when removed from food, and chapter 7 for an assessment of the potential for sperm competition in LGB).

Species are long lived as an adult:

A common character of all these species is that they are comparatively long lived as adults, and colonise hosts which once found/conditioned, can potentially support many individuals (noted by Burkholder and Ma, 1985). I believe that it is these characteristics which have facilitated the evolution of aggregation pheromones and ultimately explain why, if their function is primarily sexual, con-specifics of the same sex as well as potential mates are attracted.

The food source is capable of supporting many individuals:

Fairly long term aggregations (longer than a generation time) would only be observed if the host can support such a group. The cost of attracting conspecifics in terms of increased competition for resources will hypothetically become more and more significant if host sizes were reduced assuming, perhaps wrongly, that insects would not leave the aggregation to forage. Collective host conditioning would surely only feature when all insects investing in overcoming host defences are able to gain from subsequent utilisation of that host. The requirement of a large host is less obvious if these signals serve to attract mates. However, if for whatever quirk of history they are used primarily as a method for beetles to increase their rate of copulation, then this kind of system would surely be the perfect setting for promotion of sexual selection. The opportunity for male-male competition and female choice being greatest when many individuals are grouped together in this way.

The identity of the signaller is sex specific:

Sex specificity of signalling patterns tells us that there are or have been some sex specific selection pressures which have given rise to this system. The most obvious differences between the sexes are those directly concerned with reproduction. However, these differences can have far reaching effects on the rest of the biology of an organism. The signalling sex sometimes shows a greater response to host volatiles (Gast *et al.*, 1993) and thus sex-specific signalling could reflect sex differences in dispersal. So although we cannot assume that signalling is directly involved in reproductive behaviour, perhaps this is where we should look first for an explanation. Males are the signallers in some species and females signal in others, what are the differences between these species which could account for this? Males have the potential to gain most in reproductive output from increased numbers of mates particularly if they invest little each offspring i.e. provide no parental care and low amounts of nutrition in the ejaculate. Females could benefit by promoting all forms of sexual selection by initiating an aggregation, but will be far more limited in the number of offspring she can produce by energetic considerations.

Perhaps the key characteristic in the less common situation where females are the signallers is that these are the species that are most likely to be benefiting from a host conditioning effect. Specifically the Dendroctonus and Scolytus species of Scolytidae, many of whom are known to kill healthy trees and require this for successful egg laying (Wood, 1982). However, in the Scolytid species, *Trypodendron lineatum*, the female produces the aggregation pheromone, yet this species is not considered to be particularly aggressive and is not known to actively kill trees.

The pattern of response is sex specific:

The finding that the opposite sex to that of the signaller often shows a greater response to pheromone leads to these aggregation pheromones having more in common with conventional sex pheromones. See chapter 4 and 5 (this thesis) for an assessment of

sex specificity of response in LGB: in a laboratory bioassay (chapter 4); and in the field (chapter 5).

Production and/or response to pheromone often decreases with mating experience:

This finding is perhaps the most convincing evidence that these pheromone systems are intimately tied to sexual behaviour. Alternative explanations are possible, Fadamiro (1995) has put forward the idea that male signallers use the presence of females, detected via an involatile chemical, as an indication of population density, and thus the observed decrease in pheromone production on exposure to this chemical need not be explained in terms of any mating function.

Benefit from signalling may undulate quite rapidly for any single signaller. As mates arrive pheromone production may be cut off for a while until the signaller is free to remate again. The number of matings optimal for any individual will obviously vary both between the sexes and between species. Pheromone production should really only be sustained for polygamous males with low investment per female. Polygamous males of the maize weevil, *Sitophilus zeamais* do not show decreased pheromone production with mating (Walgenbach *et al.*, 1983). High investing males, like *Ips confusus*, who construct the nuptial chamber and actually guard this against intruders of both sexes, might be expected to decrease pheromone production as he acquires the optimum number of mates he can support. This was found to be the case (Borden, 1967). Decrease in female production of aggregation pheromone after the arrival of males has not been specifically demonstrated since the decline in attractivity recorded could be due to male produced repellents as suggested by Nijolt (1973) for *Trypodendron lineatum.*

Anti-aggregants:

The point where production of aggregation pheromones switches to the production of anti-aggregants could be used to infer exactly when and for which individuals the costs of encouraging conspecific aggregation outweigh the benefits. All the possible benefits discussed here derived from the formation of an aggregation will tend to be counter balanced by the costs of sharing resources, particularly as the aggregation grows and the host patch is depleted. Since these characteristics are common to all hypotheses it might be unrealistic to use them to distinguish between possible benefits gained. Evidence of increased intraspecific competition at higher beetle densities has been found in *Dendroctonus ponderosa* where there is a strong negative correlation between density of attacks in cut logs (i.e. defenceless ones), and pupal production per female (Raffa and Berryman (in prep.) quoted in Alcock 1982). Raffa *et al.*, (1993), suggests that, "inhibitory pheromones may function as 'pre-rivalry' signals in male-male interactions, in that both signaller and responder benefit from avoiding rivalry", (see also Pierce *et al.*, 1995 and Miller and Borden, 1992). The honesty of such signals may be ensured if response to them is dependent on their concentration and this is a truly constrained by the number of beetles

signalling. Dose-dependent inhibition of attractant by verbenone has been found in three Scolytid beetles where higher doses give most inhibition (Miller *et al.*, 1995).

2.12: FUTURE DIRECTIONS

I have discussed some of the possible selection pressures that may have shaped aggregation-pheromone systems, the two most likely being host conditioning and mate attraction. We may never know what happened in the past, but perhaps we can determine which of these influences are acting to maintain these systems today. Below I outline areas of further study that could contribute to this discussion.

One potentially profitable approach would be to apply the comparative method to this topic. A study of patterns of signalling correlated to the life history strategy and ecology of different species may yield interesting results. I have already mentioned a possible starting hypothesis, that species in which females are the signallers may be those colonising hosts with a relatively high capacity to resist attack. Similarly, a review of the mating systems of species that have aggregation pheromones might highlight interesting trends. The Scolytidae would be a good start for such a study. Kirkendall (1983) has reviewed the mating systems of this incredibly diverse family. The data available are limited, however, they do suggest some associations. Species where males produce pheromones that have been shown to attract both sexes are, without exception in the sample reviewed, the initial colonisers and they are harem-polygynous. This means that males in gallery systems are usually found with more than one female. The shut down of pheromone in response to arrival of mates appears to be variable between species of the same family and the distribution of this character among species could also provide further insights.

A contrasting approach would be to study any variation between individual signallers. This may be key to evaluating any role these pheromones play in sexual selection. Be it genetically or environmentally determined, variation in ability to attract potential mates places pheromone signalling as a prime candidate to be influenced by sexual selection. Genetic variation in pheromone production certainly exists in the Boll weevil where selective breeding for its increase has led to a 4.5 fold increase in selected strains as compared to the parent population (McCoy and Wright, 1990).

What evidence have we already that is compatible with the idea that females use pheromone production to choose between mates? Male olfactory attractiveness to females is positively correlated with a male's subsequent fertilisation success as measured by its P_2 value in double matings in the red flour beetle *Tribolium castaneum* (Lewis and Austad, 1994). So in this case two possible influences on the pattern of paternity are correlated. In *T. castaneum*, "neither males nor females exhibit aggressive behaviour towards members of their own or the opposite sex", (Lewis and Austad, 1994) so it is thought that behaviour on contact contributes little to assortative mating in this species. Assortative mating has been

correlated to pheromone characteristics in *Ips pini* where it is the ratio of enantiomers of ipsdienol which were found to be correlated to mating success (Teale *et al.*, 1994). Different females were found to have a consistent preference within the variation of ratios, and males were found to show highest response to blends similar to those that they were subsequently found to produce. The evolutionary genetics of these systems warrants further consideration since signal production and response are not analogous as might be the case in assortative mating by body size (see discussion in Teale *et al.*, 1994). No correlation was found between pheromone characteristics and body size. Teale agrees with Herms *et al.*, (1991) and infers that predation and interspecific competition may be the source of selection pressures that have resulted in this pattern of pheromone production and response.

Increased reproduction on 'conditioned' hosts is often inferred, yet has surprisingly little documentation. Perhaps a study of life-history traits for individuals reared on previously infested and uninfested hosts would give an insight into the magnitude of this possible benefit. It is very difficult to definitively list all hosts that any one species might utilise. If no collective feeding benefit can be found for an aggregating species on known hosts, the possibility of significant benefit on undocumented hosts is always possible.

2.13: CONCLUSIONS

To sum up, both sexual and host conditioning functions of aggregation pheromones are possible given the evidence currently available. It is likely that in those cases where a conditioning function is implied, both kinds of benefit are occurring for the signaller. I have looked at evidence for aggregation-pheromone function from the patterns that can be recorded now, however, it must be remembered that such pheromones may have initially evolved in a very different context and may have had a different function. For example, *Prostephanus truncatus* is currently found as a pest of stored maize and Cassava, but is also known to be a wood borer (Hodges, 1994), a habit that may have been influential in determining its use of pheromones. I will be examining the current influence of the aggregation pheromone signal on mate choice in *Prostephanus truncatus* by studying variation between individuals as suggested in the 'future work' section of this review.

CHAPTER 3: BASIC PATTERNS OF AGGREGATION-PHEROMONE SIGNALLING AND RESPONSE.

3.1 INTRODUCTION

The individual was chosen as the unit of study in order to investigate the function of aggregation pheromone in *Prostephanus truncatus*. It was hoped that by looking at patterns of signalling and response of individuals, rather than groups of beetles, trade-offs and correlations between individual variation in signalling and other traits could be investigated. Reliable measurement of the variation in pheromone signal of single insects was always going to be a difficult, but rewarding challenge. Whilst this work was in progress, three other bioassays were also designed to monitor the walking response of LGB to single signalling males (Hodges and Dobson, 1998 in press; Boughton and Fadamiro, 1996; Scholz, 1997). Previous work used groups of beetles as the unit of study.

Two main methods were employed throughout this work: first the response of conspecifics was used to detect and quantify pheromone signals; second, pheromone samples were collected directly onto filters for chemical analysis. The first method, the use of a bioassay, proved to be more productive although there can be no substitute for direct chemical analysis to answer some questions. This chapter presents the bioassay design and some initial experiments designed to map out some basic patterns of pheromone production. Three aims were addressed: first, to test the bioassay equipment; second, to obtain knowledge of the influence of some variables on pheromone production and response, which was required for the design of later experiments; third, to obtain knowledge needed to elucidate the biological function of aggregation pheromone in LGB. The main variables tested were: time of day; presentation of maize as whole grains or flour to the signaller; removal and addition of food to the signaller; type of plant host; and the presence of females.

The CTH room used in the majority of the laboratory-based experiments in this study runs on a 12 hours light and 12 hours dark cycle. The light comes on at 8am and goes off at 8pm (GMT). LGB has already been shown to exhibit a periodicity of flight activity in the laboratory that is similar to that observed in the field where beetles become particularly active around dusk and in some cases dawn (Scholz, 1997; Tigar *et al.*, 1993; Fadamiro and Wyatt, 1995). *Ips confusus* (Scolytidae) exhibits periodicity of pheromone production that varies in synchrony with the periodicity of flight activity (Borden, 1967). In fact there are many such examples of periodicity of pheromone release that coincide with general increase in activity, which are particularly common for true sex pheromones (see Burkholder and Ma, 1985, and references therein). Therefore, it was likely that LGB might

show a variable bioassay score with time of day. We needed to know whether there were any times of day when production/response to pheromone was particularly low and therefore should be avoided in the design of experiments.

Prostephanus truncatus is a tunnelling beetle and it can be difficult to extract males from their food. Also, when males fall out of grains or are busy tunnelling into a new grain this may alter their rate of food uptake, which in turn may alter their pheromone signal. To avoid such complications I tested to see if males will signal if the grain is presented as a more uniform medium such as coarse ground maize meal.

Information about the timing of initiation and cessation of signalling when males are put on food or left to starve will address the question of whether male LGB will produce pheromone when not on food. In other words, do male LGB ever cheat by signalling in the absence of an appropriate oviposition site or is pheromone production always an honest signal that indicates the location of an appropriate host? Direct collection of pheromone from 20 males placed on plaster of Paris chips instead of food showed that no signal (measured as amount of T1) was being produced after three days (Smith *et al.*, 1996).

Acoustic signals may be energetically costly, variation in call rate and duration together account for 80% of the variance in metabolism during calling in the tree frog, *Hyla versicolor* (quoted in Andersson, 1994). This has led to the idea that such signals may be used as an honest indicator of health as well as location. The discussion of whether the pheromone signal of LGB males is energetically or nutritionally constrained was investigated further by testing the influence of food type on the signal. Signallers feeding on the root crop cassava were compared to those feeding on maize. Although LGB is known to breed on cassava, it is less nutritious than maize and F1 offspring are significantly smaller (R.H. Smith, pers. comm.). Scholz (1997) found no difference in the attractivity of pheromone signals from males signalling on wood species known to support LGB reproduction (*Commiphora africana*), compared to those feeding on maize even when both these odour sources were presented simultaneously, although the response to maize was numerically higher.

Shutdown of production of aggregation pheromone was detectable after five days exposure of males to females in a Scolytid beetle (Borden, 1967). Pheromone shut down in LGB can also be induced by the presence of females (Smith *et al.*, 1996)). This phenomenon is obviously crucial to the interpretation of the biological function of this signal and was used in later experiments. Smith *et al.*, (1996) found a significant decrease in pheromone production by males just 24 hours after the introduction of live females. In Smith *et al.*, (1996), beetles were not allowed sufficient time to reach their maximum rate of pheromone output before one half of the test insects were exposed to live females. This

may reduce the time it takes for the treatments to become significantly different because the control treatment is still on the increase as the female-treated group declines. I have used my bioassay to record the timing of shut down of fully signalling males.

3.2 BIOASSAY DESIGN

The bioassay presented here provides information about the net outcome of a signal and the response to that signal. It can be difficult to ascribe variation in outcome specifically to the signal or the response. If differentiation is required, artificial sources of pheromone can be used to provide a reliable signal. Likewise, direct chemical analysis of the signal could eliminate variation arising from a variable response.

The walking response of beetles moving towards an aggregation pheromone source was chosen as the variable to be measured in the bioassay. During the initial development of the apparatus, a failure to see any observable response to a signaller could have arisen either because the male was not signalling or because the responder was ignoring the signal, or because the apparatus was not relaying the signal. The use of artificial pheromone sources to provide a reliable signal was rejected for fear of contamination with unnaturally high quantities of super attractive chemicals, which might later mask subtle differences in natural signals. Direct collection of signals to check that males were signalling was also rejected as an option since this is very time consuming and has its own technical difficulties.

A wide variety of methods were tested incorporating the use of different shaped chambers; chambers made of different materials; different aged beetles; beetles isolated from conspecifics for varying amounts of time; responding beetles kept with or without food; male and female responders; and different ambient conditions. It was not feasible to test all possible combinations. Instead, using what was already known about this signalling system the most likely combinations of insects, conditions and apparatus were tested first. All apparent success in eliciting some kind of response to the signaller was recorded and the bioassay was improved step by step until a reliable method was found.

The result was an olfactometer consisting of a choice chamber with four different air currents leading into a central arena. A reliable signal was produced when males were kept isolated on food for one week at 30 °C 70% r.h. Females were found to be more responsive than males to pheromone signals. Responders were also kept isolated on food for one week prior to use. The number of times the responding beetle placed its head and prothorax in each of the tubes leading into the central arena within a predetermined period of time was chosen as a convenient measure of the walking response. To make each tube choice independent, the responder is placed back into the centre of the arena after each choice.

3.2.1 Details of the apparatus

For a plan view of the bioassay apparatus see fig. 3.2.1a. The plastic petri dish (central arena) has a hole drilled in the lid connected to a vacuum pump which draws air from outside the apparatus through the holes in the lids of the source pots and into the arena through clear plastic tubing. Air currents are thus drawn from four pots containing either the beetle source to be assayed, or grain only (see fig. 3.2.1b). Grain-only pots act as a control for the influence of food volatiles and any inherent tendency of beetles to crawl down tubes. The petri-dish was lined with a disc of paper towel that is very slightly larger than the base of the dish so it can be made flush with the sides so that beetles are unable to crawl under it. This provides a rough surface over which beetles can move easily. All assays were performed in a constant temperature room where temperature, humidity and light regime were controlled.

Up to three arenas could be assayed together at any one time. Suction was always adjusted so that each arena received suction at a rate of 1 litre of air per minute.

3.2.2 Procedure during the assay

1. The next sources to be assayed are connected directly to suction tube for 3 minutes to remove volatiles already present in air in the container.
2. Petri-dish is wiped with 70% ethanol (water solvent).
3. New paper lining is put in the arena.
4. New tubes are used to connect up the pots to the arena (control pots are used several times).
5. Suction is checked.
6. New source pot is incorporated into the apparatus in a random position around the arena.
7. A new responder beetle, is placed in the middle of the arena, the lid is put back and the stop watch is started. All responders were kept in a holding arena similar to the arena of the choice chamber for a period of one assay prior to testing.
8. The number of times a responder places its head and prothorax into each tube is recorded for a set length of time (usually 20 minutes). Each time this occurs the responder is returned to the middle of the arena.
9. Assay is dismantled.

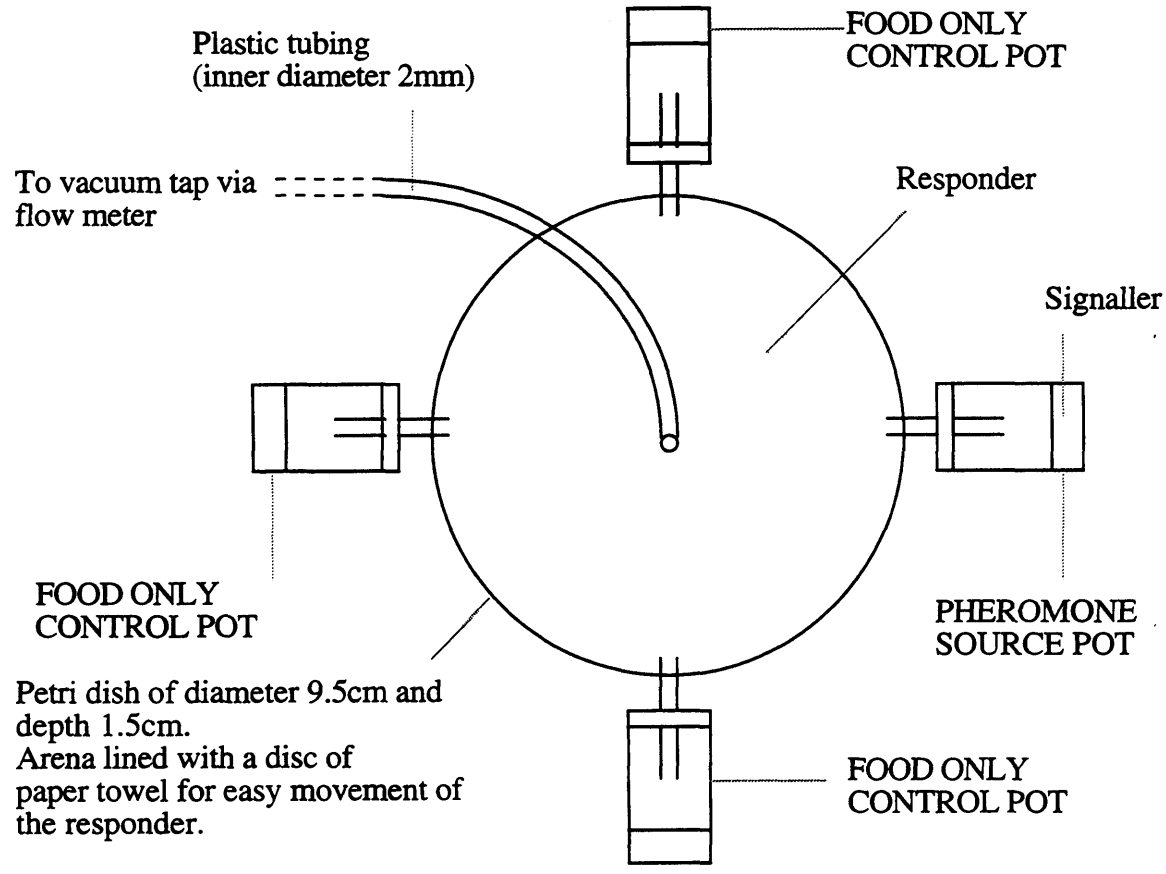


Fig. 3.2.1a: Plan view of bioassay apparatus.

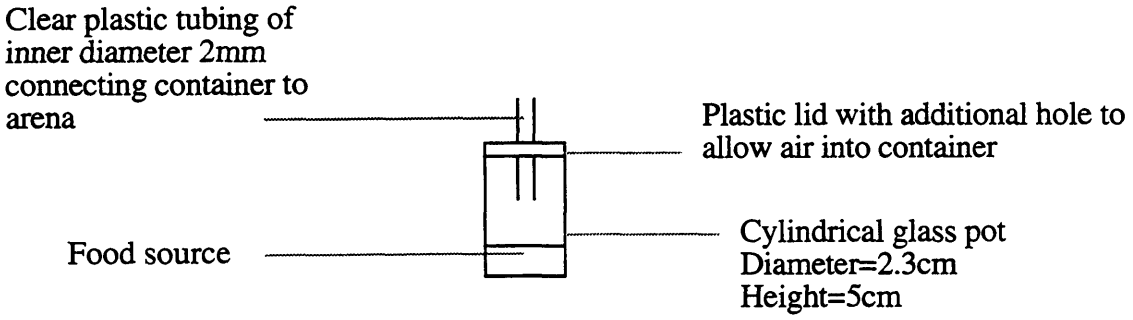


Fig. 3.2.1b: Detail of source pots.

3.3 MATERIALS AND METHODS

3.3.1 Influence of time of day

Source of insects:

The males used as pheromone sources were first isolated as pupae. Males were reared separately on flour for two weeks, sexed and then placed on fresh split grain seven days before the first assay. Fifty females were used as responders. These were sieved from a six week old culture, sexed, and then placed on fresh ground grain in a pheromone-free CTH room (30°C) six days before the first assay.

Apparatus:

Olfactometer apparatus as described in section 3.2. was used for the bioassays. Each assay was continued for 20 minutes and two arenas were run side by side.

Experimental design:

12 single male pheromone sources were used. Each source was assayed once during all hours of light in the CTH room. Four replicates were assayed every hour from 11am-2pm and 5pm-8pm on one day, and then 8am-11am and 2pm-5pm on the following day. This sequence was repeated for a new batch of four males every two days. Trials therefore continued over six days. Responders always had a break of two days between consecutive trials. Within this constraint responders were randomly allocated to trials.

Analysis:

A Kruskal-Wallis test was used to test for any significant differences of response with time of day.

3.3.2 Influence of using flour instead of whole grain as food, on the signaller

Source of insects:

16 male signallers were reared from pupae. All males were approximately three weeks old when they were placed into a treatment group.

Apparatus:

Olfactometer apparatus as described in section 3.2 was used. All assays were continued for 20 minutes. Two sets of apparatus were run simultaneously.

Experimental design:

Males were randomly assigned to one of two food regimes: first, approximately $3cm^3$ coarse ground maize meal; second, an equivalent amount of split maize. Males were left in their treatments for 10 days before the first assay. All replicates were assayed once each per day for four days. One of each treatment was assayed at the same time.

3.3.3 Timing of shut down of signal induced by removal of food

Source of insects:

20 males of mixed age were used as pheromone sources (taken from a 2 month old culture). All were placed singly in glass pots with fresh grain and left for 10 days prior to the experiment. A bank of 40 females was used as responders. The females were all placed singly in glass pots with fresh grain 10 days prior to the first assay.

Apparatus:

Bioassay apparatus is described in section 3.2. Source pots were adapted to allow food to be present in a pot, yet not accessible to test insects, see fig. 3.3.3a. Two sets of apparatus were assayed at once (one from each treatment) and assays were continued for 20 minutes.

Experimental design:

Males were randomly allocated to one of two treatments, 'with food' and 'without food' (see fig. 3.3.3a). Males were placed in test pots and all replicates were assayed once daily for four days. Replicates were assayed two at a time, one of each treatment in random order (picked out of a hat).

Analysis:

The response given to each of the treatments at the end of the trials (day 4) was compared for a difference using a Mann-Whitney U test since there was a small sample size and the data obtained did not approximate to a normal distribution.

3.3.4 Timing of onset of signalling after placing on grain

Source of insects:

Male signallers were placed individually on coarse ground grain and left in the CTH room for one week. All males were then placed in fresh pots containing small pieces of polystyrene, but no food. Three days later males were assigned to treatment pots. 35 females were used as responders. All were placed individually on fresh, ground maize meal and left for 10 days before the first assay.

Apparatus:

Bioassay apparatus was used as described in section 3.2. Source pots as described in experiment 3.3.3 were used (see fig. 3.3.3a).

Experimental design:

24 males were randomly assigned to one of three treatments:

1. x8 Kept starved
2. x8 Allowed access to grain
3. x8 Allowed access to grain and one live female.

All replicates were assayed once per day on day 0,1,2,4,6,7,9,and 13 after males were placed into treatment pots. After being assayed on day 13, all males from treatments 2 and 3 were placed on fresh grain with no females. These pots were then assayed after a further eight days (day 21).

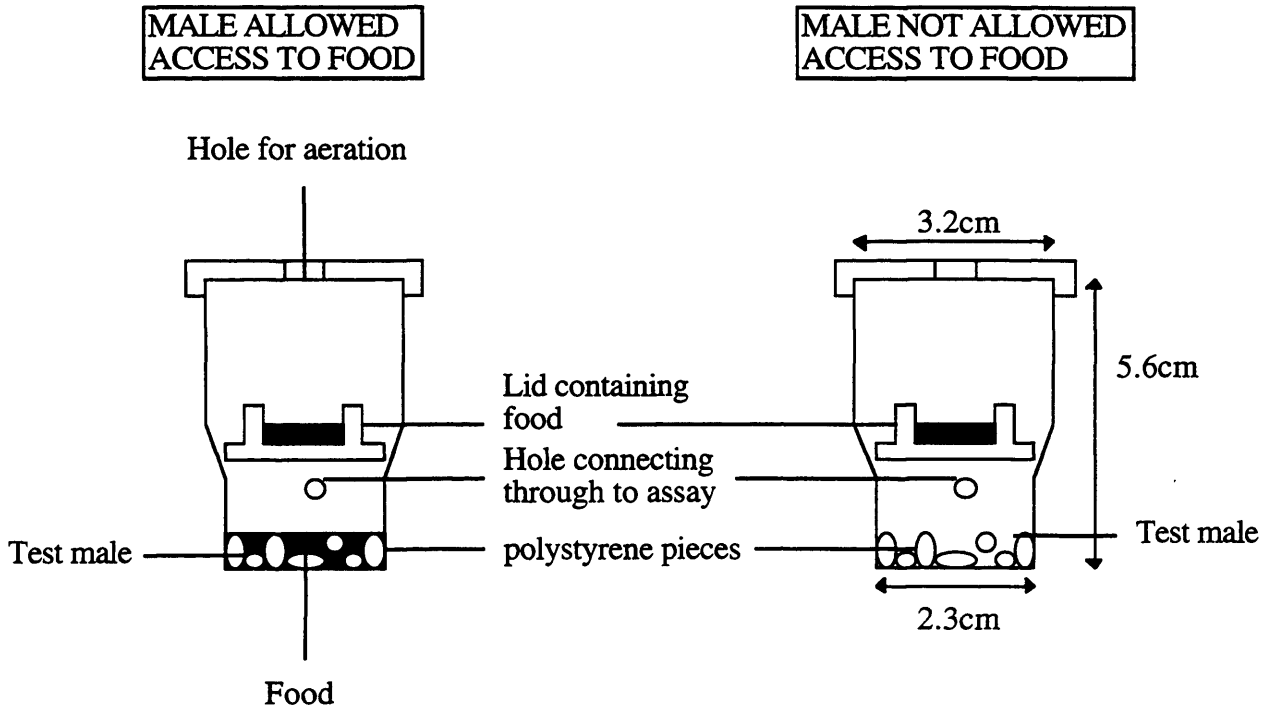


Fig. 3.3.3.a : Cross section of plastic pheromone-source pots adapted to contain food without allowing insects access to it. NB. LGB cannot walk up smooth vertical surfaces.

3.3.5 Influence of plant host on male-aggregation-pheromone signal

Source of insects:

All insects were sieved from a 10 week old culture. All insects were placed individually on food in glass pots. 50 females were placed on coarse ground maize meal. 48 males were randomly allocated to one of two food regimes:

1. x24 Three split maize grains and a small quantity of coarse maize meal
2. x24 A similar volume of cassava (supplied by NRI see section 1.5.2)

All beetles were placed in the CTH room and left for 14 days before the first bioassay trials. The quantity of food given was chosen such that it was excess to that which could possibly be fully tunnelled by the male, yet small enough to limit the possible impact of differential absorption of pheromone onto the food source on the volatiles released from the pot as a whole.

Apparatus:

Olfactometer apparatus as described in section 3.2 was used. Three replicates were run at once. Assays were 15 minutes long.

Experimental design:

All male sources were assayed singly 14 days after placement in the two food treatments. The three remaining positions in the olfactometer were occupied by food only controls. Males placed on cassava had cassava only controls and males placed on maize had maize only controls. Two replicates of one treatment were assayed at the same time as one replicate of the other treatment and then vice-versa for each group of three replicates throughout the day. Within this constraint, the order with which males were assayed was randomised.

One day later all males were randomly paired up with a male from the other treatment group. Each pair was assayed together in the olfactometer apparatus in positions opposite each other. The two remaining positions in the olfactometer were taken up by one maize-only control pot and one cassava only control pot.

Single females were used as responders and were randomly selected from a bank of 50. Each female was used a maximum of once per day.

Analysis:

A Wilcoxon signed-ranks test was used to test for a difference between the responses given to each of the treatments when they were presented simultaneously.

3.3.6 Timing of shut down of pheromone signal induced by Female Factor.

Source of insects:

Thirty males were taken from a two month old culture and used as signallers for this experiment. They were sexed and placed in individual glass pots on fresh maize meal and left in the CTH room for 10 days. At the end of this period each male was assigned to one of three treatments:

1. x10 Single male on slightly kibbled grain with some flour, total quantity equivalent to approximately two whole grains of maize.

2. x10 As in treatment **1** with a single live adult female.

3. x10 As in treatment **1** but using previously infested grain from the same culture jar that the signallers were taken from (two month old). This was first frozen for eight days and then left to acclimate to the CTH room for two days.

All females used (as responders and in treatment 2) were taken from a six week old culture, sexed and placed in individual glass pots on fresh maize meal and left in the CTH room for 20 days.

Apparatus:

Bioassay apparatus was used as described in section 3.2.

Experimental design:

Assays were started the day after placement of males in treatment pots. Assays were performed daily for four days and then every other day until 10 days of treatment had passed.

Each assay was 20 minutes long and three sets of apparatus were run simultaneously, one allocated to each of the treatments randomly. Responders were selected randomly from a bank of approximately 40 and no responder was used more than once on the same day. Control pots contained the same grain as that used in treatment 1.

Analysis:

Mann-Whitney U tests were used to compare separately both Female-Factor treatments with the males signalling on fresh grain.

3.4 RESULTS

3.4.1 Influence of time of day

The frequency distribution of responses recorded is skewed to the left since many assays recorded low or zero responses. The data are therefore represented as medians and their interquartile ranges.

The overall median response per trial given to source pots (those containing a male) was 3 visits (25% quartile=1, 75% quartile=6). The overall median response per trial given to food only control pots was 0 visits (25% quartile=0, 75% quartile=0.333). Control pots elicited a significantly lower response than source pots (Mann-Whitney U test stat. = 2337, N=144, $p<0.0001$). The response to single male pheromone sources was found to be higher than that for control tubes for all hours of the light phase of the CTH room (fig. 3.4.1). Therefore source pots retain some attractivity throughout this time and females are responsive to this signal throughout this time. No significant pattern of changing response with time of day was detected: Kruskal-Wallis test statistic = 6.62, d.f.=11, p=0.83.

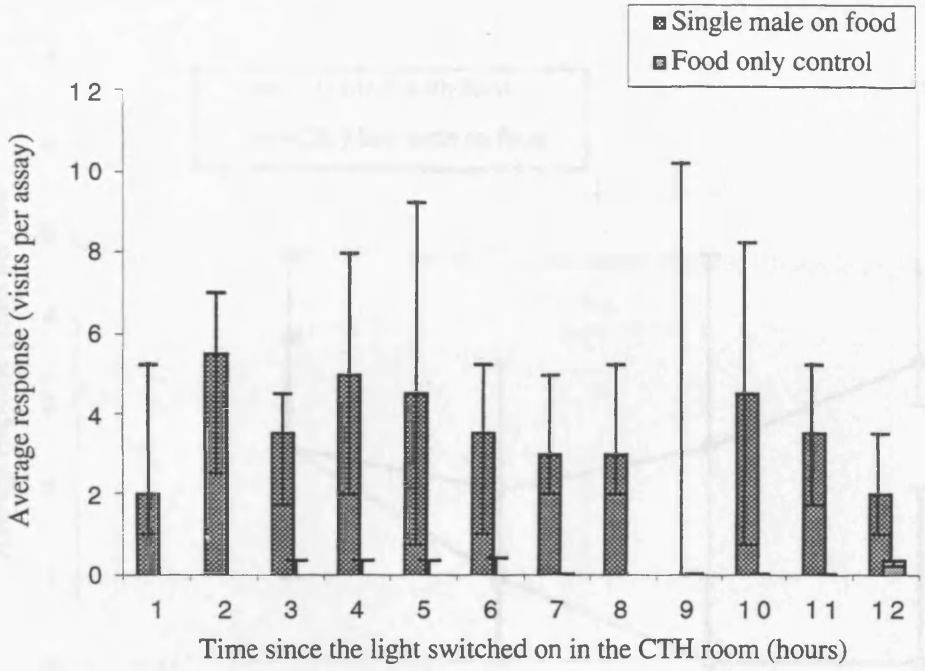


Fig. 3.4.1: Bar chart to show the median response given to single males placed on food for all hours of the light phase in the CTH room. Error bars span the 25% and 75% quartile values. N=12 for all columns. No significant pattern of changing response with time of day was detected: Kruskal-Wallis test statistic = 6.62, d.f.=11, p=0.83.

3.4.2 Influence of using flour instead of whole grain as food, on the signaller

No significant difference was found in the average response to signallers placed on whole or ground grain: whole grain median = 7 visits per trial (25% quartile = 4; 75% quartile = 8.25); ground grain median = 6.5 visits per trial (25% quartile =1.75; 75% quartile = 9) (N=8 for each treatment).

3.4.3 Timing of shut down of signal induced by removal of food

The median and inter-quartile range of response to signallers from both treatments was initially of comparable value. The median response recorded for signallers deprived of food consistently dropped each day until reaching zero by day three. All signallers from the food-deprived treatment were still alive up until day four, but two had died by day six, the rest had died by day eight. All signallers given food lived until the end of the experiment. On day four (the last day that all subjects were alive), males on food were significantly more attractive than those allowed to starve (Mann-Whitney U test stat.=84.5, 1d.f., $p<0.001$) (see fig. 3.4.3). The response given to all food only control pots remained low throughout the experiment (overall median = 0; 25% quartile = 0, 75% quartile = 0.333).

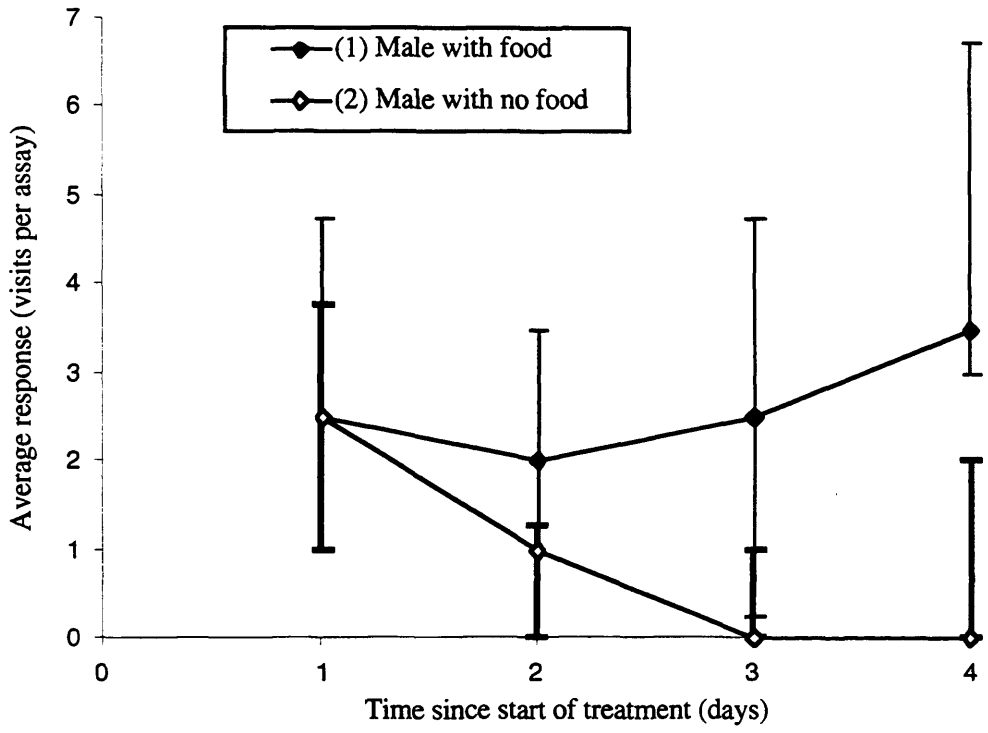


Fig. 3.4.3: Median response with time to single males either on food or without food compared to food only control pots. Error bars span the 25% and 75% quartile values. N=10 for each point.

3.4.4 Timing of onset of signalling after placing on grain

A detectable response to signallers placed on food with no female (compared to control pots) was recorded after six days and was maintained until the end of the experiment (day 21) (see fig. 3.4.4). The average response from day 6 until day 13 was fairly low at around one and a half visits per assay. Since each daily estimate has a fairly large variation, it was possible that the treatment difference could have arisen from just a few individuals. However, only two of eight replicates elicited a consistently low response and the other six contributed about equally to this result.

No detectable response was ever recorded for signallers kept without food and all replicates of this treatment were dead by day three (this data is not shown in fig. 3.4.4).

No detectable response was recorded for signallers placed on food with one live female, for 13 days. These signallers were shown to be capable of signalling since a response was recorded on day 21, eight days after the females were removed and the signallers were placed on fresh grain (see fig. 3.4.4). This response was comparable to that recorded for the males on food only treatment.

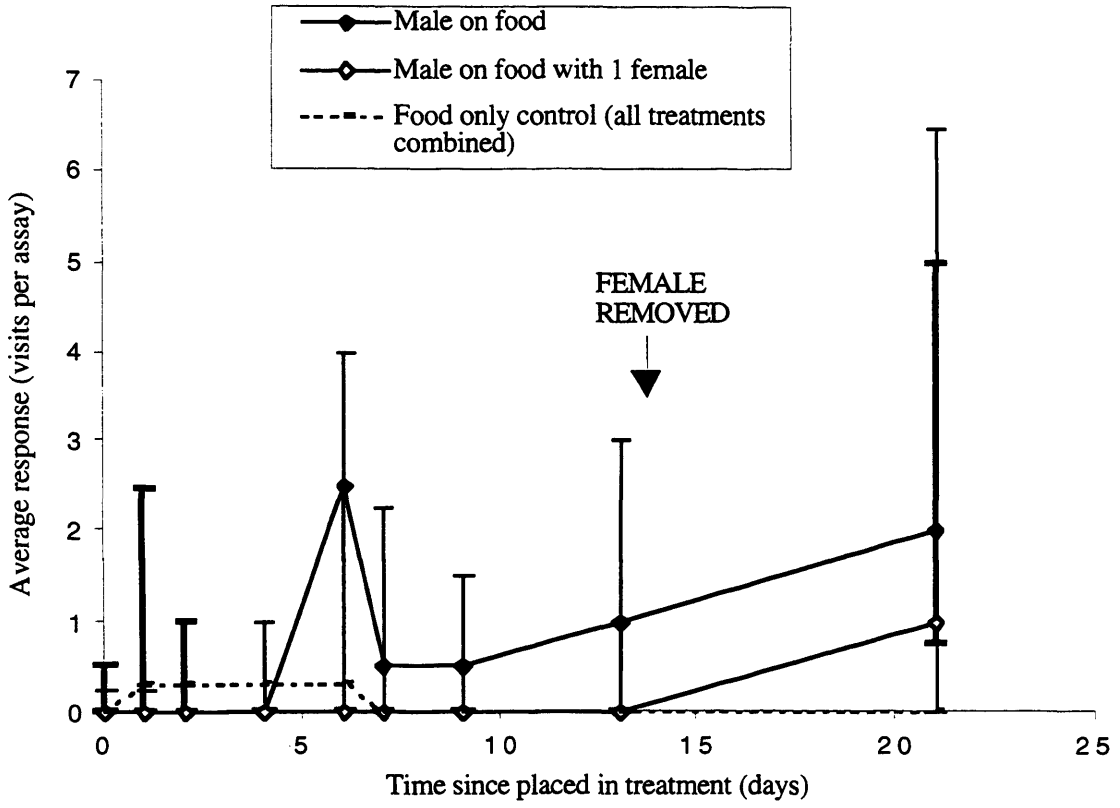


Fig 3.4.4: Median response against time to single-male pheromone sources previously starved and then placed in one of two treatments: placed on food; placed on food with one live female. The arrow indicates when all females were removed and all sources were placed on fresh grain. Error bars span the 25% and 75% quartile values. N=8 for each point.

3.4.5 Influence of plant host on male-aggregation-pheromone signal

Males assayed singly:

The median response to male sources was 5 visits per 15 minute assay (25% quartile=3.75, 75% quartile=8). As in other bioassay trials, response to the control pots was consistently low (median = 0). The median responses recorded for males feeding on maize or cassava were identical, although the mean response to cassava was marginally smaller (see fig. 3.4.5a). Cassava and maize only controls elicited a comparably low response compared to pots containing beetles (see fig. 3.4.5a).

Males assayed in pairs:

The median response given by the female responder to either of the two male sources was 6.5 visits per 15 minute assay (25% quartile=4, 75% quartile=9). Again response to the controls was consistently low (median = 0). In these trials males feeding on maize were found to be significantly more attractive to those feeding on cassava: Wilcoxon

signed-ranks test, z=-2.144, N=24, p=0.032. The median response given to males feeding on cassava was just 50% that given to males on maize (see fig. 3.4.5b).

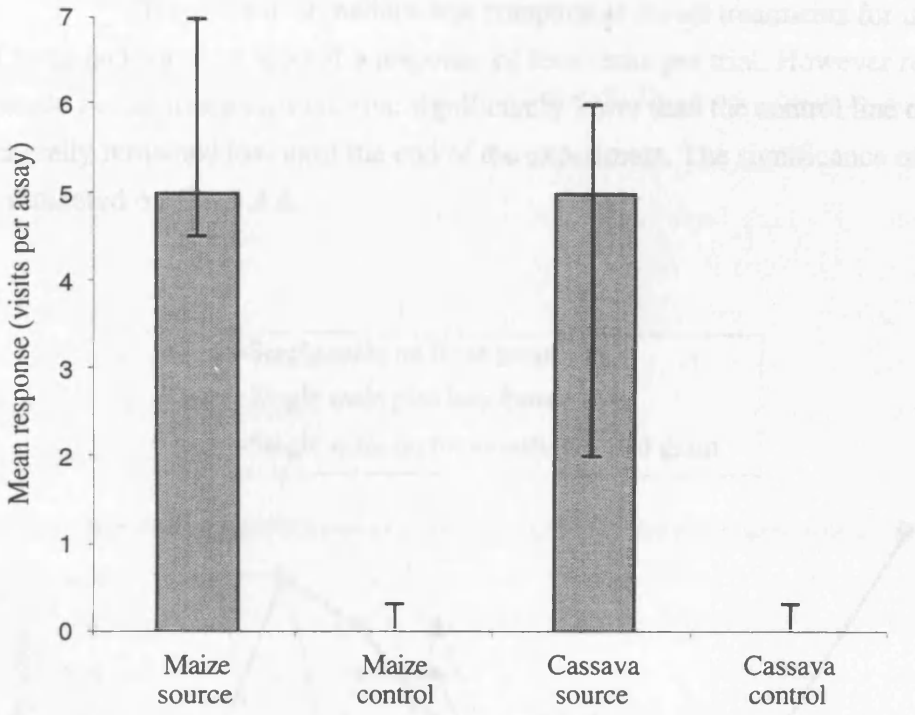


Fig. 3.4.5a: The median response to single males signalling on either maize or cassava compared to food only control pots. Males were assayed **SINGLY** i.e. one male pheromone source per assay. Error bars span the 25% and 75% quartile values. N=24 for each column. The males feeding on cassava elicited a numerically lower, but not significantly different response from males feeding on maize.

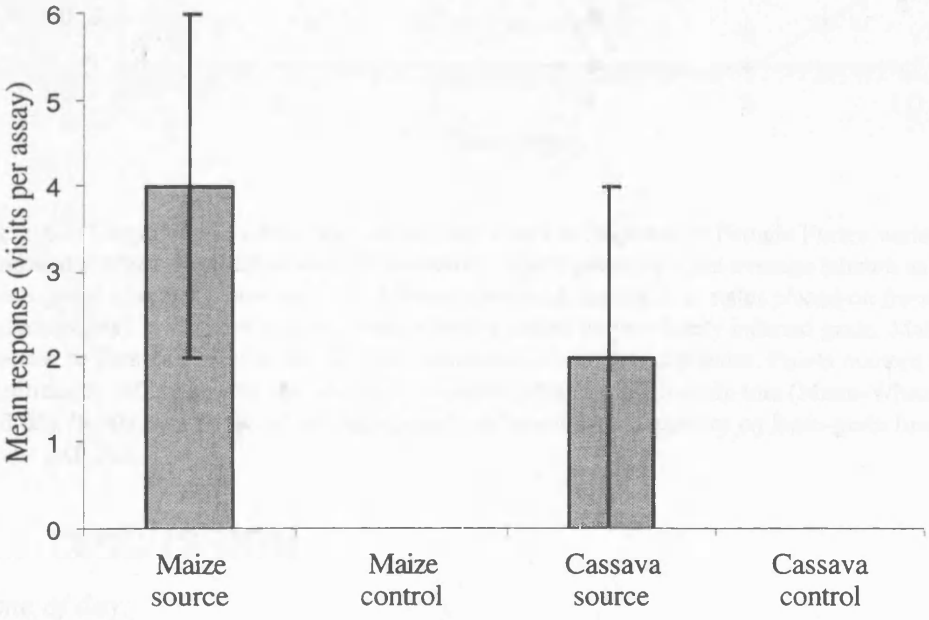


Fig. 3.4.5b: The mean response to single males signalling on either maize or cassava compared to food only control pots. Males were assayed **IN PAIRS** i.e. one male signalling on maize was placed opposite

one male signalling on cassava in each assay. Error bars span the 25% and 75% quartile values. N=24 for each column. Males feeding on maize were found to be significantly more attractive to those feeding on cassava: Wilcoxon signed-ranks test, z=-2.144, N=24, p=0.032.

3.4.6 Timing of shut down of pheromone signal induced by Female Factor.

Response to signallers was comparable for all treatments for the first four days of trials and wavered around a response of four visits per trial. However response to both Female Factor treatments became significantly lower than the control line on day six and generally remained low until the end of the experiment. The significance of this difference is indicated on fig. 3.4.6.

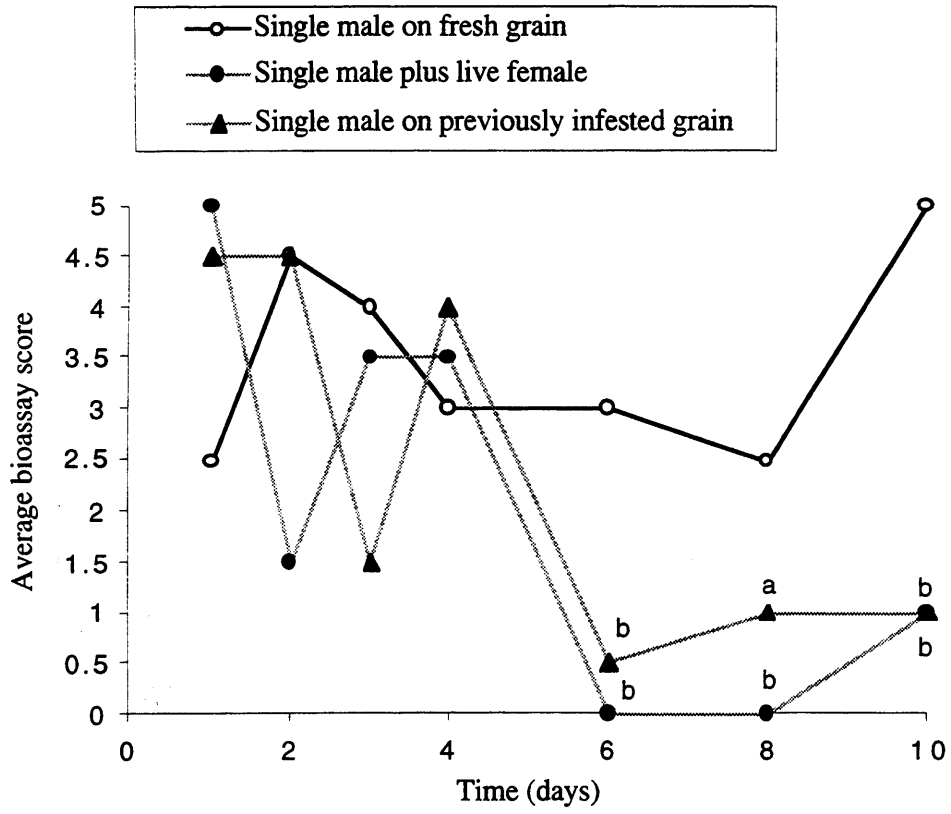


Fig. 3.4.6: Graph to show how pheromone shut down in response to Female Factor varies with time since treatment started. Bioassay score is the number of visits per assay. The average (shown as the median) scores given to males placed in three different treatments are shown: males placed on fresh grain; males placed on fresh grain with one live female; males placed on previously infested grain. Males are potentially exposed to Female Factor in the last two treatments. N=10 for each point. Points marked as 'a' are not significantly different from the points on the male placed on fresh-grain line (Mann-Whitney U test $p>0.05$). Points marked as 'b' are significantly different from the males on fresh-grain line (Mann-Whitney U test $p<0.05$).

3.5 DISCUSSION

Time of day:

Since a response to male signallers has been found throughout the light phase of the CTH room, all future experiments can appropriately be performed during this time.

No significant influence of time of day was found on the response level in the bioassay, but this potential influence will continue to be controlled for when considering treatment effects. The grain in pheromone-source pots may act as a sink for pheromone and thus buffer any daily fluctuation in production such that responders are unlikely to show a response that is linearly proportional to the size of the signal.

Effect of male starvation:

Detectable changes in pheromone signal on removal of food occur on a similar time scale to starvation, i.e. of the order of a few days as opposed to a few hours. This shows that it is possible for males to emit some signal when there is no food available. Pheromone emitted during the first two days after food was removed may have been synthesised before this time. Also, whether signals produced without food are strong enough to be attractive in a natural situation cannot be determined using this apparatus. As already noted this apparatus may be relatively insensitive to changes in signal size down to a threshold level.

The resumption of detectable signalling after a period of starvation was recorded six days after the reintroduction of food. This long time lag implies that the signal may be energetically costly and/or slow to synthesise. Males kept in the presence of females did not resume signalling for the total of 13 days, which agrees with the findings of Smith *et al.,* (1996) that females can suppress male signalling for at least 15 days. It would be interesting to see if males whose signal is repressed by the presence of a female resume signalling faster than those repressed by starvation.

Effect of different plant hosts:

The fact that males signalling on a less nutritious host plant were less attractive supports the hypothesis that the aggregation pheromone signal is costly to produce, either energetically or nutritionally. If the signal were cheap to produce then we would predict that males would either give a maximum signal or no signal at all. There is no reason to suppose that males would willingly inform potential responders of the lower quality of the host. Thus, males may only be able to produce the most effective signals when on the most nutritious plant hosts. If this is indeed the case then aggregation-pheromone signals may play a key role in host-plant selection, particularly when many potential hosts of different quality are available and responders are able to compare signals.

This experiment demonstrates the increased sensitivity of the bioassay to differences in two signals that can be gained by presenting two signals simultaneously to a responder. This increased sensitivity has two sources: first, the behaviour of the responder and second, the creation of paired data that can eliminate the effect of differences in overall response by different responders. Later experiments have used this method to detect differences between pheromone signals.

Effect of Female Factor:

The time-scale of detectable pheromone shut down recorded in response to the presence of females is approximately twice as long as that found in response to starvation. The start of detectable shut down was also at least six times slower than that recorded by direct chemical analysis in Smith *et al.*, (1996). A more sensitive method of recording shut down would be to feed the signals from treated and untreated males into the same arena as described in experiment 3.3.5. In later experiments where Female Factor was used to manipulate the pheromone signal, males were exposed for a period of at least seven days to ensure significant pheromone shut down.

CHAPTER 4: INTER-MALE VARIATION IN PHEROMONE SIGNAL (I BIOASSAY and II CHEMICAL ANALYSIS)

4.1 INTRODUCTION

The main aim of the work presented in this chapter was to test if variation in male-pheromone signals translates into a perceptible difference in the response elicited in conspecifics. As Andersson says, "For demonstration of sexual selection of a trait, it is important to show that variation in the trait leads to variation in a possible mechanism of sexual selection". It is already known that aggregation-pheromone characteristics vary with age, level of starvation, and in response to other pheromone signals in *Prostephanus truncatus* (Smith *et al.*, 1996 and chapter 3 this study). Direct collection of pheromone from groups of 10 males showed that pheromone production increased rapidly with adult age to a peak of production at 10-15 days. After this peak, production declined steadily until death (Smith *et al.*, 1996).

If the pheromone signal is a sexually selected trait then experience of other such systems leads us to predict that it will vary between individuals and such variation is often heritable (see Cade, 1984, and Andersson, 1994). Linkage disequilibrium, shifting selection pressures, the existence of many sexually selected traits and the possibility of alternative strategies may also tend to increase heritable variation (Cade, 1984). In *Prostephanus truncatus* signalling can lead to increased predation of larvae by *Teretriosoma nigrescens,* which uses the signal as a kairomone to locate its prey. Increased risk of predation is one possible cost of signalling that may influence the evolution of the signal. The large potential for changing population density arising from the formation of aggregations may represent an influential shifting selection pressure on the relative benefits of signalling.

The question of whether a suite of characters is sexually selected in LGB will be investigated in later chapters. Since males can be signallers or responders there is the possibility that two or more strategies can be favoured for different males, or the same male under different conditions or of different ages. One particularly interesting idea was suggested by Moore (1995), that, "signalling by a male may be beneficial only if he is the most attractive male present. Furthermore, signalling by a subordinate male may indicate his presence to the dominant male. Therefore the tactic of less attractive males should be to remain silent". This mirrors the situation found in Cowbirds (*Monothrus species*) (West, 1981 quoted in Moore, 1995), where only dominant Cowbirds sing. These processes would tend to increase the observed phenotypic variation in signalling.

The temporal stability of any hierarchy of attractiveness between males will indicate the relative importance of the identity of the signaller in determining pheromone characteristics. Natural variation in pheromone characteristics, be it a stable characteristic of the individual or more facultative in nature, presents an opportunity to evaluate the consequences of signalling. This opportunity is exploited in chapters 5 and 6.

The main challenge of this work was the technical difficulty of measuring olfactory signals. In this work we have not measured chemical output directly, instead it has been implied from behavioural responses. Measuring the pheromone signal indirectly in this fashion increases the likelihood of error of measuring. Many variables, such as the activity level of the responder, have been controlled for by using a contest-type method where two male signallers are simultaneously presented to a responder. Still, any preferences shown only gain credence if they are shown to be repeatable. If the variation fluctuates greatly in time then it is difficult to distinguish real fluctuating pheromone signals with large random components in the method of pheromone measurement.

Three main sets of trials designed to assess the predictability of response to individual variation in pheromone signal are presented. First, likelihood of inter-male variation was maximised by testing males originally taken from different geographic locations. Second, trials designed to compare the response of male and female responders were conducted. Fadamiro found that there was no difference in the response of males and females to an artificial pheromone source in a flight bioassay (Fadamiro, 1995), or to an artificial pheromone source in a walking bioassay (Boughton and Fadamiro, 1996). Hodges and Dobson (in press) and Scholz (1997), however, found female response to be higher than male response in walking bioassays. The discrepancy may be because insects in the trials conducted by Boughton and Fadamiro were not isolated from culture prior to testing, whereas those in the other studies were isolated prior to testing. If the sexes are isolated prior to testing then there could be differences in their culture conditions. More specifically, males may be exposed to their own aggregation pheromone signal, whereas females do not produce such a signal. If beetles can become habituated to the signal, then males may exhibit a lower response in the bioassay. Finally, we tested for the existence of detectable variation between male signals when age, strain, ambient conditions and culturing regime of the signallers were all kept constant. The temporal stability of this variation was also investigated.

Age of test insects was deliberately not always constrained to be of uniform age to get an idea of the range and pattern of variation in signal and response across real populations. Very young insects (less than 5 days old as adults) were not used since both males and females are particularly inactive and males do not produce a detectable aggregation pheromone signal at this age (Smith *et al.*, 1996). Previous work has indicated that there is no clear switch in male strategies (to be a signaller OR responder) with age

(Smith *et al.*, 1996), however, non-continuous variation in signalling could be detected by using a range of ages. No such dichotomy was found. This study focuses on the consequences of the variation in male signals on reproductive success. Future work could particularly address changes in behaviour with age of insects. Obtaining known age insects of LGB is hampered to a certain extent by the difficulty in removing parent generations from their tunnels within grains. Scholz (1997) has developed a method of using compacted flour (which gives better insect survivorship than loose flour, yet is easy to sieve) as a culture media which would make such a study more practical.

An attempt was made to correlate characteristics of the pheromone signal (absolute amounts of T1 and T2 and their ratio T1/T2) with signal success in the bioassay. This was made possible by collaborating with an experiment performed at NRI Chatham where pheromone signals from single males were collected, and whose chemical components were subsequently quantified. Bioassay-choice trials were conducted on these males immediately after the last chemical sample was taken. It was hoped that this would show which features of the pheromone signal are important for short range attraction such as that measured in the bioassay. Also, the sensitivity of the bioassay could be chemically quantified.

4.2 AIMS

- Find out if variation between signalling males was detectable using the bioassay.
- Find out if responders 'agree' as to which males are most attractive.
- Find out if response to male signallers differs between the sexes in overall magnitude and in the pattern of preference between signallers.
- Find out how stable any hierarchy between male signallers is over time.
- Correlate chemical characteristics of pheromone signals to their performance in the bioassay.

4.3 MATERIALS AND METHODS

4.3.1 Experiment to determine if variation in male signal was detectable using the bioassay.

This experiment uses signallers of different strains to maximise the likelihood that there will variation in the aggregation pheromone signal. Only females were used as responders in this early experiment since they gave consistently higher responses than male responders in preliminary trials.

Source of insects:

Six male signallers were tested. Two each from a Mexican, Togo and Tanzanian source were used. All males were removed from cultures as enclosed pupae. Hatching date of each insect was noted and fresh adults were placed individually on ground grain. These insects were cultured for a week before sexing to minimise damage. After sexing they were all placed singly on fresh grain and left for five days before the first trial (all males approximately aged 12 days as adults at first trial).

30 females taken from a Tanzanian source were used as responders. These responders were adults randomly sieved from a culture, sexed and then placed individually in glass pots with some ground grain. Responders were isolated from conspecifics in the CTH room for five days before the first trial.

Apparatus:

Bioassay apparatus was used as described in section 3.2.

Experimental design:

All six males were screened by assaying singly at age 12 days and age 18 days. This was done to check that males were signalling enough to elicit a response in the bioassay. Males (aged 19-20 days) were then placed two at a time into the assay apparatus such that they occupied positions opposite each other. Every possible pairing of the six males was assayed each day for two days. Each day the assays were performed in a random order (picked out of a hat) within the constraint of having two sets of apparatus running at once. Each assay was continued for 40 minutes and two sets of apparatus were run simultaneously. All source pots were aerated for approximately 4 minutes before each assay to reduce any influence of the timing of previous tests on pheromone levels.

Analysis:

We wanted to know if the outcome of a contest between two males is predictable from how those two males have performed in other trials. To achieve this the **outcome** of any given contest (score given to male 1- score given to male 2) was plotted against the **difference in overall scores** obtained by the two males excluding data from the contest under consideration as recorded on that day's trials:

Difference in overall scores = ((total score given to male 1- total score obtained by his opponents) - (total score given to male 2 - total score obtained by his opponents)).

Males are referred to as male 1 or male 2 in the results section. The rule for calling a male as 1 or 2 is as follows. The males were placed in an arbitrary order of: Tanzania 1, Tanzania 2, Togo 1, Togo 2, Mexican 1 and Mexican 2. In any contest

considered, the male highest up this rank was called male 1 and the lower ranking male, male 2.

Lillifors analysis (in SYSTAT) (Wilkinson, 1990) showed the data sets in this chapter to be approximately normally distributed. All correlation coefficients presented are Pearson correlation coefficients calculated using Excel 5.

4.3.2 Experiment to determine if males and females 'agree' which of a pair of males is more attractive.

In this experiment, both males **and** females were used as responders to allow a direct comparison to be made between the overall level of response of both sexes and any difference in preferences shown for particular signals. This experiment focused on one geographical source of insects (Tanzanian source). Choice of strain was arbitrary.

Source of insects:

Nine males were tested. They were sieved from culture as adults of unknown age and placed on fresh ground maize for nine days before the first trial. All males used were from a Tanzanian source. Thirty females were isolated per day for three days from a Tanzanian source. All were sexed and placed directly on fresh ground grain and were isolated from culture two days before being used in the bioassay.

Apparatus:

Bioassay apparatus was used as described in section 3.2.

Experimental design:

All combinations of male pairings were tested twice, once with a male responder and once with a female responder (total number of assays = $((9^2-9)/2)x2=72$). Assays were run on two sets of apparatus simultaneously with one set using a female as a responder and the other a male. Within this constraint assays were performed in a randomised order (picked out of a hat) over three days. Each assay was continued for 25 minutes. All source pots were aerated for approximately four minutes before each assay to reduce any influence of the timing of previous tests on pheromone levels.

Analysis:

Results using male responders were processed separately from those results using female responders. Data were processed as described in experiment 4.3.1. The preferences of the sexes were compared by correlating the outcome of each signal combination as given by a female responder *vs.* the equivalent outcome given by a male responder. Finally the average magnitude of response irrespective of preference, of each of the sexes was compared using a Wilcoxon signed-ranks test.

4.3.3 Experiment to determine if the response of females to aggregation pheromone can be depressed by increasing their exposure to the signal prior to testing.

Source of insects:

All insects were sieved from the same 10-week old culture and sexed. 25 males were placed in separate glass pots with a single pre-tunnelled whole grain and a small amount of maize meal. 50 females were placed in similar pots with approximately 3mm depth of maize meal. All pots were covered with a small piece of nylon mesh (100 holes per cm^2) held in place with tape. The shallow depth of food ensured that females were continually exposed to the air circulating around the pot.

Apparatus:

The bioassay apparatus as described in section 3.2 was used.

Experimental design:

Pots containing females were randomly allocated to one of two treatments: exposure to high or low concentrations of aggregation pheromone. Females allocated to the low-pheromone-concentration treatment were placed five at a time in a 550ml jar with five pots containing a small amount of maize only. Females allocated to the high-pheromone-concentration treatment were placed five at a time in a 550ml jar with five-male-pheromone-source pots. All jars were sealed with a filter-paper top as described in section 1.5.3. It was hoped that this method would allow air to diffuse between pots within each jar easily and thus expose the females in the high-pheromone treatment with air from the signalling males within their jar.

Insects were left in their treatments for eight days before bioassay trials were conducted. All trials were conducted during one day. Replicates were assayed three at a time, one from one treatment and two of the other. Male sources were taken straight from the high-pheromone-treatment jars. This was done such that females were always taken out of jars before males so the treatment was maintained right up until each bioassay trial. Male sources were all assayed twice, once by females from each of the two treatments. Male sources may have given off slightly less pheromone the second time they were used as any reservoir of pheromone trapped in the pot was evacuated. Therefore, the order in which males were presented to each of the two treatments was controlled so each treatment was exposed to the same number of first and second time used males. All trials were continued for 20 minutes.

Analysis:

Male identity accounted for a portion of the variation and each male had been tested with one female from each treatment, therefore the data were analysed using a Wilcoxon signed-ranks test between females exposed to the same male.

4.3.4 Experiment to determine if variation exists between males of the same source and age, and if so, whether the pattern of variation is stable over time.

Source of insects:

Thirty six males were used as signallers and 36 females were used as responders in this experiment. All were sieved from a Tanzanian culture as pupae and separated into individual glass pots of coarse ground maize flour. Date of emergence was noted to obtain adults of known age. Males were weighed and placed into pairs of similar emergence date, (kept to within at least two days). Weight was noted to allow a preliminary assessment of the correlation of weight with pheromone attractivity. All beetles were cultured singly for 10-14 days before the first bioassay trials.

Apparatus:

Bioassay was used as described in section 3.2.

Experimental design:

Bioassays were of contest type. Two males were connected up to the arena simultaneously as described in experiment 4.3.1. Eighteen pairs of males were tested. Each pair was assayed using one female responder on one day and then a different female responder on the next day (day 2). All males were then placed on fresh grain and this procedure was then repeated after a day break (on days 4 and 5) using the same females with the same male pairing. Males were placed on fresh grain to eliminate the possible influence of a slowly released pheromone reservoir coming from the grain around the signaller. Two comparisons were therefore made possible: first, the response of one female to a pair of males compared to the response of another female to that pair of males the next day; second, between the response of a female to a pair of males and her response to that pair after they have been placed in a fresh food tube and after 3 days have elapsed. Trials were staggered such that the entire experiment was conducted over 7 days. Each female responder was never used more than once in a day and each pair of males was tested a maximum of once per day. Replicates were assayed three at a time and each assay was continued for 25 minutes.

Analysis:

The outcome of all trials was calculated as in experiment 4.3.1. All associations were tested using a Pearson correlation coefficient (Excel 5).

4.3.5 Experiment to correlate bioassay response to chemical assay, for pheromone plumes from single males.

Source of insects:

The beetles used in this experiment were a Ghanaian strain collected from the field in 1996. They had been maintained at NRI Chatham in a CTH room at 27°C and 70% r.h. Males were removed from their pupal cases and placed on wheat flour for two days

(sexed according to Shires and McCarthy, 1976). Males were then placed in a pre-drilled tunnel in a maize grain.

Forty females were isolated as adults (sexed as described in section 1.5.6). These insects were used as responders. They were sexed then placed on ground maize grain in separate glass pots in a CTH room set at 27°C and 70% r.h. and left for 16 days before the trials.

Bioassay apparatus:

Bioassay apparatus as described in section 3.2 was used. Three replicates were assayed at once. A pump was used instead of the vacuum line, this was maintained at about 4.5 litres per minute ensuring that each set of apparatus received an air flow of approximately 1.5 litres per minute.

Pheromone collection apparatus:

Collection filters were made using Pasteur pipettes, packed with 200mg of porapak-Q (Phase Separations, UK), and plugged at both ends with silanised glass wool. The filters were prepared for use by flushing with 'distol' grade dichloromethane (Fisher Scientific, UK). Beetles in maize grains and control grains without beetles were placed singly in 30 cm^3 glass vessels (Fisher Scientific, UK) through which air was drawn by small electrical pumps at a rate of 1 litre per minute. A large glass round bottomed flask (1litre capacity)was also connected to the system to act as a buffer against pressure variations induced by the pump. The intake air was passed through filters containing 200mg of 'Porapak Q' (Phase Separations, UK) to collect T1 and T2 (all designed and performed by staff at NRI).

Chemical analysis:

Volatiles were desorbed from the filters by washing with 750µl of 'distol' grade dichloromethane (in three aliquots). 5µg of octylacetate was added to each sample as an internal standard. Samples were analysed by capillary gas chromatography using a CP-Wax52-CB 25m x 0.32mm column (Chrompack, The Netherlands) with helium carrier gas, at an inlet pressure of 5psi. The temperature was held at 50°C for 2 minutes, then programmed to 220°C at 6°C/min. Results were calibrated against known amounts of pure synthetic pheromone, and peak identities were confirmed using an ion-trap detector.

Experimental design:

Initially, volatiles were collected at intervals of 1,2 or 3 days for a total of 24 days. After this an additional collection was then made over 60 hours ending the on the morning of the bioassay.

Males were bioassayed in pairs. Males were initially divided into two groups: replicates 1-5; and replicates 6-10. All possible combinations of pairs within each group

were assayed. In addition to these tests, 10 other pairings were tested such that a male was taken from each group and all males were tested twice. All of these combinations were assayed in random order within the constraint that any male could only be chosen once for each set of three trials to be performed simultaneously. Only one day was available for trials.

Assays were continued for 20 minutes each. Female responders were only used once.

4.4 RESULTS

4.4.1 Experiment to determine if variation in male signal was detectable using the bioassay.

Trials where males were assayed singly:

In the first trial (males aged 12 days) no response was observed for any of the sources with the exception of one male of Mexican origin who obtained 6 visits per assay. In the second trial (males aged 18 days) a response was observed for 5 out of 6 of the sources. The average score obtained was 8.5 visits per assay. Therefore, response to the signallers was high enough to begin the choice trials.

Trials where two male sources were assayed together:

Mexican 2 escaped on the second day of the trials. The **overall score** of the remaining males predicted the winner of any one single trial in 21 of 22 cases where the responder showed a preference. In other words females 'agree' which males are more attractive and this ranking is stable over the period of each set of trials (8 hours). In addition to this, the magnitude of the choice (size of the **outcome**) was correlated to the magnitude of the **overall score difference** between the pair of males, (r^2=0.66, N=25, $p<0.001$) (see fig. 4.4.1).

When males were ranked in terms of their ability to attract females the two males taken from a Tanzanian source occupied the top two ranks. (NB The responders were also of Tanzanian origin).

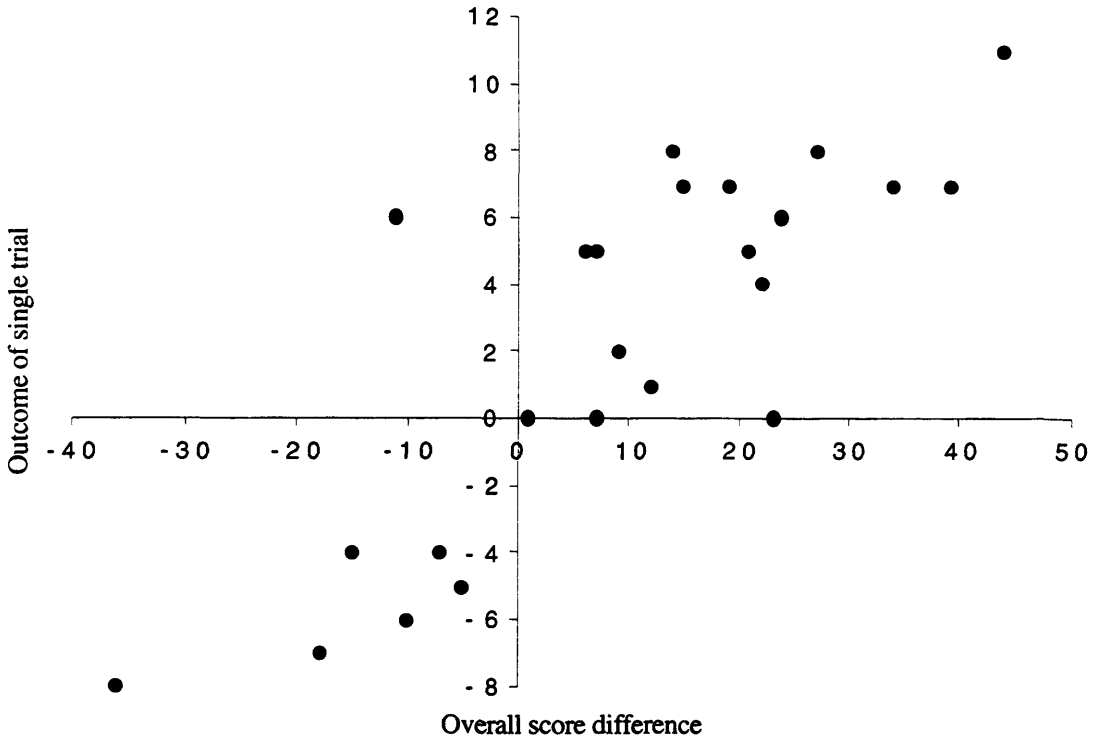


Fig. 4.4.1: Scatter plot to show the correlation between the outcome of a single trial between two males and their overall score difference as determined by all other trials they have taken part in (r^2=0.66, N=25, p<0.001). NB. Females used as responders.

4.4.2 Experiment to determine if males and females 'agree' which of a pair of males is more attractive.

The overall performance of each signaller predicted the winner of any one single contest in 48 of 62 cases where the responder showed a preference. This shows that within each sex there is some agreement as to which males are more attractive. This level of predictability was exactly the same whether male or female responders were used. As in results section 4.4.1, the magnitude of the preference was correlated with the magnitude of the overall score difference between the pair of males (for male responders r^2=0.37, N=36, p<0.001, for female responders r^2=0.26, N=36, p<0.001) (see figs. 4.4.2a and 4.4.2b). However, it can be seen that the association between these variables is not as strong as that found in fig. 4.4.1.

The association between the outcome of a trial with a female responder *vs.* the outcome of an equivalent trial using a male responder was investigated. These two variables were found to be significantly, yet loosely correlated (r^2=0.15, N=36, p<0.025) (see fig. 4.4.2c). Therefore males and females 'agree' which of a pair of signalling males is more attractive. Females showed a higher level of total response than males with an average of 8.44 visits per trial compared to 4.76 visits per trial when male responders were used. The total response (male 1 + male 2) given by a female was compared to the equivalent response

given by a male using a Wilcoxon signed-ranks test. Females consistently gave a higher total response than males: z=-3.437, N=36, p<0.001.

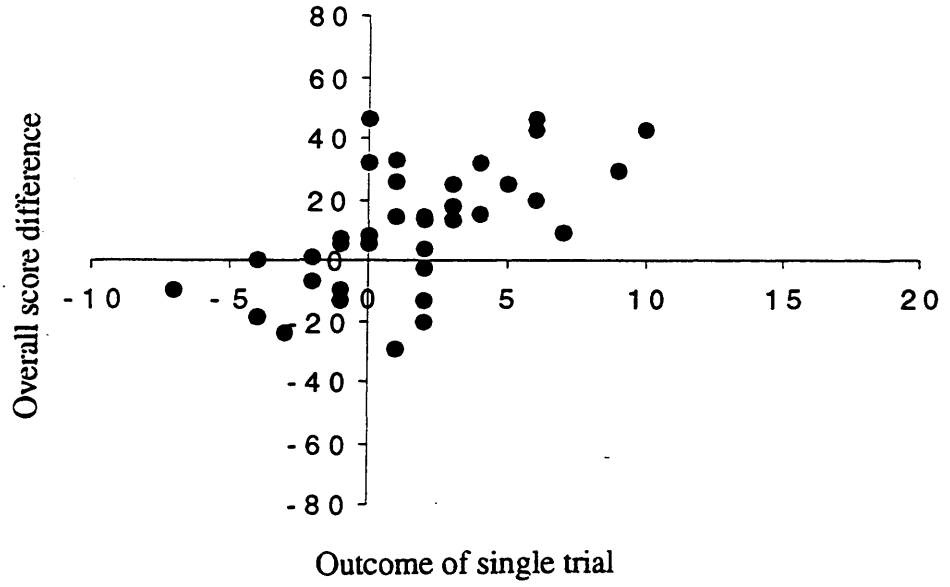


Fig. 4.4.2a: Scatter plot to show the correlation between the outcome of a single bioassay between two males and their overall score difference as determined by all other bioassays they have taken part in as determined by **male responders** (r^2=0.37, N=36, p<0.001).

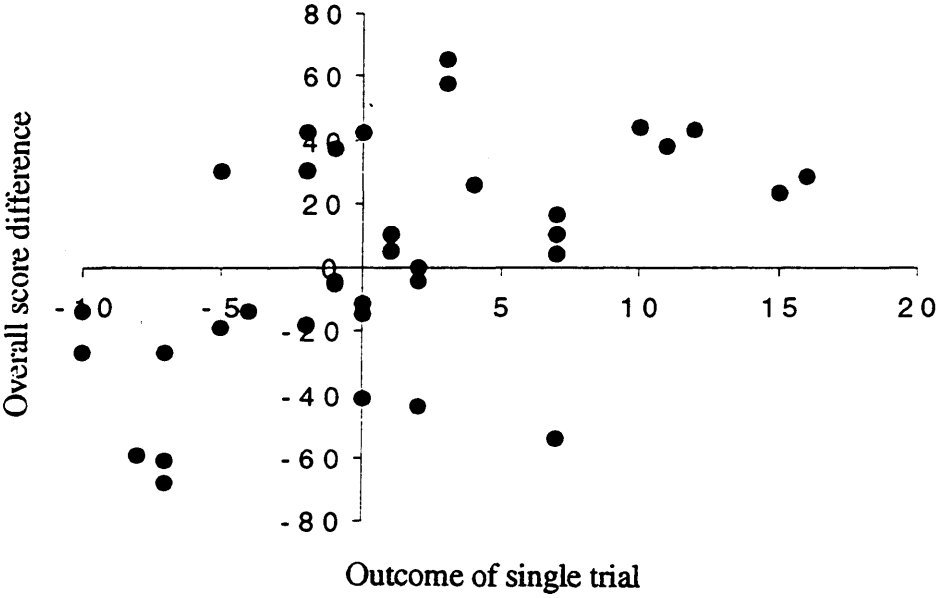


Fig. 4.4.2b: Scatter plot to show the correlation between the outcome of a single bioassay between two males and their overall score difference as determined by all other bioassays they have taken part in as determined by **female responders** (r^2=0.26, N=36, p<0.001).

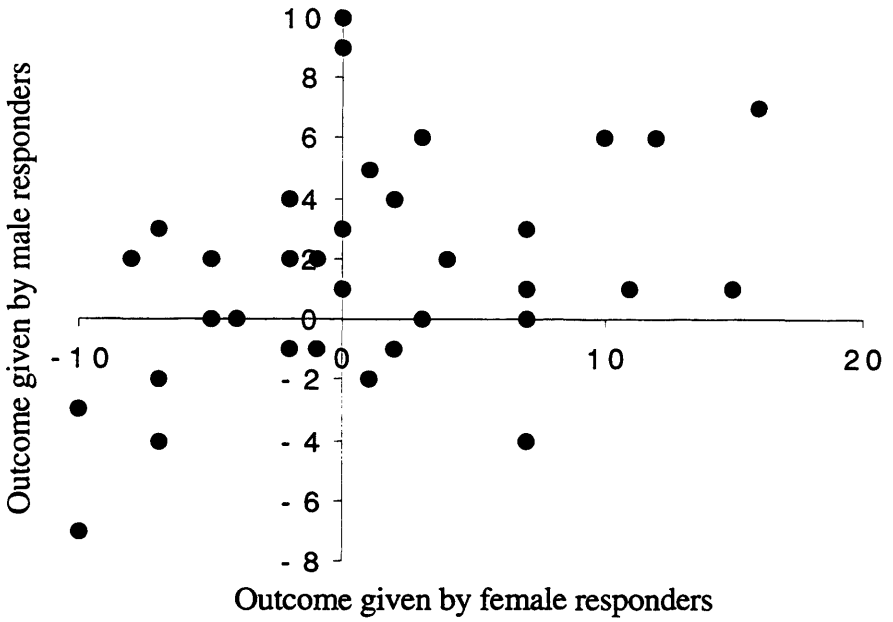


Fig. 4.4.2c: Scatter graph to show the correlation between the outcome of a single bioassay as determined by a **male** responder and the outcome of the equivalent trial using a **female** responder (r^2=0.15, N=36, p<0.025).

4.4.3 Experiment to determine if the response of females to aggregation pheromone can be depressed by increasing their exposure to the signal prior to testing.

Females previously exposed to higher levels of aggregation pheromone were found to show approximately half the level of response to an aggregation-pheromone signal in a walking bioassay compared to females kept in low-aggregation-pheromone concentration conditions (average response for high pheromone treatment = 1.71 ± SE 0.46 visits per trial; average response for low pheromone treatment = 3.52±SE 0.74 visits per trial). This treatment difference was significant, Wilcoxon signed-ranks test: z=2.523, N=21, p=0.012.

4.4.4 Experiment to determine if variation exists between males of the same source and age, and if so, whether the pattern of variation is stable over time.

For any one pair of males, the outcome given by one female on one day (response to male 1 minus response to male 2) was correlated to the outcome given by another female on the next day, (r^2=0.47, N=36, p<0.001) (see fig. 4.4.4a). Therefore variation exists between males of the same age and strain that is perceived by female responders. Also these responders agree which males are more attractive and this hierarchy is stable over 24 hours.

The response of one female on one day is not significantly correlated to her response after three days and after the males had been placed on fresh grain, (r^2=0.034, N=36, p>0.05) (see fig. 4.4.4b). The ranking of male attractivity is not therefore,

completely stable and can be disrupted either by time, disturbance, or a combination of the two.

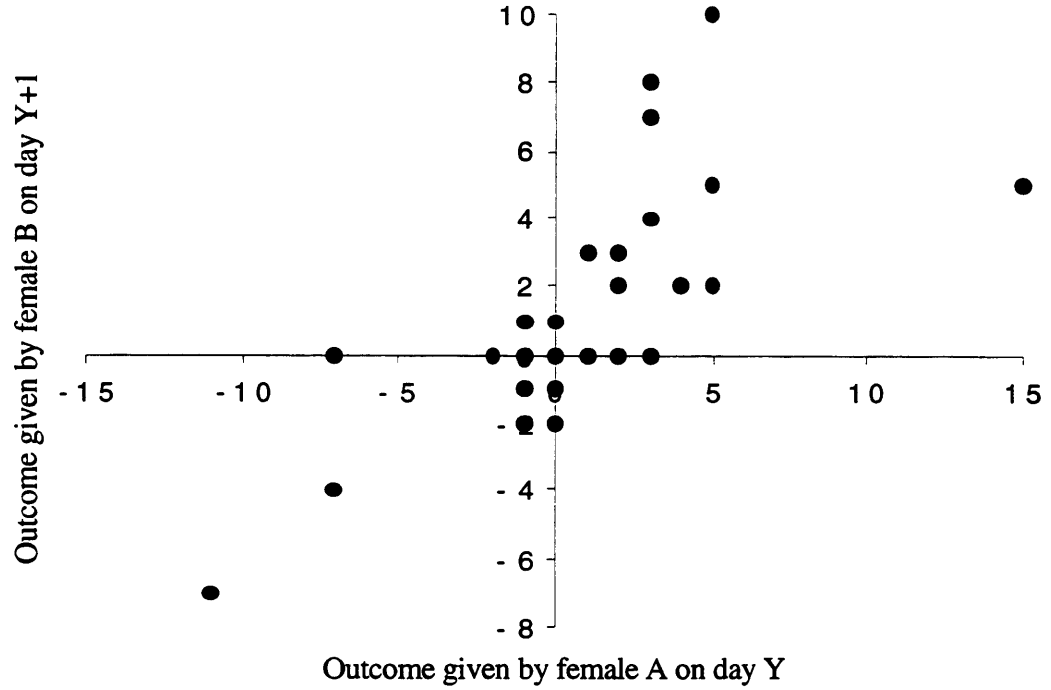


Fig. 4.4.4a: The correlation between the outcome (response given to male 1 minus response given to male 2) of a trial as determined by one female on one day *vs.* the outcome of the equivalent trial as determined by a second female on the next day (r^2=0.47, N=36, p<0.001).

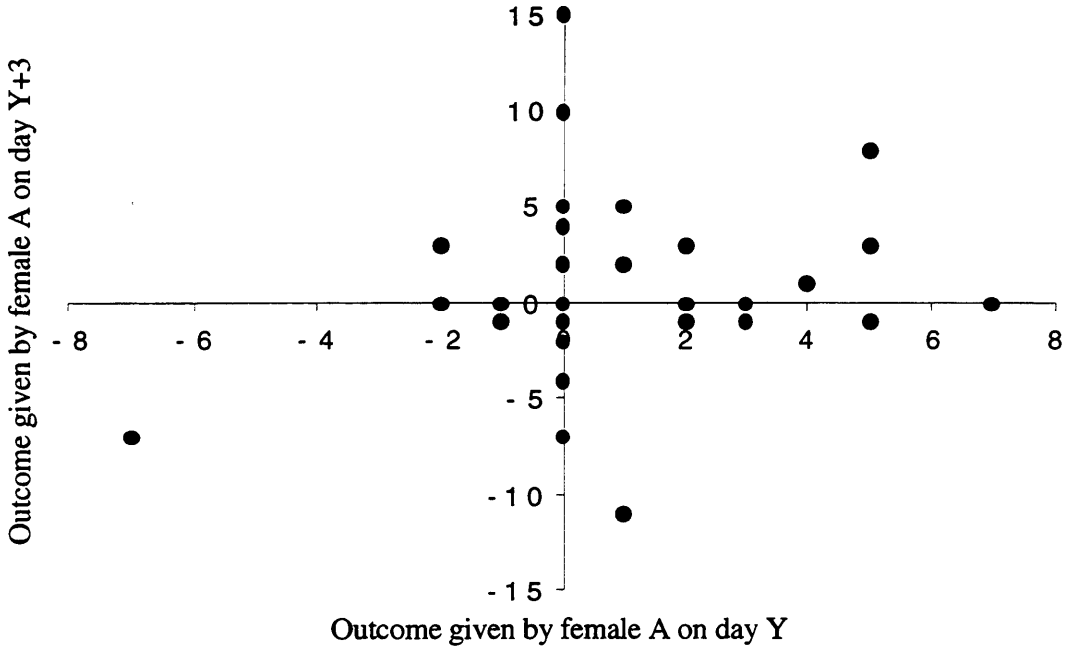


Fig. 4.4.4b: The correlation between the outcome of a trial as determined by one female on one day *vs.* the outcome of the equivalent trial as determined by the same female three days later after the male signallers had been placed on fresh grain (r^2=0.034, N=36, p>0.05).

Larger males did perform better in bioassay trials, though this difference was not statistically significant in these trials. Each pair of males was tested four times in these trials. Which of a pair of males won the most of these four contests was noted (draws were also recorded). These frequencies are shown below in table 4.4.4c.

Table. 4.4.4.c: Table to show which of a pair of males won most contests (out of the four performed by each pair), the larger male, the smaller male or an equal number of contests by each male.

Larger male won most contests	Smaller male won most contests	Each male won same number of contests
10	6	2

4.4.5 Experiment to correlate bioassay response to chemical assay of pheromone plumes from single males.

Chemical analysis:

Results from the first 24 days of pheromone collection will be reported by NRI. Here, only the last pheromone collection is reported.

Six of the ten males were found to be emitting detectable amounts of T1 and T2. Of these six males the average amounts of T1 were 6.15µg (0.103µg per hour) and the average amounts of T2 collected were 2.34µg (0.039µg per hour). The rate of T1 emission is plotted against the rate of T2 emission for each signalling beetle in fig. 4.4.5a to demonstrate the variability in the sample used. Both the amount of each component and the ratio between them is variable between individuals.

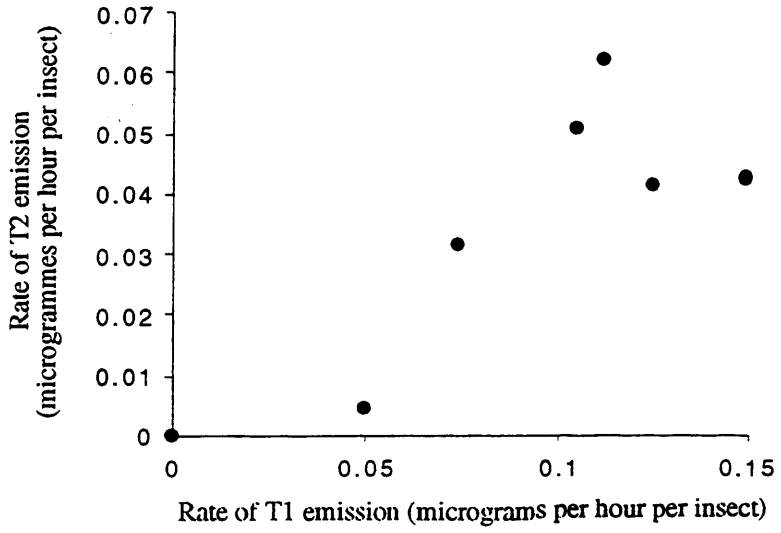


Fig. 4.4.5a: Scatter graph to show the rate of T1 emission against the rate of T2 emission for each signalling beetle.

Bioassay results:

The average response given to males that were subsequently found to have been emitting a detectable amount of either T1 or T2 was 1.64 visits per trial (N=36). The four males with no detectable signal from the chemical analysis obtained very few visits during the bioassay trials (average 0.13 visits per trial (N=24). Therefore, both bioassay and chemical methods have detected the same males as non-signallers. Further interpretation of the data is now limited by the low number of trials featuring two signalling males. Two signals are needed to compare the relative success of different features of the pheromone chemistry. Features of the bioassay-winners' pheromone chemistry are presented below in figs. 4.4.5b-d. Where no preference was shown in the bioassay and/or no difference was found between the pheromone output, the trial is scored as being neutral. The ratio of the two components of aggregation pheromone (mass of T1/mass of T2) can only be calculated for males with a detectable signal.

Table 4.4.5b: Frequencies of winners of bioassay trials in terms of relative amounts of T1 detected in their pheromone signal. Trials where the males drew in the bioassay or had the same amount of T1 are scored as neutral.

	Higher amount of T1	Lower amount of T1	Neutral
Trials with non-signallers	9	0	10
Trials with signallers only	4	3	4
All trials	13	3	14

Table 4.4.5c: Frequencies of winners of bioassay trials in terms of relative amounts of T2 detected in their pheromone signal. Trials where the males drew in the bioassay or had the same amount of T2 are scored as neutral.

	Higher amount of T2	Lower amount of T2	Neutral
Trials with non-signallers	9	0	10
Trials with signallers only	6	1	4
All trials	15	1	14

Table 4.4.5d: Frequencies of winners of bioassay trials in terms of the relative ratio of T1 and T2 detected in their pheromone signal.

	Higher ratio (T1/T2)	Lower ratio (T1/T2)	Neutral
Trials with signallers	3	4	4

To summarise, the amounts of both T1 and T2 were found to be a good predictor of the outcome of a bioassay trial, however, if those males subsequently found not to be signalling are taken out of the analysis, not enough replicates remain to determine which characteristics of pheromone emission are preferred.

4.5 DISCUSSION

Both males and females have been shown to detect fairly subtle differences in an aggregation-pheromone signal and to vary their response accordingly. Neither males or females make an absolute choice between two signals. Instead, responders bias their response away from a random one to a degree that is related to the difference between the signals. All or nothing responses are predicted by Eberhard (1996) for cases where response is governed by natural selection. This hypothesis is proposed in the context of mating cues delivered to the female in the ejaculate, but could, I think, equally be applied to other signals between the sexes. Eberhard proposes that dose-dependent responses are a characteristic of sexually selected cues since they allow females to choose between males either for 'good viability genes' or 'good attractiveness genes'. Males in this system also give a dose-dependent response to other males. Perhaps choosing the same way females do will maximise their female encounter rate.

In this series of experiments we have shown that a predictable outcome of trials can be obtained using this bioassay method (fig. 4.4.1). Therefore, concern regarding sources of error that could obliterate any pattern in the data arising from the method have been allayed. We have also shown that the response of one beetle to two signals can be used to predict the response of other beetles given the same choice. The accuracy of these predictions is highest when the sex of the responders is kept constant and time between trials is kept to a minimum. Observable variation in male signal remains when strain, age, ambient conditions and culturing conditions are kept constant.

In some experiments presented in this chapter all possible combinations of male pairings were trialled and consistency was measured by comparing the outcome of single trials with the overall-score difference of the two males. Although this method does give satisfactory results, perhaps a better experimental design is that employed in experiment 4.3.4. In this design consistency of result was measured by simply repeating trials. The design used in 4.3.4 has two advantages over the earlier design. First, more individuals can be screened for the same number of bioassay trials. Second, should any signaller die/escape during the trials, a smaller portion of the data is lost. The calculation of overall score requires all trials to be completed successfully, the loss of any single insect therefore, automatically eliminates all data derived from all trials involving that insect.

Male response was found to be only 56% that of the average female response in LGB (compare fig. 4.4.2a to fig. 4.4.2b). This would seem to indicate that the opposite sex to that of the signaller is more responsive to the signal, thus supporting the sex-function hypothesis. However, in a similar study of the saw-toothed grain beetle, *Oryzaephilus surinamensis* (L.), White and Chambers (1989) highlighted that, "as males produce the pheromone, females would not have been exposed to pheromone prior to the tests, in contrast with the males, and this could be responsible for any observed differences in their responses". White and Chambers (1989) controlled for this possible habituation of responders by culturing males and females together up until the point of testing. They found that females always showed a higher response to the male-produced pheromone whether cultured individually or in a mixed sex culture. However, changing the culturing environment did have a significant influence on the difference in response of the sexes since males cultured individually gave a lower response than those in a mixed culture with the reverse being true of females. The observation that a male's response is increased by the presence of the opposite sex suggests that perhaps males decrease their signal in response to the presence of females, a possibility not mentioned by White and Chambers (1989).

Unfortunately their experimental design incorporates mating as a confounding variable. Mixed cultures will not only allow males and females to share the same physical space, they also allow for mating to occur prior to testing. In this study this possibility was eliminated by keeping insects separate, yet allowing free exchange of gases through the use of gauze pot lids. Here we have concentrated on the influence of prior exposure of pheromone on females and have not investigated the possibility of increased male response due to removal the signal he is making.

Borden (1967) also investigated the effect of previous exposure to pheromone on subsequent response to pheromone in the bark beetle, *Ips confusus*. It was found that female response to aggregation pheromone was significantly lowered after just 15 minutes exposure to male frass (presumably emitting pheromone signal) compared to a clean air treatment. Although the response of males was also lower after previous exposure, this change was not significant. Perhaps the males used as responders in this experiment were still signalling and therefore the clean air treatment they received became contaminated by their own signal.

In summary, White and Chambers (studying Curculionidae) found female response to be lowered by 9% by rearing females in a mixed as opposed to single sex culture. Borden (studying Scolytidae) found female response to drop by 73% after just 180 minutes exposure to male aggregation pheromone (presented as male frass). LGB females in this study gave a 51% drop in response if exposed to higher rather than lower levels of pheromone for 8 days. This large drop in response is more than enough to explain the earlier observation that males exhibit only 56% of the level of response that females do to

the pheromone signal (44% drop relative to the female response). Therefore, no sex differences in perception or reaction to aggregation pheromone have been demonstrated in this study. Scholz recorded a similar habituation of female LGB exposed to artificial sources of T1 and T2 (Scholz 1997), however in this study, habituation was only recorded when females were removed from maize. Perhaps starved females are even more readily habituated than feeding females.

Bioassay trials linked to chemical collections of pheromone plumes showed that the bioassay reliably picked out which males of the 10 used were not signalling. Unfortunately, not enough replicates remained to give any further information about the chemical characteristics of preferred signals.

To conclude, conspecifics are sensitive to variation in the pheromone signal. Aggregation-pheromone characteristics could therefore easily be selected as a component of mate choice in this species. The degree to which conspecifics actually choose between two competing signals, in the field, is investigated in the next chapter. Male signallers can vary their investment in signalling with time, therefore this character is very flexible. Cade (1984) suggests that, "...facultative alternatives and underlying genetic variation are not mutually exclusive possibilities...individuals may differ in the tendency to change behaviour under different conditions". This means that the total investment in aggregation-pheromone signalling per male could be fairly complicated to calculate. Any attempt in constructing a hierarchy of attractiveness between males is therefore only really practical over a short time scale (in the order of a few days). This knowledge helped to design later tests to determine some of the consequences of signalling during courtship.

CHAPTER 5: INTER-MALE VARIATION IN PHEROMONE SIGNAL (III: FIELD TRIALS)

5.1 INTRODUCTION

Synthetic aggregation pheromone is used to bait flight traps that are widely distributed to monitor the spread of *Prostephanus truncatus* across Africa (see chapter 1). Considerable inter-male variation in naturally produced aggregation pheromone signals has been found both by chemical analysis of signals (Prof. David Hall pers. comms.), and through the use of bioassays in *Prostephanus truncatus* (chapter 4 this study). It was shown in chapter 4 that test females generally agree which males are most attractive and males show the same preferences as females. It was not known how well these patterns of preference found in the bioassay apparatus would translate into patterns of dispersal in a natural situation. Also, there is always the worry that populations maintained for many generations in the laboratory may be inbred, or exhibit characters that do not reflect those found in natural populations. Field trials were conducted in order to address these problems.

Single males were used as lures to bait flight traps in a woodland habitat in Ho, Ghana. A funnel-trap design was used since this design has been shown to be relatively effective at trapping both LGB and *Teretriosoma nigrescens* compared to some other popular designs (Key *et al.*, 1994). Traps were arranged in pairs and as such acted as mini choice-tests. This allowed collection of data on the level of choice conspecifics make between the pheromone signals of two males signalling side by side. Fadamiro noted that *P. truncatus* is often seen to hover over a pheromone source (Fadamiro, 1995). It is possible that this behaviour could allow dispersing beetles to evaluate aggregation pheromone signals in some way. By sexing all beetles caught in traps, sex differences in preferences could also be assessed. Volatile samples were collected from male signallers in the field with a view to correlating pheromone output and component ratios with signal success.

The numbers of the predator, *Teretriosoma nigrescens,* caught in traps were also of interest. The predatory beetle *Teretriosoma nigrescens*, released as a biocontrol agent against LGB, first arrived in Ho around 1996 (R.J. Hodges, pers. comm.). *Teretriosoma nigrescens* follows LGB's aggregation pheromone to locate its prey. The predator had already been found in traps baited with artificial sources of pheromone (Tigar *et al.*, 1993), but no estimates of numbers attracted to natural sources were known. Ghana was chosen as a study site, mainly for logistical reasons. There is already an established programme of research on Larger Grain Borer at the Ministry of Agriculture in Ho, Ghana.

This field work was also timed to coincide with trials planned by Dr. Rick Hodges travelling from the UK.

Two studies already exist where single male *Prostephanus truncatus* have been used as lures in flight traps. Wekesa (1994) placed single-male baited flight traps about 60m away from a source of *P. truncatus* in Kenya. Significant numbers of conspecifics were not attracted, but there were only relatively low numbers of flying insects present. Significant trap catches were, however, obtained using single-male lures in a study performed in 1995 in Togo (Scholz, 1997). In this study single males were allowed to tunnel into maize cobs that were then hung inside delta traps with the glue removed, and left for varying lengths of time from one to four weeks. After the trapping period, numbers and the sex of beetles colonising the cobs were determined. By letting beetles accumulate on cobs, this experiment was able to assess the changes in attractiveness of a growing aggregation of beetles. Traps left for a week attracted a mean of 59 beetles per week (median 39). The sex ratio of beetles attracted was female biased (64% females). The work reported in this chapter allows an estimation of the number of conspecifics a single signalling male can attract each day, and thus gives an idea of the efficiency of signal and response in this species in the field.

5.2 AIMS

- Estimate the number of beetles that can be attracted to the immediate vicinity of a single signalling male per day in a woodland site in Ghana.
- Estimate the sex ratio of beetles attracted by a signalling male.
- Estimate the level of choice being made by responders. Are some signallers more attractive than others if they are presented side by side? If so, what is the magnitude of their advantage in the field?
- Evaluate any sex differences in the level of choice being made by responders between signals.
- Estimate the variation in the quantity and quality of pheromone emitted by males taken from a Ghanaian population.
- Test for any correlation between natural variation in pheromone signal (chemically determined) and natural variation in attractiveness of these emissions (trap data).

5.3 MATERIALS AND METHODS

5.3.1 Trapping trials:

Study site:

An area of teak plantation situated on the side of a south facing ridge on the outskirts of Ho, Ghana, was used as a study site. This study was performed in February 1997. Daytime temperature ranged from approximately 25-35 °C. Humidity was generally low for the area and ranged from approximately 40-70% r.h., peaking at night.

Insects used:

Males used as signallers in these experiments were taken from two sources. Initially all males were trapped directly from the study site using a trap baited with an artificial pheromone lure (polythene vial containing 1mg T1 and 2mg T2). Subsequent waves of signallers used were taken from cultures of locally caught beetles reared on maize at the Ministry of Agriculture in Ho. Beetles were taken from the top of three month old culture jars.

Trapping equipment:

Traps: Flight traps were used to catch beetles in these trials. Initially both a simple funnel trap and a funnel trap with a baffle were tested (see fig. 5.3.1a). Subsequently, only the simple funnel traps were used. Where traps were presented in pairs, they were suspended from a single point and separated using a length of stick (see fig. 5.3.1b and fig. 5.3.1c). The wires used to attach traps to trees were coated in insect glue to prevent walking insects such as ants gaining access to the male-on-food lure.

Lures: Grains containing male signallers were placed individually in open small glass pots, covered in fine nylon mesh (100 holes per cm^2) to avoid insects getting in or out. One extra perforation of approximate diameter 2mm was added to facilitate the emission of any pheromone through the mesh (see fig. 5.3.1d). Lure pots could easily be swapped between traps since they were attached using Velcro tape.

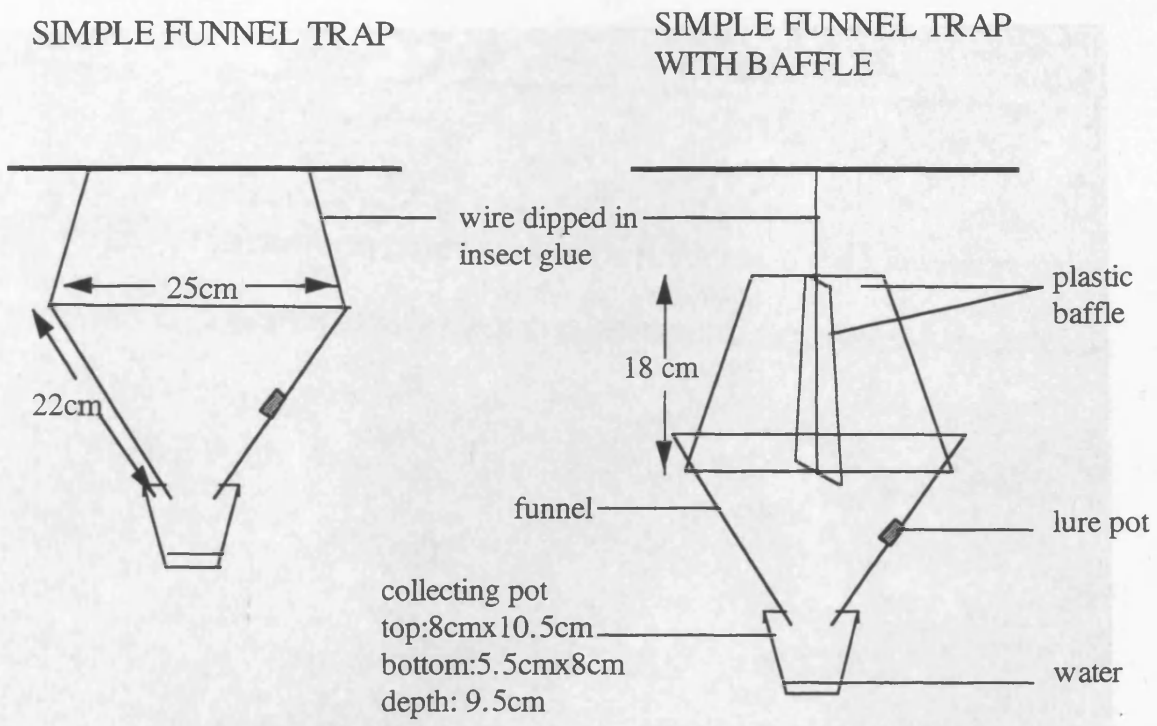


Fig. 5.3.1a: Diagram to show the two trap designs that were tested: simple funnel trap and simple funnel trap with baffle.

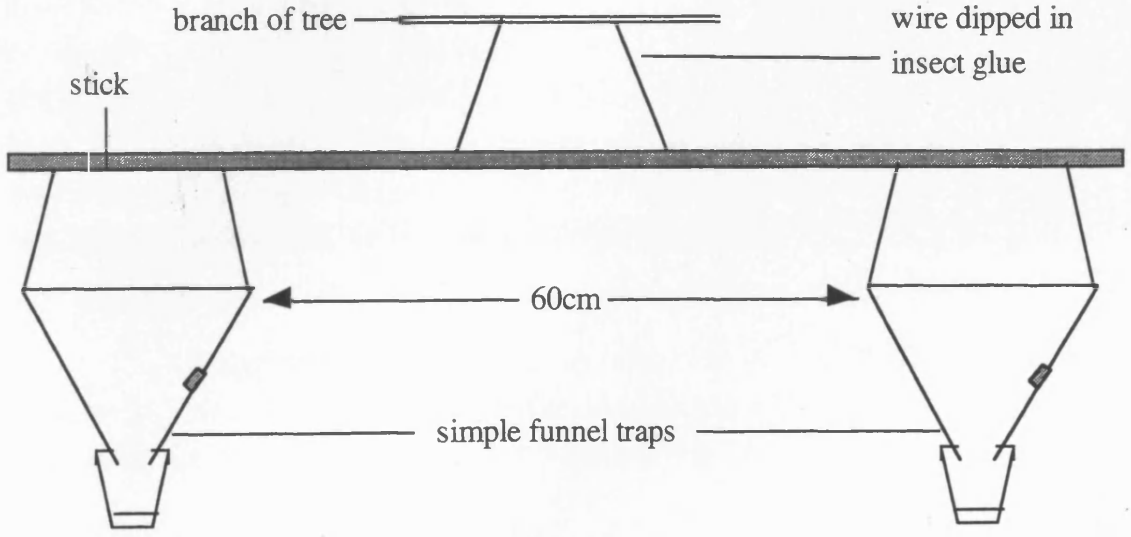


Fig. 5.3.1b: Diagram to show how trap pairs were suspended from trees separated by 60cm using wooden sticks.

Fig. 5.3.4c: Photograph of one trap pair in the study site (Teak plantation in Ho, Ghana).

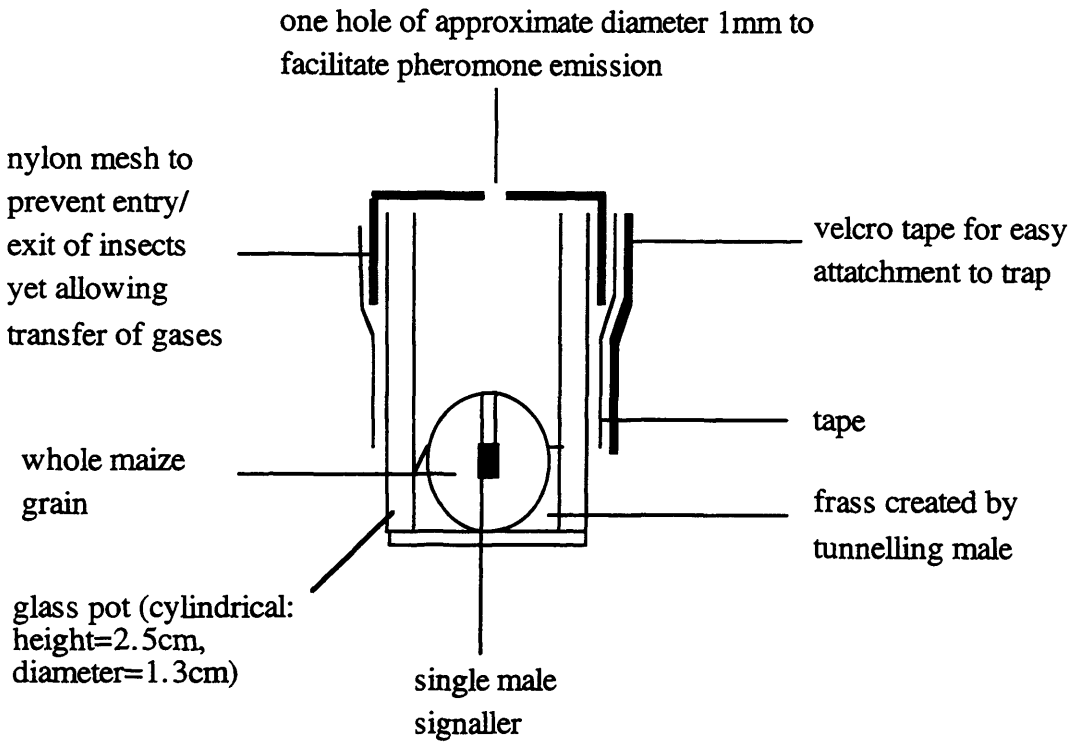


Fig. 5.3.1d: Lure pot containing a single signalling male feeding on a small quantity of maize.

EXPERIMENTAL DESIGN:

Preliminary trials

Initially, 21 traps were hung singly approximately 25m apart in the study site in two parallel lines. Each trap was hung from branches of trees in the teak woodland approximately 1-2m above the ground. Each trap was randomly assigned to one of three treatments: simple funnel trap with baffle and single male lure; simple funnel trap with single male lure; and simple funnel trap with baffle but no lure. All males used in lures were wild-caught males who had been previously placed in fresh grain for three days. After one day of trapping, all traps were emptied and all *Prostephanus truncatus* and *Teretriosoma nigrescens* were counted. All individuals of *Prostephanus truncatus* were sexed as described in section 1.5.6.

All traps had to be temporarily removed from the study site away from the threat of bush fires one day after they had been put in place. Traps were redeployed after a delay of one day in pairs as required for the main choice trial.

Choice trial

Adding baffles to the trap design did not perceivably increase trapping efficiency, so the simple funnel design was used for all subsequent trials. The simple design has the advantage that beetles are less likely to be knocked into a trap while possibly hovering around comparing pheromone signals.

Traps were hung in pairs with a distance of 0.6m between traps and at a height of about 1.5m above the ground (see fig. 5.3.1b). Initially eight pairs of traps were set up in a line parallel to the road. Pairs of traps were never closer than 25m from each other and were generally approximately 30m apart. Both traps were baited with a single male lure in four pairs and only one of a pair of traps was baited in the remaining four replicates.

The aim of having replicates where one trap was baited and the other left empty was to assess whether beetles might blunder into a trap while following the lure of an adjacent trap just 0.6m away. In order to maximize numbers of beetles attracted to these replicates, one lure that caught no beetles was replaced with a fresh one. After four days of trapping (5th Feb.) all eight replicates were given lures in both traps. On the 9th of Feb., all traps bar one had to be removed as a bush fire swept through the site. The bush fire was fueled by leaves and dry brash mainly on the woodland floor and only caused superficial damage to most of the trees. After a one day delay, nine pairs of traps were redeployed. These were in place for 11 consecutive days of trapping.

Traps were emptied once each day between the hours of 8am and 3pm and in the majority of cases between 8am and 10am. Numbers of *Prostephanus truncatus* and *Teretriosoma nigrescens* caught per trap were recorded and all *Prostephanus truncatus* caught were sexed.

Lures were generally assigned to a pair of traps and remained there until removed to be placed in the volatile-collection equipment. Each pair of lures was switched between the two possible positions in a pair of traps each day in order to allow an assessment of position effects within a trap pair. As trapping data and volatile samples were taken from pairs of males, new pairs of males were introduced in their place. A total of 28 wild caught males were used as lures, of which 18 were sampled for volatiles. In addition, 21 males taken from local insect cultures were used as lures, 12 of which were sampled for volatiles.

Analysis:

Details of the analysis are presented in the results section. Generally, chi-squared analyses were used to test hypotheses regarding the distribution of beetles caught among the traps. In some cases some low trap-catch data were not used in the analysis in order to fulfill the recommendation that at least four fifths of the expected values should be above five (Sokal and Rohlf, 1981).

5.3.2 Pheromone samples:

Collection:

Collection filters were made as described in section 4.3.5.

Pheromone samples were collected using the apparatus shown in fig. 5.3.2. Note that volatiles from each of a pair of males were collected simultaneously. Each sample consisted of 23-25 hours of continuous collection. Air flow through each filter was approximately 1 litre per minute, however electricity supply to the pump did fluctuate enough to affect its performance and there were a few brief power cuts. The activated charcoal was replaced twice throughout the collection period. Once collected, volatile samples held on filters were stored in one of two freezers at approximately -20°C. Two pairs of control samples with no lure added to the collection chamber were taken. Samples were then transported to the Natural Resources Institute, Chatham, Kent, UK for analysis.

Chemical analysis:

The volatiles collected were analysed as described in section 4.3.5.

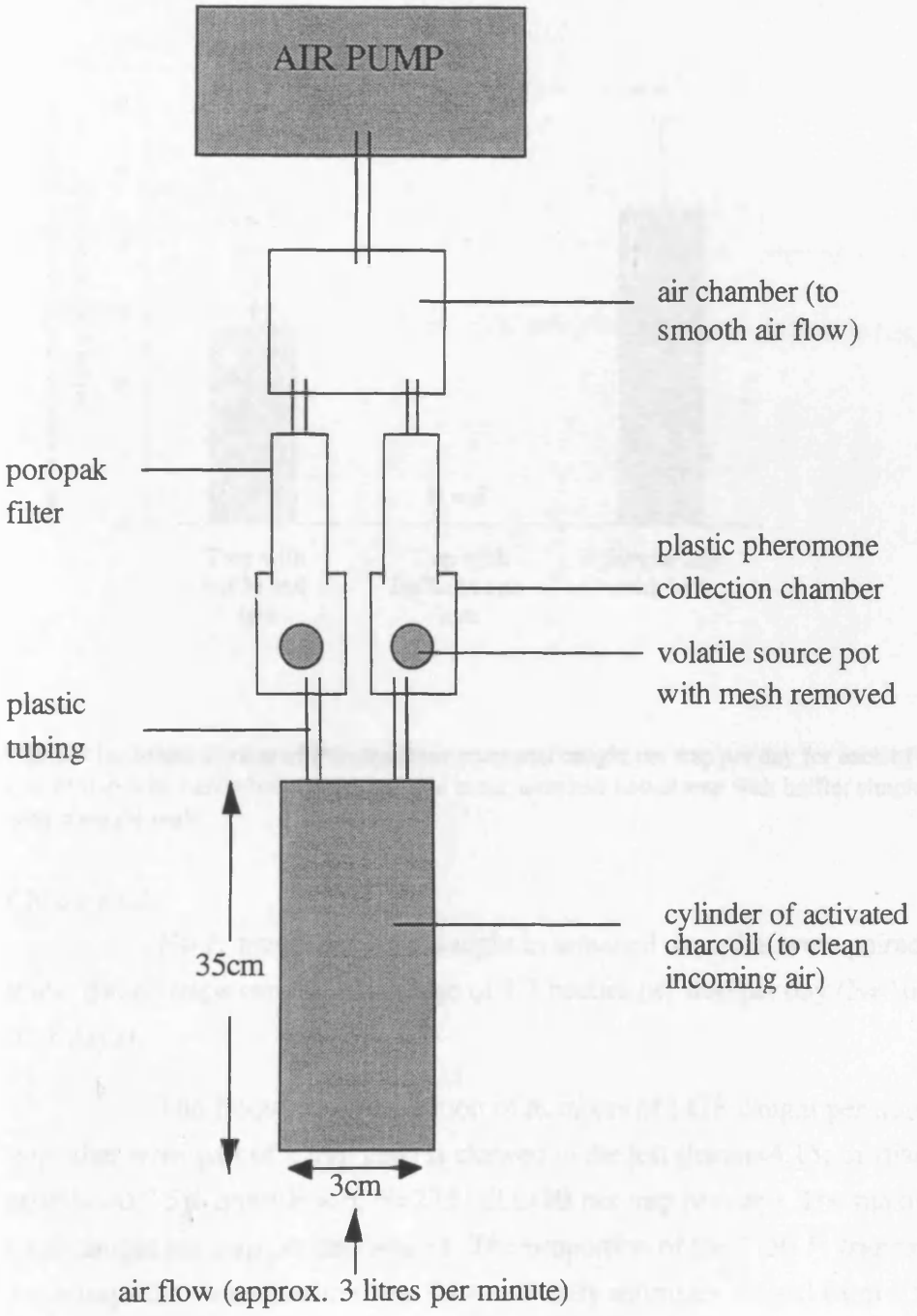


Fig. 5.3.2: Plan view of the volatile collection apparatus.

5.4 RESULTS

5.4.1 Trapping trials

Preliminary trials

Prostephanus truncatus were caught in 10 of the 13 baited traps. No *P. truncatus* were caught in any of the unbaited traps. Mean catch per trap and the associated standard errors for all treatments are shown in fig. 5.4.1a. Simple funnel traps caught comparable numbers of beetles as traps with a baffle.

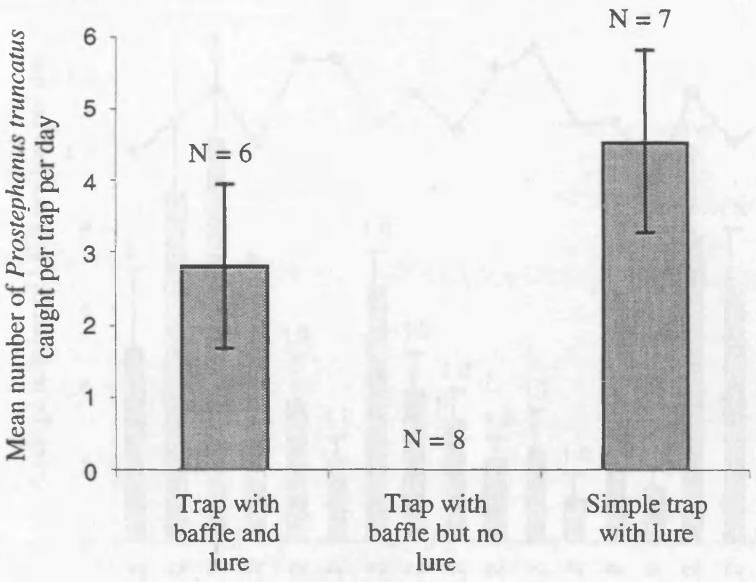


Fig. 5.4.1a: Mean number of *Prostephanus truncatus* caught per trap per day for each of three trap types: funnel trap with baffle baited with a single male; unbaited funnel trap with baffle; simple funnel trap baited with a single male.

Choice trials

No *P. truncatus* were caught in unbaited traps that were paired with baited traps. Baited traps caught an average of 4.7 beetles per trap per day (N=16 : four traps for four days).

The frequency distribution of numbers of LGB caught per trap per day (for traps that were part of a trap pair) is skewed to the left (mean=4.15; median=2, 25% quartile=0, 75% quartile = 6, N=275, all LGB per trap per day). The maximum number of LGB caught per trap per day was 31. The proportion of the 1150 *P. truncatus* caught in these traps that were females was 0.64 and daily estimates ranged from 0.59 to 0.73. Average daily trap catches and their associated sex ratios are shown in fig.5.4.1b. Beetle numbers were not randomly distributed between the two traps of a trap-pair. A nx2 chi square analysis with Yates correction combining data from all trap-pair catches that caught at least two beetles gave a sum of chi-squared of 207 with d.f.=90 and therefore $p<0.001$.

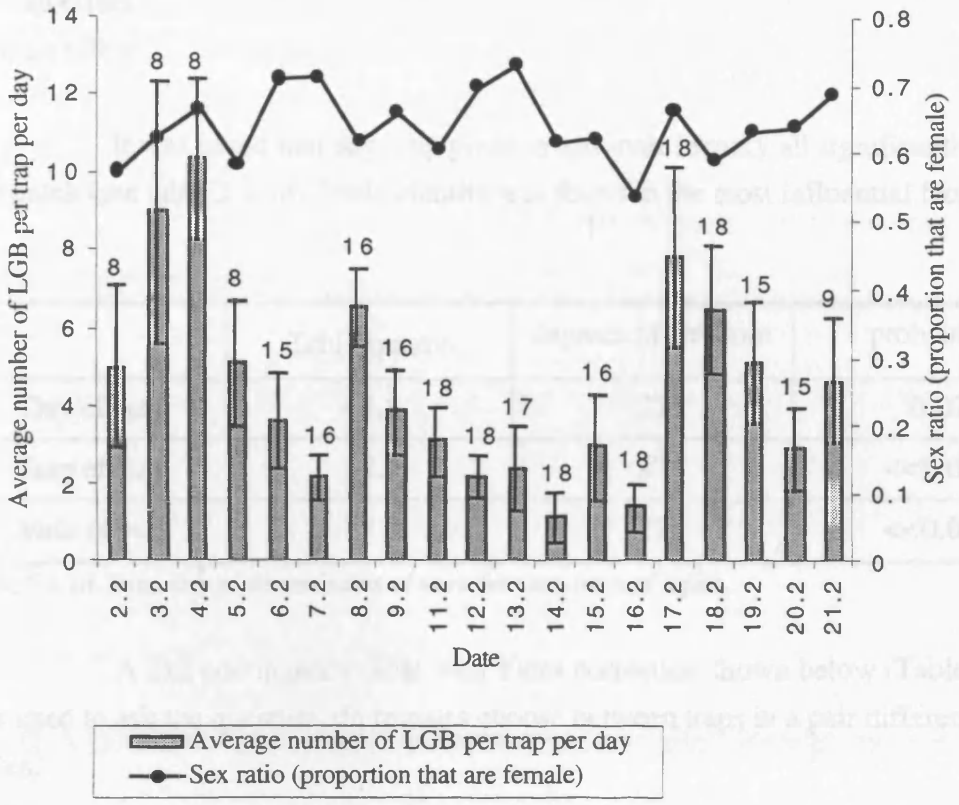


Fig. 5.4.1b: Chart to show the average number of *Prostephanus truncatus* caught per trap per day and their sex ratio, for traps set in pairs. The number of traps sampled is indicated as a number above the error bar.

Given that one trap in a pair catches more beetles than the other, how much of this skew is associated with the signaller? Data where total trap catch for a pair of traps was at least eight individuals for two consecutive days, and where the pair of signallers was swapped over between the two trap locations at the end of the first day were used for this analysis. 27 sets of data fulfilled these criteria. The data comprise results from 11 different pairs of males and nine trapping sites. The data were tabulated as a series of 2x2 tables, (see table. 5.4.1c).

Table. 5.4.1c: Example of 2x2 table used to distinguish day, trap and male effects on trap catch distribution between two traps of a pair.

	TRAP A	TRAP B
DAY 1	Total caught by MALE 1	Total caught by MALE 2
DAY 2	Total caught by MALE 2	Total caught by MALE 1

For each 2x2 table of data three nx2 chi-squared statistics with Yates correction were calculated to distinguish between the three possible effects detectable using this table:

1) Day effect
2) Trap effect
3) Male effect

It was found that day, trap position and male identity all significantly affected trap catch (see table 5.4.1d). Male identity was found to the most influential factor.

	Σchi-squared	degrees of freedom	probability
Day effect	44.8	27	0.02
Trap effect	67.9	27	<<0.001
Male effect	87.5	27	<<0.001

Table 5.4.1d: Summary of determinants of skew between traps of a pair.

A 2x2 contingency table with Yates correction shown below (Table 5.4.1e), was used to ask the question, do females choose between traps in a pair differently from males.

	Male Signaller 1	Male Signaller 2
No. of Males	a	b
No. of Females	c	d

Table 5.4.1e: Example of 2x2 contingency table used to test if males and females choose between two traps of a pair differently.

Combining all cases where the trap catch per day was at least nine males and nine females and at least one beetle was caught by each trap gives nine replicates. The sum of chi-squared for the interaction between sex and trap catch is 2.4 (d.f.=9 and $p>0.95$), which shows that no significant difference between the choice of males and females has been demonstrated.

Differences in the choice of males and females can also be studied on the next spatial scale up, between pairs of traps. Since there is variation between total catch per pair of traps it would be interesting to see if the sex ratio of beetles caught varies with changing size of trap catch per pair. A greater proportion of females in high trap catches would indicate the possibility that females show a higher level of choice than males. Data collected from the 6th Feb. to the 20th Feb. were used since trapping during this time was relatively consistent between days. Trap catches were ranked in terms of the numbers of beetles caught. The tally of beetles was then divided up into eight consecutive sections of about 110 beetles. The sex ratio and average trap catch per pair of traps were calculated for each

section. Using the null hypothesis that sex ratio does not change with size of trap catch, expected numbers of females caught in each section were calculated. A chi squared test with 8 d.f. gave a sum of chi-squares of 5.0, p=0.76, which shows that no significant variation of sex ratio could be detected with changing trap-catch size.

5.4.2 Pheromone samples

Unfortunately collection of volatiles was unsuccessful. T1 was difficult to quantify due to large amounts of impurities in the samples. The amount of T2 was quantified and ranged from 0.005-0.09µg per hour, but control samples contained comparable amounts of T2 to test samples: Test sample average = 0.033µg per hour; control sample average = 0.031µg per hour (with no male signaller in the collecting chamber). Therefore these samples could not be used to evaluate chemical variation in pheromone signal.

5.4.3 Additional observations

An average of 0.5 *Teretriosoma nigrescens* were caught per baited trap per day. Trap catch was very unevenly distributed between days with approximately one third of the total caught on a single day. No *Teretriosoma nigrescens* were caught in unbaited traps.

Approximately 20 hours were spent searching for *Prostephanus truncatus* tunnelling in vegetation in the study site. Although other Bostrichid species were located, no *P. truncatus* were ever found. Test tunnels were however, found in the sticks used in the construction of trap pairs. A total of seven tunnels were found, five of which were into the cut end of the stick. Tunnels were found in both types of wood used for trap construction, which were shoots of teak and another unidentified tree species. Two live *P. truncatus* were removed from these tunnels, all others were empty.

5.5 DISCUSSION

Considerable variation exists in trap catch both between traps and between days. Mean trap catch was approximately four beetles per trap per day. This shows that males are able to attract significant numbers of beetles even when signalling alone in Ghana. It is thought that there is a sizable population of beetles away from maize utilizing an unidentified wood host or more likely, hosts (see chapter 1). Males signalling whilst feeding on such hosts may be less effective signallers than the test beetles in this study (see effect of host on signal in chapter 3). Here, the males used were feeding on maize, which is likely to be a relatively nutritious food source. The success of single-male trapping first demonstrated by Scholz (1997) and confirmed here opens up the possibility of a very profitable area of research into the actual patterns of signalling and response in the field.

It is apparent, however, that knowledge of the spatial distribution of this pest away from storage will become more and more crucial to any interpretation of the population dynamics of this species. It is still not known whether *Prostephanus truncatus* is widely scattered or forms large aggregations in the bush akin to those occurring in maize stores.

The overall sex ratio of beetles attracted in this study was 64% females. This is exactly the same sex ratio reported by Scholz (1997), attracted by male-baited lures in Togo. A female bias has also been found in flight traps baited with artificial pheromone lures (Hodges *et al.*, 1998; Scholz *et al.*, 1997a). This implies that females predominate in the dispersing population, and/or that dispersing females are more likely to follow aggregation-pheromone plumes than dispersing males. Laboratory studies have shown that females show a higher response to naturally produced pheromone in a walking bioassay (Hodges and Dobson (in press); chapter four this study), but this may be due to male habituation to pheromone which would be less likely to influence dispersing beetles. Fadamiro (1995) reported no differences in flight activity or duration of flights between the sexes. This result is contradicted by the female-biased flight activity recorded by Scholz (1997). Fadamiro also found that there was no sex specific differences in response to artificial pheromone sources in wind tunnel trials when insects had already been preselected for flight activity. Li (1988) found that the sex ratio at emergence was highly significantly female biased (58.5% females), however Scholz reported a 1:1 sex ratio in insects cultures (Scholz, 1997). It is therefore unclear whether females predominate in samples attracted by pheromone simply because they are more abundant in the overall population, or because they show any sex-specific patterns of dispersal or response to the signal.

The female bias in the dispersing population may be dependent on habitat type. Scholz highlights that sex ratios in wooded habitats away from stores are often of almost equal sex ratio (Ramirez-Martinez *et al.*, 1994 quoted in Scholz, 1997). She proposes that, "the higher nutritional value of stored food products may act as an arrestant, making males (for reasons unknown) less likely to react to other cues... that would otherwise have stimulated them to migrate" (Scholz, 1997).

Males could increase their reproductive success by employing one of two strategies. They can signal for mates by emitting aggregation pheromone or they can follow other males' signals. In this study males caught using an artificial aggregation-pheromone signal were subsequently successfully used to provide a pheromone signal. This shows that males cannot be divided up into two mutually exclusive categories of signallers OR responders, but may utilise both strategies.

The female bias in trap catch supports the idea that aggregation pheromone in *Prostephanus truncatus* may be maintained through an increased mating advantage for signalling males. A highly significant skew in trap catch between two traps of a pair was found. Identity of the signaller was found to be the most significant determinant of trap catch. The large influence of male identity on trap catch implies two things: that variation exists between male signals; and that this influences the response of conspecifics. This result agrees with equivalent bioassay trials in the laboratory (Chapter 4 this study). In bioassay trials, males and females were found to be equally discriminating between signals from two competing males. The field trial carried out here has given the same result. Males and females therefore seem to invest equally in the assessment of signals and there may be no special sex-specific adaptations to choose between aggregation-pheromone signals.

One sex-specific difference in choice between signals does exist: this is the significant increase in female bias in trap catches in traps baited with artificially produced T1 and T2 (1mg of each) compared to traps baited with each component alone (1mg total) (Scholz, 1997). Scholz proposes that this could arise if males land at a lower threshold of response to concentration of pheromone. This could be the case if males follow pheromone primarily to locate new hosts and females locate the point of highest concentration to locate the signalling male as well as a host. Indeed males may actively avoid landing exactly where another male competitor has tunnelled if the chances of him silently gaining access to mates is low (see the discussion in chapter 6). It is difficult, however, to extrapolate from results obtained using synthetic pheromones at abnormally high concentrations to the natural situation.

Collection of pheromone was required to quantify chemically inter-male variation in aggregation-pheromone signal. T1 peaks were masked by large peaks of impurities. During the trials, many bush fires were lit in the area which may have swamped the samples with impurities. The highest peaks of impurities masking the T1 peak were collected on the same day as rubbish was burnt about 20m away from the volatile-collection apparatus. A similar amount of activated charcoal was used to clean incoming air as that used by Prof.D. Hall at NRI, but this may have been insufficient for the conditions found in Ghana.

T2 was quantified, but samples contained comparable amounts to those found in the control samples (no male present). The equipment designed for collection of pheromone in the field may have been at fault. Although the basic design was copied from apparatus used by Prof.D. Hall (described in section 4.3.5), which has been successful, some glass parts were exchanged for more robust plastic equivalents that may have retained some of the pheromone and/or created too much turbulence in the airflow, leading to inefficient absorption of pheromone onto the filter. Lastly, the ability of poropak filters to trap volatiles decreases with temperature. Although samples were kept refrigerated in

freezers (-20°C) whilst in Ho, they were exposed to much higher temperatures during transportation back to the UK, especially during the journey to Accra.

Aims one to four were successfully completed. This shows that field studies of natural pheromone plumes are relatively easy. Any future studies in this area can also be expected to be fruitful. Collection of volatiles from single signalling males was unsuccessful. Possible remedies for this include: an increase in the efficiency of cleaning incoming air; avoidance of the bush fire burning season; improvements in the design of the collection chamber; and refrigeration of samples during transport back to the UK.

CHAPTER 6: MATE CHOICE ON CONTACT

6.1 INTRODUCTION

Work has already been presented in previous chapters that describes some of the factors influencing how beetles will come in contact with each other. This chapter presents work on how they then interact, and the role of aggregation pheromone in these encounters. In other words does aggregation pheromone in LGB also act as a courtship pheromone? The focus is on behaviours that determine differences in mating success between individuals. In any aggregation of reproductively active conspecifics, both interactions within the sexes and between the sexes are possible factors leading to mating preferences. Since this thesis is concerned with differences in males (more specifically their aggregation signal), female choice and male contests were studied. Female choice is used in this study to mean any process, be it involving her nervous system or not, that leads to female-determined selection between potential mates (see chapter 1 of Eberhard, 1996). Male contests are also defined in general terms and need not be mediated through physical contact. Sexual selection between females (male choice and female-female contests) has not been specifically investigated in this study.

Long-range pheromones produced by males that are attractive to females have been found to influence contact courtship behaviour in the cockroach, *Nauphoeta cinerea* (Moore *et al.*, 1995 and 1997, and Moore, 1988). Manipulation of the pheromone signal has shown that dominance amongst males is determined by characteristics of this pheromone (Moore *et al.*, 1997). Female preferences of male odours also correlate with those that determine the male hierarchy (Moore, 1988). Moore's studies show that a long-range olfactory signal can continue to be used as a social cue at close range. Close-range pheromone cues have already been documented as sexually-selected characters in Lepidoptera (refs. in Andersson, 1994).

Since male *Prostephanus truncatus* have the ability to detect aggregation pheromone signals, it is possible that the pheromone plays a part in male-male interactions. Both visual and acoustic signals have frequently been shown to be instrumental in interactions between members of the same sex in other species (see chapters 13 and 14 of Andersson, 1994). Acoustic signalling in Anurans (frogs and toads) and Orthopterans (crickets and grasshoppers) has been shown to influence the spacing of males (see refs. quoted in Andersson, 1994).

Little was known about the courtship behaviour of *Prostephanus truncatus*. Li (1988) never observed *Prostephanus truncatus* mating and concluded that this must occur out of view, in tunnels. Fadamiro (1995) however, observed mating in an open arena with

no food present. Fadamiro reported that, "the male was the mate-finding sex: females did not attempt to locate mates". LGB courtship both inside tunnels in a potential host and away from the host are described in this thesis. Quantification of variables associated with mate choice has been limited to the situation where adults are away from the plant host. The open arena provides a uniform platform where two males can easily be presented to the female at once, and where starting position is unlikely to be very influential on the outcome of the mating trial. Male position and behaviour within the tunnel system in a host is an area that warrants its own study, but incorporates unwelcome confounding variables to an investigation into the effects of pheromone signal and male size on courtship performance. Time-lapse photography and artificial hosts sandwiched between plates of glass (for easier observation) were used to describe adult behaviour in tunnel systems in the host.

Male size is a common determinant of mating success in arthropods (Crespi 1989). Both advantages for larger males and smaller males have been documented (see Andersson 1994). Smaller males may sometimes be more manoeuvrable and agile in obtaining mates, particularly if copulation takes place in the air or in other three dimensional media. Larger males may perform better where contests of strength, either between males or between the male and female, are involved. Larger males may also be selected visually. Females may benefit by selecting large males if this leads their female offspring to be larger as larger females are often more fecund (see Holloway and Smith 1987). Male size was often controlled in the experiments presented in this chapter such that each of a pair of potential suitors presented to a female were of different sizes. This has a two fold advantage: first, it increases the likelihood that there will be variation in the aggregation pheromone signal between the pair of males; second, it ensures there is variation in size between the pair of males.

When adult *Prostephanus truncatus* encounter each other in an open mating arena they will often push each other (pers. obs.). This behaviour was particularly prevalent when females came in contact with males (see phase 1, section 6.3). Pushing is easy to observe, is widespread, variable, and demands explanation since it appears to be energetic and time consuming. For these reasons pushing behaviour was investigated further. Data describing which beetles initiate physical encounters and the outcome of such encounters helped unravel the motivations that result in mating preferences. Behaviours occurring early in courtship have the advantage that data are more often available for both males of a pair of possible suitors, males can thus be compared directly, and variation in female behaviour can then be eliminated from the analysis.

Pushing behaviour between males and females could enable beetles to assess each other's quality, prevent copulation behaviour, and/or serve to stimulate sexual behaviour. Pushing between members of the same sex may be a form of intra-sexual selection, performed either to increase direct access to mates or to defend resources like

tunnel systems. Courtship behaviours are not necessarily all sexually selected traits, as Andersson (1994) points out, "many aspects of courtship...may reduce escape responses ...synchronise endocrine reproductive functions, or coordinate the behaviour of mates in space and time for copulation".

This chapter starts with a verbal description of *Prostephanus truncatus* courtship behaviour observed in open mating arenas and in tunnel systems within an artificial plant host. Preliminary mating trials in an open arena are then presented. The aim of these trials was to test whether measures of courtship behaviour are characteristic of different individuals. The relative influence of the two sexes was also investigated i.e. does female identity determine most of the variation in courtship behaviour, or does male identity? A certain level of predictability of courtship measures is required to justify any attempt to relate these measures to characteristics of pheromone signalling. The next two experiments were designed to unravel any interaction between courtship success (mediated through female choice and/or male-male interactions), male size, male pheromone signal, and male identity. In these experiments two possible male competitors are presented to a female. Hypotheses were tested using two main types of data in this chapter: ultimate choice of male mate, and behavioural measures during courtship. The basic questions, 'do females preferentially mate with some males rather than others during courtship?' and if so, 'is this influenced by male aggregation pheromone emission?', can be most directly answered by looking at ultimate mate choice between males. Measures of behaviour were then used to suggest possible mechanisms for patterns of mate choice.

6.2 AIMS

- Describe behaviour between adult *P. truncatus* within the plant host and in an open arena.
- Evaluate whether behaviour during courtship leads to non-random mating in *Prostephanus truncatus.*
- Test whether male's success during courtship correlates with his performance in the pheromone bioassay.
- Test whether manipulation of the male pheromone signal influences courtship behaviour.
- Describe male-male interactions during mating trials where two males are presented to one female.

6.3 DESCRIPTION OF COURTSHIP BEHAVIOUR IN *P. TRUNCATUS* WHEN OUTSIDE THE PLANT HOST.

All the following observations were taken from beetles placed in a mating arena with a flat surface with no food available (a petri dish, diameter 9.5cm, with its floor lined with filter paper).

PHASE 1

Both the male and female walk around the arena. Encounters often appear to happen by chance, others result from directed movement of both sexes towards each other. Females appear to be more aggressive (in terms of physical pushing) than males at this stage. Females often accelerate towards males and push them with their prothorax/head. If a male approaches a female then this is most likely to result in female aggression if the male approaches her head to head. If a male approaches from behind then this can result in female aggression or phase 2.

PHASE 2

The male approaches the female from behind and starts to antennate (flap his antennae) against the base of her elytra. Antennation often occurs in bursts of a few seconds at a time with about one second break between bursts. This typically continues for about half a minute. The female often starts to walk off, at which point the male may try a new round of antennae flapping or go back to phase 1. If the female remains relatively stationary the male may proceed to phase 3.

PHASE 3

The male then starts to climb onto the female's back. He starts to vibrate his legs as well as his antennae on the female elytra. He then manoeuvres himself so that his genitalia are placed opposite that of the female. At this point the male occasionally loses balance on the female's back and can end up wrapped around her side or head. At any point during this phase the female can start to walk away, this is especially likely if the male has lost his position. If the male remains in position this may lead to phase 4.

PHASE 4

The male will continue to vibrate his front two pairs of legs and antennae for anything from a few seconds to a few minutes. Then the male and female become very still for anything from a few seconds to a maximum of about half a minute but most usually around 10 seconds, I propose that it is during this time that sperm is delivered, although this remains to be proven. Often during this time the male will sweep his antennae back to lie against his prothorax.

PHASE 5

After phase 4 the female will usually start to walk off forcing the male to dismount. Sometimes the male will dismount before the female starts to move. Occasionally the female may become aggressive towards the male, but generally they separate and both walk off.

6.4 MATERIALS AND METHODS

6.4.1 Experiment to test if *Prostephanus truncatus* is capable of mating within its plant host.

Males taken straight from culture were placed singly in pre-bored whole maize grains and left to tunnel for one week. At the same time, adult females were placed singly in glass pots on maize meal and also left for one week. Grains containing tunnelling males were then placed singly in the centre of a mating arena (a petri dish, diameter 9.5cm, with its floor lined with filter paper) and one of the females was introduced at the edge of the arena. The arena was observed continually for 45 minutes after which time the beetles were removed from the grain and the female's reproductive organs were dissected and searched for fresh spermatophores. Eight replicates were performed. In four replicates an additional male was introduced into the arena to see if the resident male would expel this possible competitor.

6.4.2 Observations of *Prostephanus truncatus* behaviour in tunnels within an artificial host (recorded using time-lapse photography).

Construction of artificial host:

An artificial host medium was created from a recipe adapted from that used by Marange, Floyd and Hodges (submitted). Wheat was milled using a hammer mill with a sieve size of 1mm. Fine flour was then obtained by sieving the milled wheat through a 4.25µm brass sieve (Endecotts). Maize flour was obtained by sieving medium ground maize meal through a 4.25µm brass sieve (Endecotts). These maize and wheat flours were then mixed in a ratio of 14 parts maize: 2 parts wheat: 3 parts water by volume to produce a firm dough. The dough was sandwiched between two glass microscope slides (2.5cm x 7.5cm) which were compressed until the dough was approximately 1.5mm thick. The sandwich was then baked in an oven at 90 °C for two hours. Slides were then left to equilibrate to the temperature and humidity of the CTH room for two days before beetles were introduced. Each group of beetles observed were released in a square plastic-container (10.5cm x 10.5cm x 2cm) containing two artificial host sandwiches placed side by side.

Video camera set up:

Camera and video equipment described in section 1.5.7 were used to record beetle behaviour in the artificial hosts. A time-lapse video recorder was used to convert 12

hours of real time observation into nine minutes of video tape played at normal speed. In this preliminary investigation, behaviour was only recorded during the 12 hour light phases of the CTH room although far red lighting could be used in the future to observe behaviour during the dark phase.

Observations recorded:

Initially two male and two female beetles were observed for five days, then this was repeated. Eight females and two males were then observed for six days. Lastly eight males and two females were observed for six days.

6.4.3 Method for recording pushing behaviour

Four aspects of pushing behaviour were recorded: first, the frequency of pushes; second the sequence in which pushes occurred; third, the identity of the pushing beetles: and last, the identity of the beetle who initiated the encounter (who approached whom). The identity of the beetle who initiated the encounter was of interest since it could indicate active pursuit of mates which, in the case of females, might be influenced by pheromone characteristics of the males. Note that strength of push was not quantified. All this information was recorded as a series of vertical lines as shown in the annotated diagram below (fig. 6.4.3a).

6.4.4 Preliminary investigation of courtship behaviour.

Source of insects:

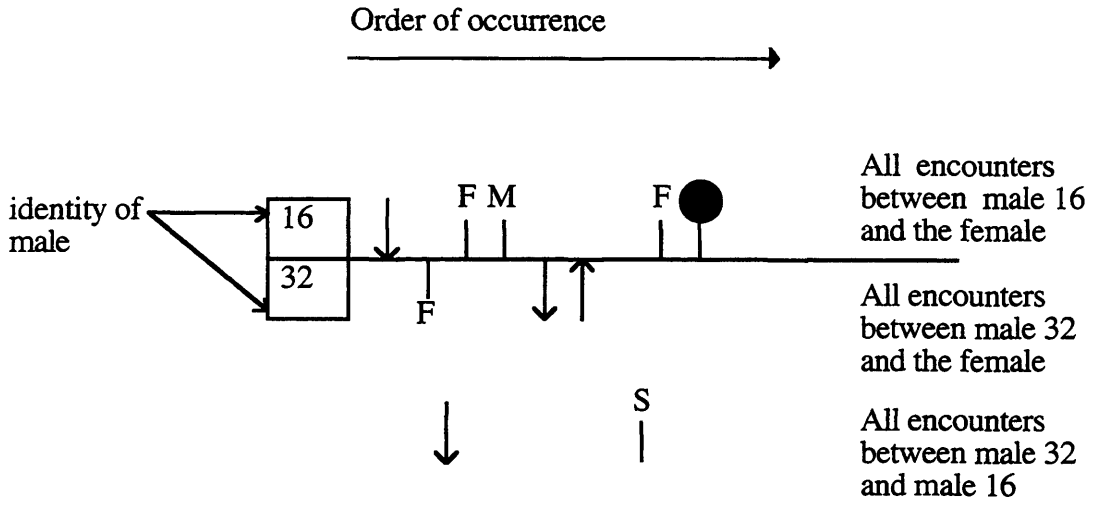
Six males and five females were isolated as adults randomly selected from a culture. All beetles were separated into glass pots containing food and left in the CTH room. Beetles were isolated for three and a half weeks to maximise the likelihood that they would be ready to mate.

Apparatus:

An arena was used for all mating trials, which consisted of a petri dish (diameter 9.5cm) with its floor lined with filter paper. A video camera was used to film the behaviour from above (see section 1.5.7 for details of camera and video equipment). All tests were performed in the CTH room.

Experimental design:

All possible combinations of male-female pairs were tested. Trials were conducted over four days. In each trial the beetles were placed into the area at the same time and observed for up to 30 minutes or until 5 minutes after the first copulation. Filter papers were removed after each test and the arena was cleaned with IMS. Beetles always had at least 1 hour and 45 minutes between tests and for the majority of cases this time was longer. Beetles were tested for a maximum of two times per day. Within these constraints, the order of trials was randomised (picked out of a hat).



Key to symbols

Male/female encounters:

= Female approached but no push

= Male aproached but no push

F = Male approached and female pushed

F = Female approached and female pushed

M = Male approached and male pushed

M = Female approached and male pushed

= Male approached and copulated (phase 4)

Male/male encounters:

= Large male approached but no push

= Small male approached but no push

L = Large male approached and pushed

S = Small male approached and pushed

Fig. 6.4.3a: Annotated diagram of method of recording pushing behaviour between one female and two males (numbered 16 and 32) in a mating arena.

6.4.4 Experimental design cont:

Test subjects were identified by making a note of any distinguishing features of the beetle and the relative size of the subjects in the trial. The initial position of each beetle in the arena was also noted to allow easy identification of the beetle on film. Video tapes were analysed and each push was recorded in the sequence that it occurred and any copulation attempts were also recorded as described in section 6.4.3. Potential matings

were inferred when males mounted females and their abdomens touched in the region of their genitalia. Time elapsed from the start of the trial to the start of the first copulation was noted.

Analysis:

A GLM model was used to test for consistencies in behaviour of both male and female individuals (Minitab).

6.4.5 Investigation into the relationship between pheromone attractiveness and success in mating trials.

Source of insects:

All insects were randomly selected from culture as adults, sexed, and placed singly on fresh medium ground maize in a glass pot. Insects were then left in the CTH room for 12 days before the first assay. All males were weighed five days after being sexed.

Apparatus:

The olfactometer apparatus described in section 3.2 and mating arena described in experiment 6.4.4 were used.

Experimental design:

Insects were grouped into replicates of two males and a female. Males were paired up such that all pairings had a standard size difference of 0.4- 0.5mg. This allowed us to investigate how any preferences between males of a standard difference in weights varied with the average weight of the pair i.e. are larger males always preferred or are smaller males preferred when both test insects are relatively large? The smell of the two males was presented to the female in the olfactometer, then all three beetles were given the opportunity to interact in a mating arena. All 36 replicates were assayed over five days.

Pheromone bioassays: Bioassays were performed as described in section 3.2. Replicates were bioassayed in groups of three replicates. Each male of a pair of males was connected up into the olfactometer in a position diametrically opposite the other. Position in the arena was randomised (coin flip). Each bioassay was continued for 20 minutes.

Mating trials: Mating trials were conducted for each replicate straight after bioassay trials (the three replicates bioassayed together were observed for mating behaviour in sequence). Courtship behaviour was observed for 30 minutes or until one male copulated with the female (phase 4). Behaviour was recorded as described in section 6.4.3.

This experiment was then repeated one month later using beetles from a different, but similar culture. 45 replicates were performed in the second set of trials. This increased the total number of replicates to 81.

Analysis:

For each replicate a volatile score (number of visits to source tube per assay), a behaviour sequence record until the first mating, and the weights of each male were recorded. From this information questions were asked about the relationship between volatile preference and mating preference; volatile preference and weight of male; and mating preference and weight of male.

The frequency of the six recorded behaviour types occurring between the female and each of the two males was used to assess which aspects of pushing behaviour between males and females are associated with mating success. Males were then categorised as the larger or smaller male of the pair, and behaviour frequencies between these two groups were compared. All frequency data were compared using chi-squared tests.

Since male pairs were taken from the range of weights found in a culture sample, it was also possible to investigate how variables changed with changing average weight of a pair of males. Total bioassay scores for pheromone activity and behaviour measures of courtship were plotted against absolute weight of the pair of males.

Behaviours recorded during male-male interactions were also analysed. Differences in behaviour between the larger and smaller of a pair of males and the winner and loser in a mating trial were assessed. All differences between categories of males were tested using a Wilcoxon signed-ranks test.

6.4.6 The influence of manipulating pheromone signal on courtship behaviour.

Source of insects

All insects were those used in the second wave of experiment 6.4.3. One of each pair of males was randomly allocated to one of two treatments:

- One fresh split grain and a small quantity of flour.
- An equivalent amount of grain previously infested with males and females presumably containing Female Factor. This grain was taken from a two and a half month old culture jar that was frozen for five days and then allowed to defrost for two days.

All males were left in their treatment pots for 12 days prior to the first mating trial.

Apparatus

The olfactometer apparatus described in section 3.2 and mating arenas described in experiment 6.4.4 were used.

Experimental design

All trials were conducted over three days. The first and last days were used for mating trials and volatile bioassays were performed on the second day.

Mating trials: Females selected randomly were placed in a mating arena with a pair of males. Male pairings were the same as those used in experiment 6.4.5. Courtship behaviour was recorded as described in section 6.4.3 for up to 30 mins or until one male secured a mating.

Pheromone bioassays: Fifteen pairs of males not used in mating trials were randomly selected to take part in bioassay trials. Both males of a pair were connected up to the arena at once providing responding females with a direct choice between them. Female response was recorded for 20 minutes per trial (see section 3.2 for bioassay procedure). These pheromone bioassays were carried out to check that the manipulation of pheromone signal had been successful.

Analysis:

Wilcoxon signed-ranks tests were used directly to compare measures of behaviour after treatment with equivalent trials before the males were allocated to treatments.

6.5 RESULTS

6.5.1 Experiment to test if *Prostephanus truncatus* is capable of mating within its plant host.

In all eight trials females entered the grain containing the male within 12 minutes of being introduced into the arena. Generally, once entering, neither the male nor the female left the grain before the end of the observation period. In one trial however, the female did briefly leave and then re-enter the grain. In no case did both insects leave the grain at the same time, therefore no matings occurred outside the grain during these observations.

Dissection of the females revealed that six out of the eight replicates had mated within the observation time since fresh spermatophores were found. This proves that *Prostephanus truncatus* will mate within the plant host. Two intact spermatophores were found in one of the replicates where an additional male was allowed to enter the grain. In the other three cases where a second male was allowed entry, only one spermatophore was found in the female. In no case were males expelled from the grain.

6.5.2 Observations of *Prostephanus truncatus* behaviour in tunnels within an artificial host (recorded using time-lapse photography).

The artificial plant hosts used here were successfully colonised by test beetles. Tunnel systems were constructed, eggs laid and larvae developed within this medium. The

glass/food medium sandwich was also successful in allowing observation of tunnelling behaviours. The width of tunnels was approximately the same as the width of the glass/food sandwich, so beetle position could be seen clearly through the glass. Details of behaviour such as antennation and direct confirmation of penetration during copulation were not possible using this method. The time lapse setting chosen was fast enough to allow an easy appreciation of the activities of the beetles yet slow enough to record copulations.

Overall distribution of beetles within the plant host:

Generally beetles of the same sex constructed tunnel systems separately and two beetles were only observed in the same tunnel system as a male/female pair. In the female biased set up (8 females, 2 males), the two males still co-habited with only one other female. Occasionally one tunnel system broke through to another resulting in more than one male or female occupying the same system.

Some roving of beetles over the host surface (around the edge of the sandwich) was seen. Roving beetles did enter other beetle's tunnel systems, but generally only remained when the system was occupied by one member of the opposite sex. Once males had constructed a short length of tunnel (approx.1-2cm) they spent the majority of time positioned at the entrance to that tunnel. Lone females generally spent more time at the head of their tunnel system throughout the whole observation period.

Pushing behaviour:

Some pushing behaviour was observed between beetles in tunnel systems and at least one male was expelled by a female using this method. Beetles did not appear to push each other within the plant host as much as they do in an open arena. This is difficult to confirm, however, as beetles are more unsteady on a flat surface and therefore pushing may be more likely to result in a greater displacement of beetles.

Male/female interactions including copulation behaviour:

Male-female pairs were observed to remain together for at least one 12 hour light period. It was impossible to be certain whether male/female pairs were the same individuals between days in these trials since beetles were not marked and filming was not performed during the dark phase. Males generally remained in the tunnel entrance whilst the female constructed blind-ending tunnels into which she laid eggs. Males occasionally returned to the female without copulating. Beetle pairs were also observed copulating within the plant host. In all set ups bar the male biased one (8 males and 2 females), pairs of beetles typically copulated approximately 1-3 times per 12 hour light period. In the male biased set up, male/female pairs were observed to copulate approximately 20 times per day. At least two different male/female pairs copulated at these high rates.

The typical behavioural sequence resulting in copulations is shown in fig. 6.5.2. Males approached females and enticed them to back up into a widened region of the tunnel system such as the junction between two tunnels or back to the tunnel entrance, where there was enough space to assume the mating position. After mating the female generally returned to the end of the tunnel, presumably to continue laying eggs and the male generally returned to the tunnel entrance.

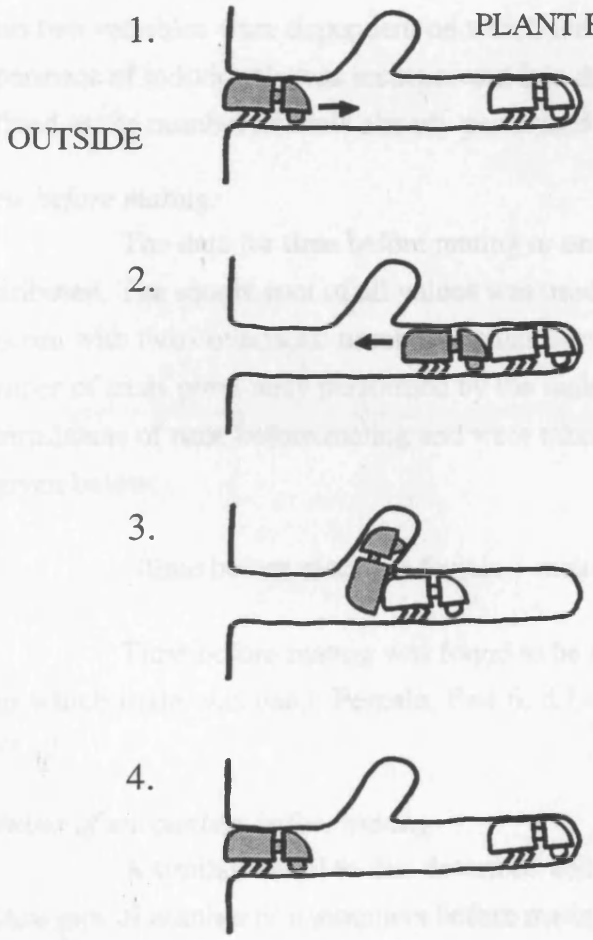


Fig. 6.5.2: The behavioural sequence of a typical copulation between a pair of LGB within a tunnel system in an artificial plant host. The male is shaded in grey and the female is represented as the white beetle. Note how the male remains in the entrance to the tunnel system before and after copulation.

6.5.4 Preliminary investigation of courtship behaviour.

General observations:

Matings occurred in 22 out of 30 trials. Of those replicates that mated, the median time until mating was 8.4 minutes (average=10.1 minutes).

Both males and females pushed each other using their prothorax. The pushing beetle kept its prothorax low and accelerated against the beetle it was pushing. Pushes

varied in magnitude from a small nudge to the extent where a beetle repeatedly rolled another around the mating arena. In this sample of individuals, females pushed males 10.5 times (mean), 6 times (median) before mating. On average, males pushed females just 1.2 times (mean) 1 time (median) prior to mating. This difference between males and females was highly significant: Wilcoxon signed-ranks test, $z=-4.00$, $N=30$, $p<0.001$.

Time before mating and the number of encounters before mating were chosen as two measures of readiness of the pair to mate. GLM models were used to determine if these two variables were dependent on which individuals were used in the trial. Previous experience of individuals was incorporated into the model. Previous experience was defined as the number of trials already performed.

Time before mating:

The data for time before mating or until the end of the trial were not normally distributed. The square root of all values was used to normalise the data. Initially the GLM was run with two covariates: number of trials previously performed by the female and number of trials previously performed by the male. These were found to be insignificant determinants of time before mating and were taken out of the final analysis. The model used is given below:

$$\sqrt{\text{time before mating}} = \text{female} + \text{male}$$

Time before mating was found to be more a feature of which female was used than which male was used: Female, $F=4.6$, $d.f.=4$, $p<0.01$; male, $F=2.04$, $d.f.=5$, $p=0.12$.

Number of encounters before mating:

A similar model to that described above was applied to the dependent variable, square root of number of encounters before mating, or until the end of the trial. Again, the previous experience of males and females were not found to influence the dependent variable and were taken out of the model. The model used was:

$$\sqrt{\text{number of encounters before mating}} = \text{female} + \text{male}$$

Again, female identity was a more significant determinant of the measure of readiness to mate: Female, $F=7.17$, $d.f.=4$, $p=0.001$; male, $F=3.43$, $d.f.=5$, $p=0.021$.

6.5.5. Investigation into the relationship between pheromone attractiveness and success in mating trials.

Behavioural measures of courtship:

The nature of the design of this experiment allows us to investigate whether differences in pushing behaviour are associated with success in being the first of a pair of

males to mate with a female. To this end, behavioural data for replicates where a mating occurred were divided up into those performed by the mated and those performed by the unmated male. All encounters observed were categorized according to which beetle initiated the encounter (who approached whom) and what the result of that encounter was (male push; female push or no obvious push). Each category of encounters was then averaged across trials and plotted as a bar chart (see figs. 6.5.5.a and b). Averaging proportions instead of absolute frequencies enables each trial to contribute equally to the chart.

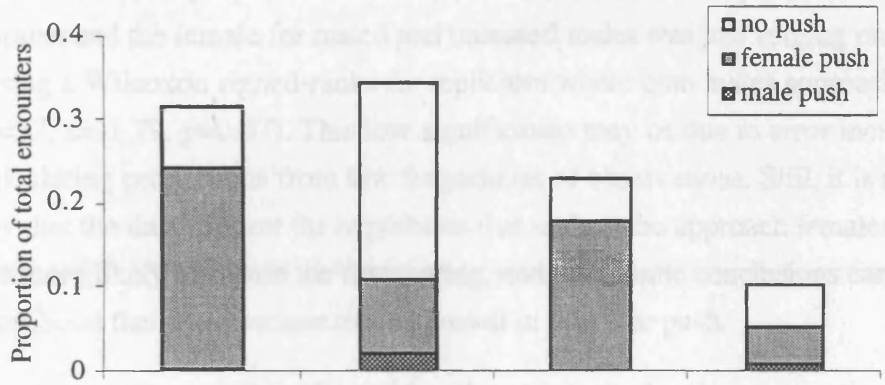


Fig. 6.5.5a: First set of trials (N=27). See legend below.

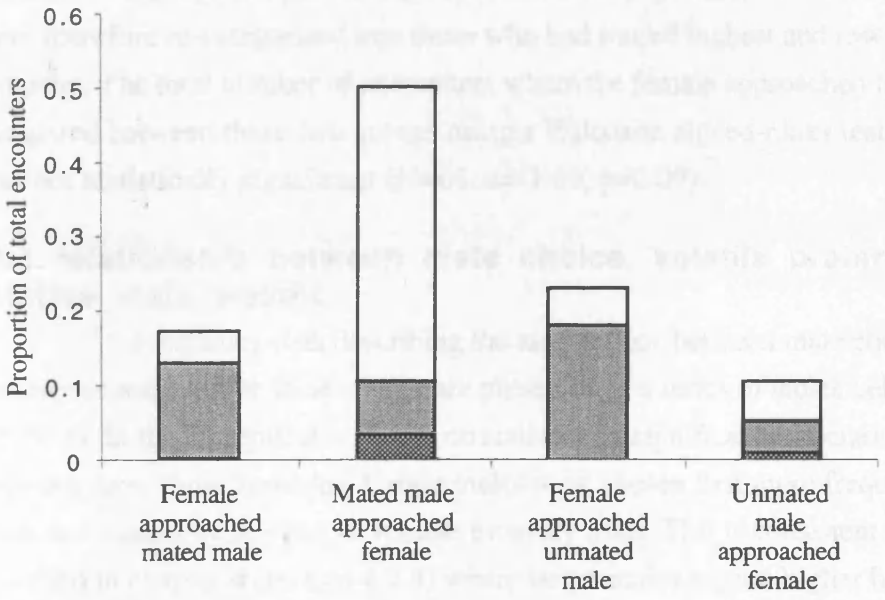


Fig. 6.5.5b: Second set of trials (N=34).

Fig. 6.5.5a and b: Bar charts to show the proportion of encounters leading to three different behaviours (male push; female push; no obvious push) for all encounters between males and females in a mating arena. Two males were presented to a single female and the males have been subsequently categorised according to whether they were the first of the pair to mate with the female. Encounters have been divided up according to which beetle initiated the encounter (who approached whom).

Sex differences in behaviour are clearly shown in figs. 6.5.5 a and b. Females are more pushy than males as found in section 6.5.4. No consistent difference between male and female approach rate has been found: females initiated more encounters in the first trials and males initiated more encounters in the second trials.

Raw counts of each behaviour scored were compared between the mated and the unmated males using a Wilcoxon signed-ranks test (the data do not approximate to a normal distribution). Only the number of encounters where the male approached the female and no push ensued was significantly different between mated and unmated males of a pair: 1st trial, N=27, z=-3.39, p=0.001; 2nd trial, N=34, z=-4.59, p=<<0.001. However, the difference in the proportion of encounters that resulted in no push where the male approached the female for mated and unmated males was just verging on significance (using a Wilcoxon signed-ranks for replicates where both males approached at least once: N=37, z=-1.79, p=0.07). This low significance may be due to error incurred from calculating proportions from low frequencies of observations. Still, it is more accurate to say that the data support the hypothesis that males who approach females most frequently are more likely to secure the first mating, and no definite conclusions can be made about the likelihood that these encounters will result in a female push.

The number of times females approached males was not found to be significantly associated with winning a mating contest (Wilcoxon signed-ranks, N=61, z=0.384, p=0.70). However, this variable might indicate whether females continue to orientate to aggregation pheromone at the distances experienced in the mating arena. Males were therefore re-categorised into those who had scored highest and lowest in the volatile bioassay. The total number of encounters where the female approached the male was then compared between these two groups using a Wilcoxon signed-ranks test. This difference was not statistically significant (N=66, z=-1.69, p=0.09).

The relationship between mate choice, volatile preference and relative male weight:

Frequency data describing the associations between mate choice, volatile preference and relative male weight are presented as a series of tables below (see tables 6.5.5c-e). In the 81 replicates tested, no statistically significant associations were recorded between these three variables. Larger males were chosen first more frequently in mating trials and visited more often in volatile bioassay trials. This is consistent with the tendency recorded in chapter 4 (section 4.4.4) where larger males gained higher bioassay scores. However, even combining these results does not produce a significant association between male weight and bioassay performance.

Tables 6.5.5 c, d and e: The association between males preferred in pheromone bioassays, those that mate first in mating trials, and male size.

C. Volatile preference vs. success in mating trial:

	Preferred volatile = First to mate	Preferred volatile ≠ First to mate	No preference
1st Trials	15	8	13
2nd Trials	10	17	18
Total	25	25	31

D. Volatile preference vs. relative weight of males:

	Preferred volatile = Heavier male	Preferred volatile ≠ Heavier male	No preference
1st Trials	21	11	4
2nd Trials	18	16	11
Total	39	27	15

E. Success in mating trial vs. relative weight of male:

	First to mate = Heavier male	First to mate ≠ Heavier male	No preference
1st Trials	16	11	9
2nd Trials	22	12	12
Total	38	23	21

Absolute weight of males, volatile preference and behavioural measures of courtship:

Pairs of males of fixed weight difference have been randomly sampled from the range of weights found in a culture of LGB. This means that it is possible to ask the question, does the average weight of a pair of males influence the total response and/or pattern of preference for the larger or smaller male?

Absolute weight of male vs. volatile response/preference:

Total bioassay score was not found to vary significantly with average weight of the males presented to the female. Bioassay score given to each male was also found to vary randomly with individual male weight.

The bioassay response given to the larger male minus that given to the smaller male was plotted against average weight of the males. Trials 1 and trials 2 were plotted separately in an attempt to visualise any consistencies in trends in the data. A moving

average of 10 replicates was used to smooth the data. Neither a consistent direction of size preference nor an optimum size preference model are supported by the data.

Absolute weight of male vs. measures of courtship behaviour:

No significant differences were found between the behaviour of the larger and smaller male of a pair. However, there is some indication that the heavier male of a pair is more likely to win a mating contest. We tested behaviour variables against the average weight of the pair of males to see if they were correlated.

The proportion of encounters in a trial classed as 'male approach, no push' was not significantly correlated to the average weight of the two males used in the trial: Spearman's rank N=46, rs=0.173, p>0.1 (see fig. 6.5.5f). This variable was tested since this was most correlated to mating success when males within a pair were compared. There is some suggestion of two populations of points in the data. Closer examination of the data revealed that this apparent separation is not due to the data being taken from two sets of trials. No variable measured has been found to account for this apparent pattern.

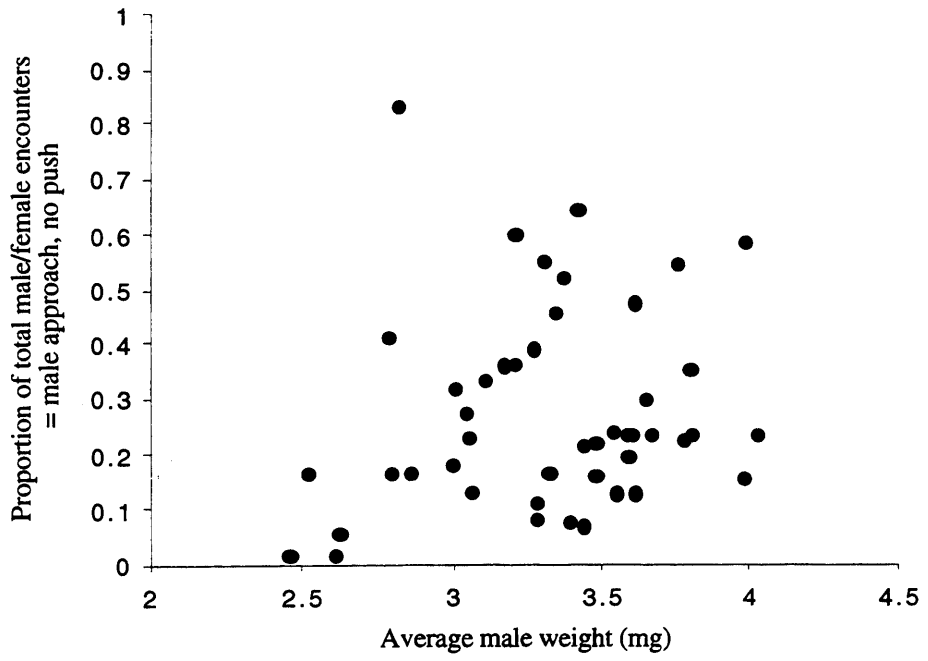


Fig. 6.5.5f: Graph to show the proportion of total male/female encounters arising from a male approaching the female that resulted in no push, against the average weight of the pair of males presented (Spearman's rank N=46, r_s=0.173, p>0.1).

Body size could conceivably influence the likelihood that females may push a male, however proportion of encounters resulting in a female push was not correlated to average body weight of the pair of males. Another possibility is that the number of encounters prior to mating may be correlated to body size, this was also found not to be the case.

Male-male interactions:

21% of all interactions recorded during mating trials were between the pair of males (Trial 1 (179/911+179); Trial 2 (326/992+326). In the first trials 30% of male-male interactions resulted in an obvious push compared with 34% in the second set of trials. Males are less aggressive towards females, the percentage of male-female interactions that resulted in the male pushing the female was 3.5% (first trials) and 5% (second trials).

The influence of male size on male-male interactions was tested. Larger males were pushier in both sets of trials but this difference was only statistically significant in trial 1 (see table 6.5.5g).

Table 6.5.5g: The average number of pushes for smaller and larger males of a pair during male-male encounters for two sets of similar trials.

	Average number of pushes per trial			
	Larger male	Smaller male	Test stat.	N
1st Trials	1.11	0.39	z=-2.20 p=0.03	36
2nd Trials	1.80	0.67	z=-1.59 p=0.11	45

To investigate the possibility that the outcome of male-male interactions is correlated to the outcome of male/female interactions, the data were divided into pushes performed by the male who subsequently secured the first mating and pushes performed by the other, losing male (trials where no mating occurred were eliminated from this analysis). Winners of mating trials were significantly more pushy in male/male interactions in the second set of trials, no difference was found in the first set of trials (see table 6.5.3h).

Table 6.5.3h: The average number of pushes given by winners and losers of mating trials to each other during mating trials. Data from two sets of similar trials are presented.

	Average number of pushes per trial			
	Mated male	Unmated male	Test stat.	N
1st Trials	0.52	0.56	z= 0.07 p=0.94 NS	27
2nd Trials	1.68	0.45	z=-2.48 p=0.013	38

6.5.6 The influence of manipulating pheromone signal on courtship behaviour.

Pheromone bioassay trials

Males in the fresh grain group were at least an order of magnitude more attractive to females in bioassay trials than males kept in the Female-Factor treatment (average response of 3.13 visits per trial compared to 0.2 visits per trial). A Wilcoxon signed-ranks test between each pair of males revealed that this difference was significant, $z=2.809$, $N=30$, $p<0.01$.

Mating trials

No significant effect of treatment with Female Factor was recorded on overall courtship success (see table 6.5.6a) or on specific measures of pushing behaviour during courtship (see behaviour measures section below). In fact, the data are more notable for their consistencies between observations made before and after treatment than for any demonstration of effect of the treatment. The same male mated first in both trials conducted on each replicate significantly more times than not ($N=23$, chi-squared = 9.78, $p<0.01$) (see table 6.5.6b). This shows that mating preferences are consistent between females since different females were used before and after treatment. Even though no significant effect of treatment was demonstrated, in all four cases where the first male to mate changed after treatment, the switch was from the untreated to the treated with Female Factor male.

Table 6.5.6a: The association between treatment with Female Factor and success in being the male to mate first in mating trials.

Female Factor male mated first	Untreated male mated first	No mating
14	12	4

Table 6.5.6b: Table to show the numbers of replicates where the same male was the first to mate in trials before and after treatment.

Consistent result	Inconsistent result	Cases where no result one or both trials
19	4	7

Behavioural measures (encounter data):

Behavioural measures are likely to be more sensitive to changes induced by the treatment than ultimate mate choice. Therefore, the behaviour highlighted as that most closely associated with mating success (number of times each male approached the female resulting in no push) was examined for treatment effect. Since male pairs were kept constant before and after one of the males was treated with Female Factor, influence of the treatment could be accurately measured by comparing the pair of males before and after

treatment. The measure of the relative amount each of a pair of males pursued the female used is shown below:

$$\frac{\text{(No. approaches by Treated male = no push) - (No. app.by Untreated male = no push)}}{\text{Total number of times the female was approached by a male}}$$

A Wilcoxon signed-ranks test was used to test if there was a consistent difference in the relative behaviour of pairs of males before and after treatment. No difference was found, N=30, z=1.91, p=0.056, but the trend indicated is for Female-Factor treated males to pursue females more successfully. This measure of behaviour was surprisingly consistent before and after treatment (Spearmans rank: N=30, rs = 0.61, p<0.005) (see fig. 6.5.4 c).

Earlier results had indicated a trend for females to approach the male they had preferred in a bioassay trial more than the other male in a mating trial. However, when the pheromone signal was manipulated in this experiment, females did not then orientate less often to the males who had shut down their pheromone signal (those treated with Female Factor) (Wilcoxon signed-ranks test for replicates where females approached at least once in each trial: N=14, z=1.269, p=0.20).

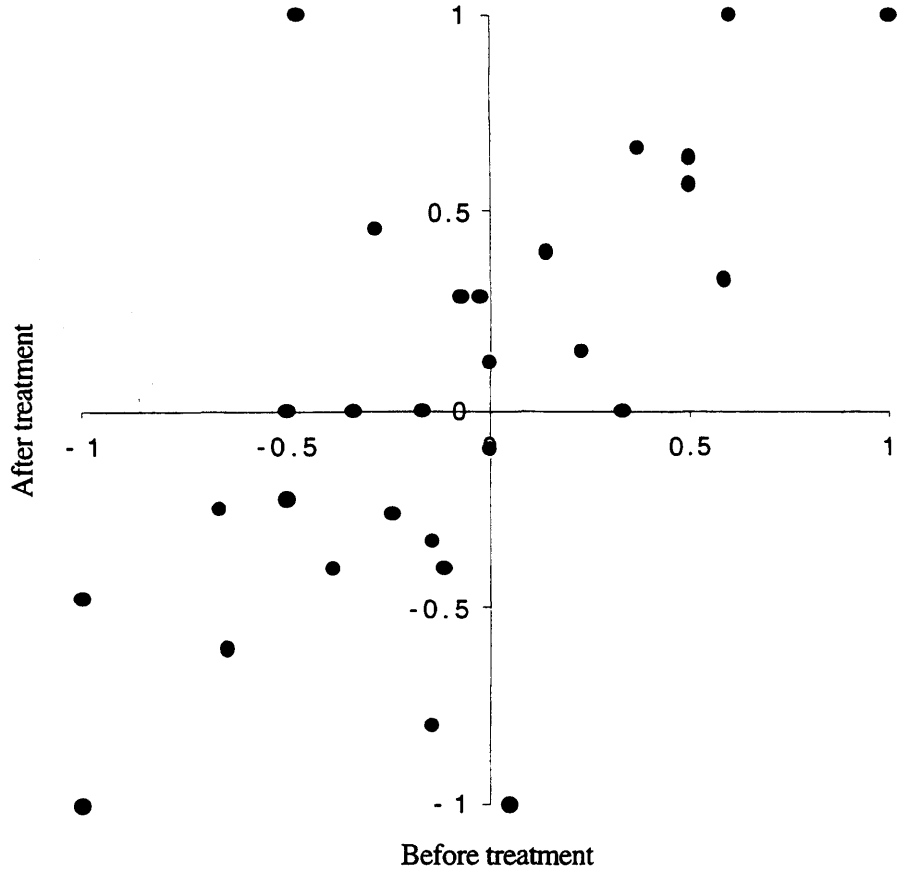


Fig. 6.5.4c: Graph to show the relative amount each male of a pair approached the female and no push ensued, before and after one of the males was treated with Female Factor (Spearman's rank: N=30, rs = 0.61, p<0.005).

Male-male interactions

No effect of treatment with Female Factor on male-male pushing behaviour was found. The proportion of male-male encounters resulting in a push remained approximately the same (mean before treatment = 0.33, mean after treatment of one male = 0.31, Wilcoxon signed-ranks test of replicates where males had at least one encounter in trials before and after treatment: N=26, z=-0.143, p=0.89). The relative pushiness of each male of a pair was also unaffected by treatment of one of the males with Female Factor. The relative pushiness was calculated as:

$$\frac{\text{Number of pushes by male to be treated-No. pushes by untreated male}}{\text{Total number of pushes between the two males}}$$

Only seven replicates were suitable for this comparison since there were many instances where no pushes were recorded between the pair of males and where there was no change in the relative pushiness with treatment. Of the seven replicates where the relative pushiness changed after treatment, treated males became more pushy in four replicates and less pushy in three replicates.

6.6 DISCUSSION

Variation in courtship and copulation behaviour between species is bewilderingly vast (see Eberhard, 1996 for a review). Therefore an initial description of such behaviours can very quickly rule out the possibility of some of the more extreme behaviours found in other species, and suggest avenues for further study. Outside the plant host, LGB courtship behaviour is characterised by a particularly variable pre-copulatory period of pushing behaviour, a period of stereotyped rubbing of the female using male antennae and legs, a short copulation, and little evidence of any post copulatory interaction between male and female. This is in contrast to behaviour within tunnel systems, where male and female pairs were found to remain together, in some cases probably for the entire observation period (5-6 days), and to mate repeatedly during this time. In these preliminary observations only one female was observed to co-habit with each male, unlike many polygamous Scolytid species (Kirkendall, 1983; Schmitz, 1972).

Fadamiro's observation that males are, 'the mate finding sex', is not convincingly supported by the data presented in this chapter. In open arenas and within the plant host, both male's and female's movements bring pairs together for copulation. General reluctance to mate by females, observed as pushing behaviour, results in female choice between potential mates. Stereotyped rubbing between mates as described in phases two and three of courtship (6.3) (usually performed by the male on the female) is widespread in insect courtship (Eberhard, 1996). Copulation duration in LGB is very short whether insects mate in or outside a plant host. This contrasts with that found in *Sitophilus oryzae* (Holloway and Smith, 1987), and in the milkweed leaf beetle (Dickinson, 1988).

Leaf beetles remained coupled for an average of 0.75 days in Dickinson's study. Presumably the female constrains sperm uptake and or displacement rates, or promotes an extended period of sperm delivery by some other form of cryptic female choice in this leaf beetle. This is predicted since risk of interruption to mating should otherwise favour males who deliver their ejaculate as fast as possible. There is little evidence that *Prostephanus truncatus* males continue to court females after intromission begins. In fact both male and female are remarkably still during this phase and the pair part company directly after this. Continued courtship after intromission has begun can indicate that males can benefit from continued stimulation of the female presumably by influencing variables of cryptic female choice (Eberhard, 1996). Therefore cryptic female choice is not suggested by the observable copulation behaviour of this species.

Two measures of readiness to mate, time before mating, and number of encounters before mating, were found to be variable and characteristic of which individuals were involved in the mating trial. Number of encounters before mating is perhaps a better measure of readiness to mate than time before mating, since time before mating includes periods when beetles are relatively inactive which can unduly influence the data. Female identity was a much greater influence than male identity on readiness to mate. The effect of variation in female behaviour has not been investigated further in this study as differences between males are the focus here, but female variation in stubborness to mate (level of choice) would be an interesting line of investigation.

Different females were found to mate preferentially with the same males when given a choice of two different males. Neither male variation in aggregation pheromone signal nor fresh weight were found to be clear determinants of the non-random pattern of mating observed in LGB. Manipulation of the pheromone signal confirmed that this signal does not greatly influence mate choice during contact courtship. Pheromone manipulations also did not influence pushing behaviour between males. However the sensitive nature of the experimental design did pick up a light trend for males treated with Female Factor to increase the behavioural measure most correlated with mating success (male approach, no push). Therefore, although the aggregation pheromone signal is not used by females to distinguish between mates at close quarters, the female produced pheromone, Female Factor, which is already known to alter male aggregation pheromone emission, may also induce behavioural changes in males.

Male size is correlated with pushing behaviour between males, with the larger male of a pair being more likely to push the smaller male than vice-versa. Such behaviour is similar to that reported for *Monochamus scutellatus* (Cerambycidae) (Hughes, 1981), where larger males win more often, but males do not apparently actually damage each other. Direct male-male conflict is reviewed for Scolytidae in Kirkendall (1983). Pushing behaviour such as that described here for LGB is also a feature of Scolytid behaviour.

Pushing between male Scolytids has been observed on the bark surface. Generally it is noted that once a male is within a gallery he cannot be forced out (Oester and Rudinsky, 1975, quoted in Kirkendall, 1983). Size differences were not found to influence the largely age dependent aggressive interactions of the cockroach *Nauphoeta cinerea* (Moore *et al.*, 1997).

These results indicate that all mate acquisition benefits from pheromone signalling occur from manipulating female distribution. It has already been shown that pheromone perception of distributing beetles in the field has a high resolution, and beetles can distinguish between two male signals placed 0.6m apart. Therefore aggregation signalling is likely to be a major determinant of mating success when males occupy distinct tunnel systems. However the pheromone signal does not apparently influence mate choice between two or more males occupying the same system. This means that males employing a satellite strategy of following other male's signals may successfully mate with any females they intercept even if they are not signalling (which is likely if they are not feeding). However, once females enter a signalling male's tunnel system she becomes relatively easy to guard, which could limit the risk of lost paternity to satellite males.

If LGB has only recently expanded its host range to include stored products and is indeed a relatively poorly adapted storage pest (Hodges pers. comm.) then LGB tunnel systems may be poorly adapted to the limited space of a maize grain. The long term association between pairs of LGB observed in artificial hosts may break down in maize since first, there is a greater risk that tunnels will break through to the surface of the grain, (although there is evidence that LGB can detect the thickness of the medium it is tunnelling and may stop in time) and second, other beetles can break into the tunnel system from all directions, which is not possible for a much larger host.

Mate guarding in LGB appears to be flexible and dependent on the location of mating. Couples apparently only remain together for any appreciable length of time when mating occurs in the context of tunnel systems in a plant host. It may only be feasible for males to attempt to monopolise a female when she is inside a tunnel that limits her movements, and is easy to defend. LGB behaviour within tunnel systems is startlingly similar to that described for some Scolytid species. Kirkendall notes of Scolytids that, '..males that stay during gallery construction remain blocking the entrance hole, occasionally leaving their post to copulate, feed, or remove accumulations of frass..' (Kirkendall, 1983).

Male LGB guard females by the fourth method described by Alcock (1994); 'monitor a mate without physically grasping her following completed copulation,' and possibly also by the second method; 'donate mating plugs after insemination,' since the spermatophore may act at least temporarily as a mating plug. Post copulatory guarding can

also be seen as pre-copulatory guarding since male LGB often mate repeatedly with the same female. Male LGB presumably incur costs of staying with the female from lost time searching either physically or through pheromone signalling (since the signal is shut down in the presence of a female). The original starting hypothesis for this thesis proposed that males shut down the pheromone signal to limit male-male competition. It is, however, possible that on an evolutionary time scale, females could potentially force males to continue signalling by preferentially mating with signalling males. This idea is similar to that discussed in Andersson (1994) of, 'female incitation of male competition'. In the elephant seal, *Mirounga angustirostris* , females protest loudly against being mounted especially if the male is young, leading to attraction of competing males (Trivers, 1972 quoted in Andersson, 1994). Therefore the question, 'should males shut down the pheromone signal in the presence of females?', can be viewed from both a male and female perspective and as such, the answer will represent the outcome of potential conflicts and concordance of interests of males and females. I have summarised the main pros and cons of continued signalling below:

Possible selection pressures acting on MALES to continue signalling in the presence of a female

- Increase the number of potential mates.

Possible selection pressures acting on FEMALES to force male to continue signalling whilst they are together

- Promote male-male competition from the arrival of males responding to the pheromone signal.

Possible selection pressures acting on MALES to cease signalling in the presence of a female

- Decrease the potential for male-male competition from other males following the pheromone signal.

- Decrease the level of predation of possibly adults, but more likely young offspring, from predators that follow the aggregation pheromone signal to locate their prey (pheromone acting as a kairomone).

- Energetic/nutritional gains if signalling is costly in these respects.

Possible selection pressures acting on FEMALES to either favour males who shut down their signal or at least be unprejudiced towards non-signalling mates.

- Decrease competition for oviposition site from females following the pheromone signal.

- Decrease the level of predation from predators following the aggregation pheromone signal.

Why, then, do females not continue to use the pheromone signal to choose between mates? Perhaps the answer is that natural selection has favoured males that shut down their signal in the presence of ovipositing females in order to limit predation. If this was an important selection pressure then females who continued to remain with such males would also be favoured and mate selection during courtship on the basis of pheromone signal would not be promoted. Blocking the entrance to a gallery system may not only limit conspecific access, it may preclude access to parasites and other predators. Some evidence that male presence increases numbers of live offspring produced through decreased predation in the Scolytidae is cited in Kirkendall (1983).

The alternative explanation is that males cease signalling to limit attraction of other males; this assumes that females, who could benefit from increasing the level of male-male competition, cannot reverse this trend by continuing to select for signalling males. This scenario is further complicated by the fact that as long as males signal, extra females are also attracted. Depending on the size of the food resource, competition for oviposition sites may also contribute to the benefits incurred from cessation of signalling. Alternatively, it may just be that pheromone signals are difficult to assess at very short range. Volatile chemical signals may merge with each other and may not be a reliable source of information. However, Moore's studies of cockroaches would tend to indicate that this need not be the case (Moore 1998).

Surprisingly, previous mating experience (in terms of number of matings already performed up to six possible matings) was not found to be correlated with readiness to mate (experiment 6.4.4). Thus no reduction in willingness to mate of either males or females was demonstrated for the average recovery time of 12 hours used in this study. Although transfer of sperm was not directly confirmed in these trials, later observations have shown that phase 4 of courtship is a reliable indicator of sperm transfer. Observations of beetles tunnelling in the plant host confirmed that LGB adults mate repeatedly (commonly once or twice each 12 hour light period). These observations are more reliable indicators of possible mating rates in the wild since beetles have the opportunity for other behaviours such as feeding and oviposition.

LGB females were found to mate repeatedly with the same partner. Petrie (1992) discussed multiple mating of the same partner in birds and proposed three possible

adaptive explanations: 1) to ensure fertilization (not much evidence for this in birds); 2) to increase paternity assurance for the male such that the female might gain from consequently increased parental care; 3) to decrease the probability that the male will mate with other females and therefore the female will keep good territory associated with the male. Dewsbury (1982) cites costly ejaculates as a possible reason for multiple mating with the same mate, "...because of the stimulus requirements for pregnancy initiation, sperm competition, female choice and control, and the costs and risks of searching, the males of many species may be selected to copulate repeatedly with a single female". Dewsbury proposes that the balance in this optimality theory depends on the operational sex ratio. Multiple mating with the same partner may represent a form of male parental investment. Fox (1993) suggested that benefits incurred by female *Callosobruchus maculatus* (Coleoptera) are largely through nutritional benefits from the large ejaculate delivered by the male (but see some limitations discussed in Fox *et al.*, (1995)). Multiple mating thus suggests that sperm competition, cryptic female choice and/or male paternal investment are possibly important determinants of variation in male reproductive success in *Prostephanus truncatus*. The next chapter investigates some of these phenomena.

CHAPTER 7: SPERM COMPETITION

7.1 INTRODUCTION

The influence of pheromone-directed dispersal and courtship behaviour on mate selection in *Prostephanus truncatus* has already been considered in chapters 4-6. This chapter touches on the next step that determines relative male reproductive success: processes that occur between the onset of copulation and the fertilization of eggs. *Prostephanus truncatus* is likely to be a good subject for the study of sexual selection within the female tract since it has already been shown that both males and females will mate several times. Also, males and females are likely to encounter many possible mates during their relatively long adult lifetime, especially given the aggregation behaviour of this insect. It has long been recognised that both inter- and intra-sexual selection are possible within the female tract in the form of sperm competition and cryptic female choice (Parker 1970). Sperm competition is defined by Parker (1970) as, "the competition within a single female between the sperm from two or more males for the fertilization of the ova". Cryptic female choice is a term used to describe, "female processes that affect male reproductive success and occur after the male has succeeded in coupling his genitalia with those of the female", (quote from Eberhard, 1996). Thus the race for fertilization is a battle between male ejaculates whose outcome can be influenced by preferential treatment of some ejaculates relative to others by the female.

Reproductive morphology/chemistry can tell us an enormous amount about the likely determinants of fertilization success between males in polyandrous species like LGB. Reproductive structures are notoriously diverse, complex, and often surprisingly extreme in form. Extreme forms are likely to have resulted from sexual selection (Andersson, 1994) and can therefore indicate fruitful areas for further study. For these reasons the reproductive organs of male and female LGB were dissected, described and discussed in this chapter. Scholz (1997) has since given a basic description of the reproductive organs of *Prostephanus truncatus,* I will therefore concentrate on reporting details of those parts of the reproductive tract which could influence/have been influenced by sexual selection.

The proportion of offspring sired by the second male to mate in a double mated female (P_2 value) is a useful parameter often measured in studies of sperm competition. It is unusual for the first male to mate to secure 100% of paternity if the female remates (Ridley, 1989). Last-male sperm precedence is common in insects. The extent to which males can secure paternity of offspring from multiply mated females is interesting to the discussion of the potential costs incurred by male signallers who attract other male competitors. Paternity can be traced using genetic markers, or the so called 'sterile male technique' (Eady, 1991).

No genetic markers currently exist for LGB so the sterile male technique was attempted in this study. In this method one of a pair of males allowed to mate with a female is sterilised. Eggs laid are then scored as viable or non-viable. Careful control of mating order allows possible influences of differing fertilization success between sterile and non-sterile males to be eliminated from the calculation of P_2 (see Boorman and Parker, 1976, quoted in Eady, 1991).

One of the most striking features of patterns of sperm/ejaculate investment is that it is extremely variable, both between individuals (Lewis and Austad, 1994; Simmons and Parker, 1992); for the same individuals of different ages (Fox *et al.*, 1995); physiological condition (Simmons and Parker, 1992); and, facultatively, in response to social cues (Gage, 1995; He and Miyata, 1997). Previous work has found that males sometimes alter the size of their ejaculates in concordance with predictions derived from sexual-selection theory (Gage, 1991 in a fruit fly). For instance, male Mediterranean fruit flies were found to increase their sperm number per ejaculate by almost three fold in response to increased levels of perceived male competition (presence of another male) (Gage, 1991). Siva-Jothy and Tsubaki (1989, cited in Eberhard 1996), found that male damselflies of *Mnais pruinosa* delivered less sperm per ejaculate when they were territorial compared to when they were non-territorial. Female damselflies tend to oviposit directly after mating with territorial males (thought to be before sperm mixing becomes important) and territorial males often have other possible females to mate soon afterwards, which could limit the number of sperm they have available. Therefore there are two possible differences in the selection pressures on the size of ejaculate in territorial *vs.* non territorial male damselflies that could explain this observed difference in ejaculate size.

Such short term facultative adjustment of ejaculates might allow us to determine why ejaculates are the size they are, and what cues males are responding to when altering their ejaculate size. This chapter investigates whether LGB males alter the sperm number in their ejaculates in response to male crowding such as that found by Gage (1995), and also in response to Female Factor (see chapter 3 for a description of Female Factor). It has already been shown that LGB males use Female Factor as a cue to decrease their level of pheromone signalling. Thus, LGB males already use Female Factor as a source of information about their current sociosexual environment and adjust their pheromone output accordingly, so it is perfectly feasible that this might be a cue that triggers facultative adjustment of sperm investment.

It was initially envisaged that males might invest more in ejaculate production when exposed to Female Factor as it has been proposed that amount of Female Factor could be used as a measure of local population density (Fadamiro, 1995). Total pheromone shut down takes approximately six days (chapter 3) and initially, males were not observed to stay with females after mating (in open arenas, chapter 6). Exposure to Female Factor was

therefore likely to be determined by the local population density and not so much by the chance association of males with a single female. Observations made using time-lapse recordings of LGB behaviour in tunnels within the plant host, however, show that males do in fact co-habit in tunnels with the same female for extended lengths of time and so a single female could trigger pheromone shut down in a male. Apart from increasing our understanding of the mating system in LGB, being able to trigger adjustment in sperm investment using a chemical cue could be a useful tool for more general studies of sperm competition and cryptic female choice.

Ejaculate and sperm investment are broad terms that encompass a number of variables. This study measured sperm number per ejaculate and approximate ejaculate size and weight since these measures are relatively easy to obtain and have already been shown to be variable. Ejaculates usually contain many other substances as well as sperm (see chapter 6 on male sexual products in Eberhard, 1996), which may alter in proportion between ejaculates. Another measure of ejaculate investment is speed to mating, since males may continue to deliver full ejaculates, but may mate at a lower rate when overall investment in ejaculates is lower. Females are often assumed to be limited in the number of eggs they can produce. This is suggested for LGB since eggs are resorbed by females in times of food shortage (Scholz, 1997). Contrary to early ideas, males have also often been demonstrated to be limited in their gamete production. Each sperm cell may indeed usually be relatively cost free, but ejaculates as a whole, which often contain thousands of sperm and other materials, may be costly to produce (Fox *et al.*, 1995 in a beetle; and see references cited in Dewsbury, 1982). Differences in ejaculate investment may only become apparent if males are given the opportunity to mate twice or more in fairly quick succession. Comparing first and second ejaculate sizes and readiness to mate, can also show whether males are at all limited in ejaculate constituents and how they partition investment (full ejaculates less often *vs.* smaller ejaculates at the same rate).

To summarize, the main questions are: do the first males to mate females risk losing paternity if females remate?; and do males adjust their investment in sperm in response to such a threat? It has already been shown in chapter 6 that females do not use the aggregation pheromone signal in inter-sexual selection for mates during courtship, so males who cease signalling should not decrease their reproductive success at this point. Whether female *Prostephanus truncatus* show cryptic female choice that is influenced by the pheromone signal is unknown and still a possibility.

7.2 AIMS

- Make a preliminary assessment of the potential for inter and intra-sexual selection at the level of the gamete in *Prostephanus truncatus* from dissection and description of reproductive organs.

- Describe the pattern of sperm precedence in doubly mated females.
- Determine if males manipulate their investment in ejaculates in response to the presence of other males or the female produced pheromone, Female Factor.

7.3 METHODS

7.3.1 Dissection of reproductive organs

Reproductive organs of male and female *Prostephanus truncatus* were dissected out under a stereo microscope (Nikon model SMZ-1) using fine forceps. Specimens were mostly dissected floating free in a drop of insect buffer as specimens were generally too small to pin into wax.

7.3.2 Sterilization of males for calculation of P_2

Source of insects

All insects used were removed from 4-5 week old cultures as cased pupae. Virgin females were obtained by isolating pupae before hatching.

Apparatus

A 5 mv linear accelerator (X-ray machine; Medical Physics department, Leicester Royal Infirmary) was used to administer controlled doses of radiation. Pupae were housed in a 5 x 5 divided petri dish, with one pupa per division. Perspex blocks provided the appropriate build up and scatter of the incident radiation.

Experimental design

In the course of two separate sets of trials, cased pupae were exposed to a range of radiation doses: 120GY, 60GY, 32GY, 30GY, 15GY, 8GY, 2GY and 0GY controls. 25 pupae were exposed to each dose (approximately half males and half females). All surviving males hatched from these pupae were allowed to mate with virgin females on split grain. Grain was subsequently dissected and all offspring produced were recorded.

7.3.3 Determination of sperm number per ejaculate for two consecutive matings of males kept in different sociosexual environments.

Preliminary observations showed that male LGB are capable of producing two spermatophores within five hours. A time of five hours between double matings was chosen since this allowed one batch of insects to be tested in a nine hour working day.

All dissections were initially carried out in an insect buffer solution as described in Gage and Cook (1994). Buffered solutions reduce damage to sperm structure during dissection and dispersion. However, using buffered solutions incurs the disadvantage that sperm can sometimes still be obscured by salt crystals even after washing. Distilled water was therefore used to disperse ejaculates in some experiments.

LGB sperm can become damaged during this process (axoneme unravels), but a very clear sample is obtained. Possible overestimation of sperm number by counting the two strands of the tail as separate sperm was prevented by limiting sperm counts to those with a head.

Source of insects

All insects used in trials were recently hatched adults (2-5 weeks old). Tunnelled kernels with no adults were taken from 30 day old culture jars. These were then placed in a fresh culture jar and left to incubate for two weeks. Each wave of insects used was sieved from such a culture, sexed and placed into treatment pots. Only fully sclerotised, active insects were used (likely to be at least five days old). Therefore insects used were unlikely to be virgin, but were mostly of a similar age.

Apparatus

The mating arena consisted of a petri dish base lined with paper towel with a clear plastic pot placed upside down on the towel. Insects were introduced under the plastic pot. This provided a circular walking arena of diameter 3cm. This is a much smaller arena than that used for the mating trials conducted in chapter 6 (diameter 9.5cm). It was hoped that this would increase the encounter rate between the male and female and subsequently decrease the time before mating, yet still allowing courtship behaviour to proceed.

Sperm-count protocol

Ejaculates were dissected out of recently mated females. Intact spermatophores were often expelled from the female during dissection. If not then they were dissected straight out of the *bursa copulatrix*. The *bursa* was always checked for sperm that might have been expelled from the spermatophore. When the spermatophore burst or no spermatophore was found, all clumps of sperm were retrieved.

All dissections and dispersions were carried out in distilled water. Debris was cleared away from the sperm sample leaving it in a clear drop of water. The sperm were then dispersed in this drop by teasing apart clumps of sperm using fine forceps and then gently mixing the sperm into the drop. When no large clumps of sperm remained, the ejaculate was then made up to 15ml with distilled water by using this water to wash the sample into a 25ml beaker. The sample was then mixed for four minutes using a blunt metal rod, in the beaker. Four 20µl samples of this mixture were then placed as drops on a glass slide using a 20µl micropipette (Gilson). The mixture was stirred directly before each sample was taken and each sample was taken from a point half way up the sample and half way from the center to the outside of the beaker to limit any influence of sperm settling through the sample.

Drops were left to dry under a dust cover. Sperm were examined using a compound microscope set to dark phase contrast under a magnification of x8x10 (Olympus

BH-2). The numbers of sperm per 20µl drop were counted by systematically scanning down each drop. An estimation of the total number of sperm per ejaculate was then calculated by multiplying up the average value per sample x 750 (15/0.02ml). By taking more than one sample from the diluted ejaculate an estimation of the sampling error was made. All implements were rinsed after use and new pipette tips were used for each ejaculate sample.

Experimental design

All sexed females were placed in individual glass pots containing fresh ground maize meal. All males were randomly allocated to one of three treatments:

- **Single, fresh**: Single male in pot containing fresh ground maize meal.
- **Single, conditioned**: Single male in pot containing one part fresh ground maize meal and two parts flour taken from a 6 week old culture.
- **Crowded, fresh**: X10 beetles per pot containing $5cm^3$ of fresh ground maize meal.

Insects were kept in treatment pots for 7-10 days before being used in trials.

Four cohorts of insects were used in successive trials. For each replicate a male was given the chance to mate with a female. As soon as they separated after mating the female was removed, the ejaculate was dissected out and the number of sperm estimated. If no mating occurred within 25 minutes the trial was abandoned. Males that mated were then placed back on food and left for five hours. After this time they were given the opportunity to mate with another female. Again, if they mated within 25 minutes, the female was dissected and the sperm number per ejaculate was estimated. The sizes of all intact spermatophores were measured under a compound microscope. Generally six males were tested each day, two from each treatment. The order of trials was changed each day to eliminate time of day effects from the treatments. A total of 62 such trials across the three treatments were performed. For each trial the following variables were measured:

- Time taken to mate (or if mating occured)
- Spermatophore length and width at the widest point (if spermatophore still intact)
- Sperm number delivered in the ejaculate
- Male weight one day after the trial (In the last wave of trials male and female weight before and after each mating were measured instead)

Additional controls:

In successive mating trials, all first matings were performed in the morning and all second matings were performed in the afternoon. 21 additional trials were therefore set up where first matings were performed in the afternoon to check for time-of-day effects. Equal numbers of males used in these trials were taken from each treatment category.

To estimate the number of residual sperm, five females selected randomly from the bank of those being used in trials were dissected for sperm without being allowed to mate with a male. The spermatheca of these females were purposely opened up to obtain an upper estimate of the number of sperm that could contaminate ejaculate samples.

7.4 RESULTS

7.4.1 Dissection of reproductive organs

Female reproductive organs

Mature females of LGB possess one pair of ovaries. Each ovary has five ovarioles (only three are shown in fig 7.4.1a, for clarity). Ovarioles contain a string of up to about four eggs at various stages of development with the largest and most mature attached to the ovary base (see fig 7.4.1a). The oviducts are lined with muscle tissue (fig. 7.4.1b) and they open into a vagina enlarged to form a *bursa copulatrix.* Also leading off the *bursa copulatrix* is a duct leading to a spermatheca (sperm storage organ). The *bursa copulatrix* is continuous with the outer body wall and terminates as a yellow sac, possibly a store of fatty tissue and/or of secretory function at the point where eggs are squeezed out of the body.

The spermatheca consists of a spherical sperm reservoir (*receptaculum seminis*), a leaf shaped accessory gland and a blind-ending duct (see fig. 7.4.1c). The sperm reservoir is sclerotised and spherical in shape. Some contractions were observed around the blind-ending ducts after some dissections, however the exact structure of this muscle tissue was not discernible. Dissection of reproductively active females revealed the sperm reservoir to be full of active sperm, but no such sperm were found in the blind ending duct. In some dissections there appears to be a broken connection between the end of the blind ending duct and the sperm reservoir (see fig. 7.4.1c). The spermatheca is commonly surrounded by fat globules.

Male reproductive organs

Male *Prostephanus truncatus* have a pair of testes, each divided up into five follicles. The male reproductive system in LGB also contains four pairs of lobes of varying structure, all converging at the ejaculatory duct. These include the seminal vesicles and accessory glands (see Scholz, 1997). The sclerotised intromittent organ is continuous with the exoskeleton and can be manually everted by applying pressure to the abdomen. The

Fig. 7.4.1a: Diagram to show plan view of female reproductive tract including the orientation of the spermatophore (shaded in grey) immediately after copulation.

Fig. 7.4.1b: Photograph of LGB oviducts showing muscle tissue (light microscope low power).
O-ovary base, LO-lateral oviduct, B-bursa.

Fig. 7.4.1c: Photograph of LGB spermatheca (light microscope, low power).
SR-sperm reservoir, BED-blind-ending-duct, AG-accessory gland, B-bursa, F-fat globules.

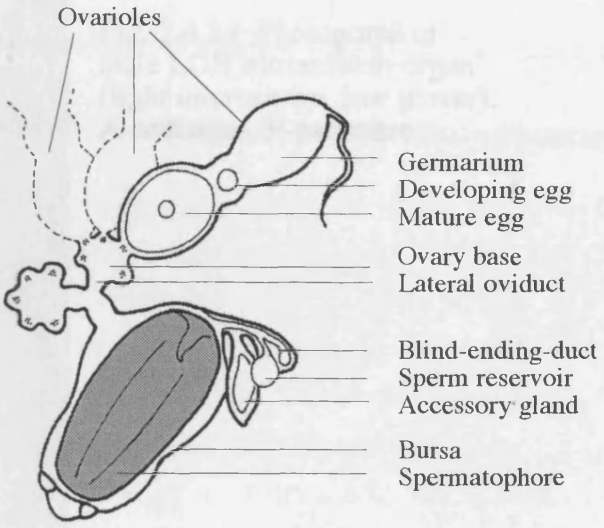


Fig. 7.4.1a.

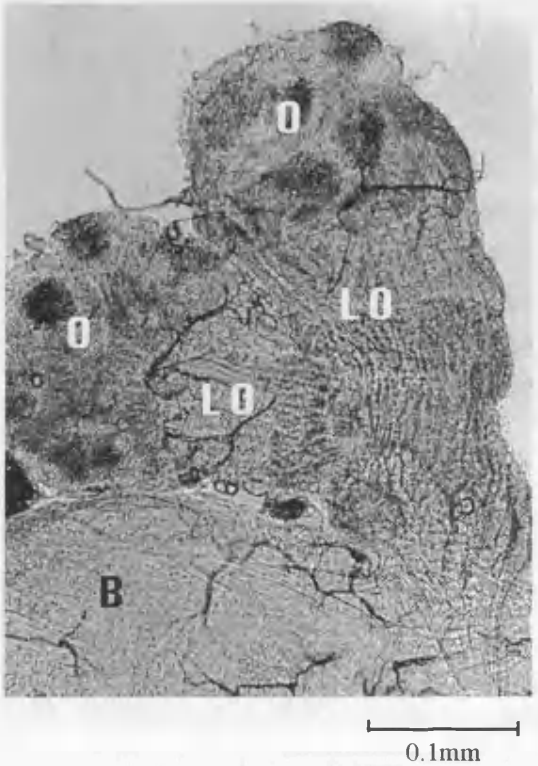


Fig. 7.4.1b.

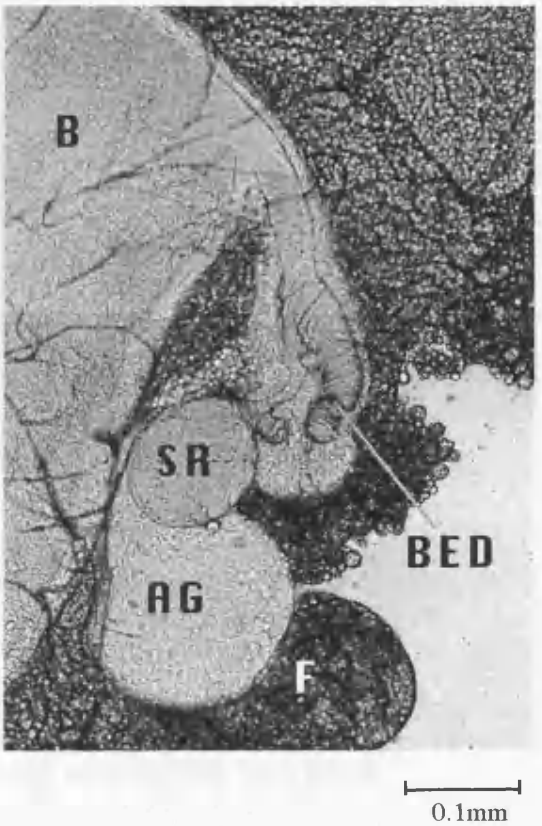


Fig. 7.4.1c.

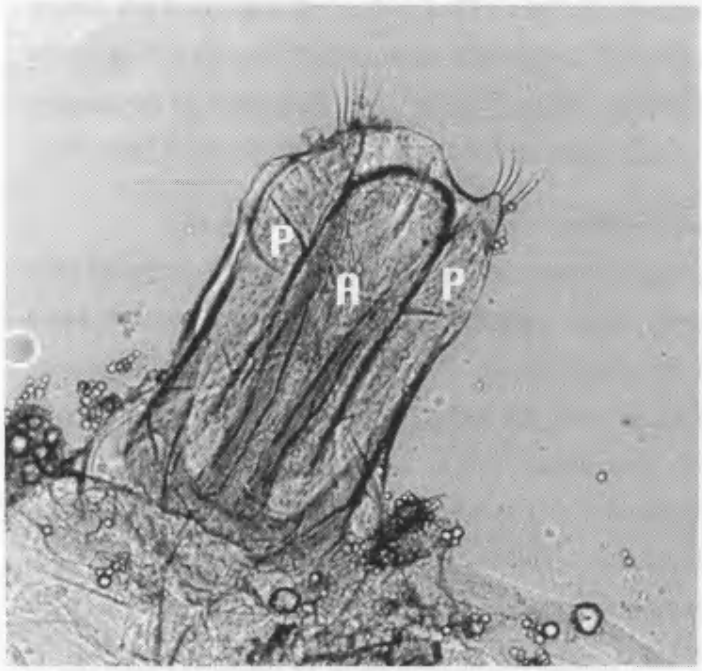
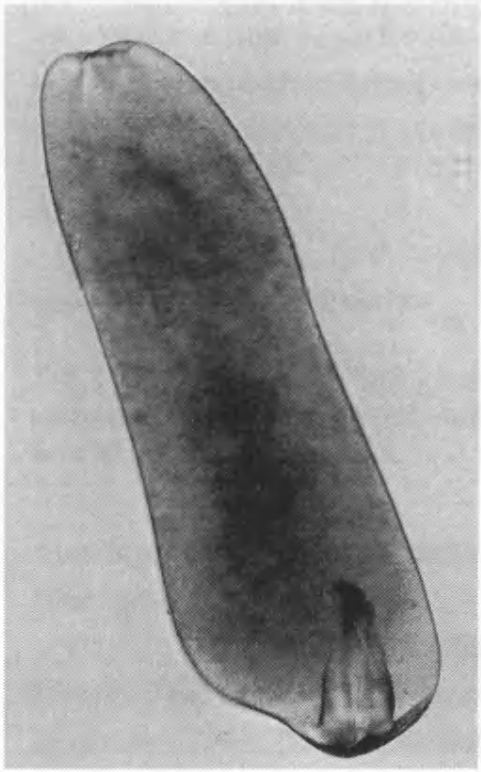


Fig. 7.4.1d: Photograph of male LGB intromittent organ (light microscope, low power). A-aedeagus, P-paramere.



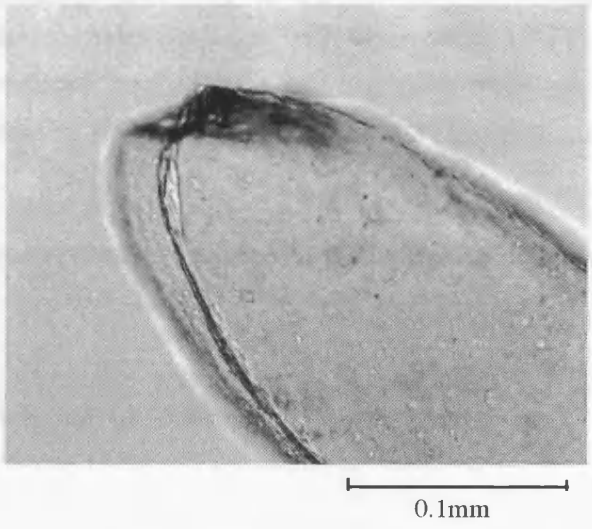


Fig.7.4.1e (left): Photograph of LGB spermatophore (light microscope, low power). NB: V-shaped invagination at its base.

Fig.7.4.1f (above): Close up photograph of the tip of the spermatophore (light microscope, medium power), showing fine tube inside.

three lobes splay out of a cylindrical sheath that surrounds them when they are contained within the body (see fig. 7.4.1d). The central lobe (*aedeagus*) is roughly cylindrical in shape with a central duct running through it. The two lateral lobes (*parameres*) are also cylindrical up until their ends where they are tapered and possess five hairs. The lateral lobes tend to be slightly longer than the central shaft.

Male LGB deliver sperm in the form of a spermatophore see fig.7.4.1e. This oval shaped package of sperm has a roughly triangular shaped infolding part, which can evert. A very long thin tube is also folded inside the spermatophore (see fig.7.4.1f). The spermatophore is delivered into the *bursa copulatrix* of the female during copulation. The spermatophore is orientated such that the inverted parts are opposite the duct leading to the spermatheca (see fig. 7.4.1a). It is difficult to determine if there is any matrix delivered in the ejaculate. On some occasions the sperm seemed to be contained within a matrix, however, dispersing the sperm causes this to disappear leading to the conclusion that a high density clump of sperm may have physical properties like a matrix.

7.4.2 Sterilization of males for calculation of P_2

No adults irradiated at the two highest doses (60GY and 120GY) survived beyond two weeks after the dose was administered. All other treatments had approximately 50% survivorship up until this time. No further trend in survivorship with dose was apparent from these trials. Viable offspring (those developing at least to the larval stage), were produced from virgin females mated to males irradiated with doses under 15GY. Only 1 dead egg (no viable offspring) was produced from all matings with males irradiated with 15GY or over. Dissection of a male irradiated with 15GY revealed healthy looking, active sperm in the testes.

Since survivorship was low and females mated with sterilized males did not lay eggs this work was abandoned.

7.4.3 Determination of sperm number per ejaculate for two consecutive matings of males kept in different sociosexual environments.

Average measures of male mating investment

Time before mating

The time taken for a male to mate was constrained by experimental design to be between 0 and 25 minutes. Ninety two out of 126 mating trials resulted in a mating before 25 minutes (73%). The range recorded was from 2-24 minutes and the median (calculated including individuals that did not mate within 25 minutes) was 8.5 minutes.

Weight changes in males and females

Both males and females lost weight in replicates where no mating occurred: males lost a mean of 0.033mg (SE = 0.0051, N=12); females lost a mean of 0.039mg (SE = 0.0075, N=12). In trials where copulation occured males tended to lose more weight relative to their unmated counterparts and females tended to gain weight relative to their unmated counterparts.

Males that mated in their first trial gained a mean of 0.044mg (SE = 0.004mg, N=15) during the five hour period between mating trials.

Spermatophore size

Of 90 matings, 54 intact spermatophores were recovered, 19 burst spermatophore were found and in 17 cases no spermatophore was found. In two cases mistakes were made in the dissection and ejaculate data from these replicates have been excluded from all further analysis. In no cases were more than one spermatophore per mating recovered. Of the 54 intact spermatophores the mean width was 0.23mm (SD ± 0.064mm) and the mean length was 0.59mm (SD ± 0.173mm).

Sperm number per ejaculate

From all ejaculates sampled the mean estimate of sperm number per ejaculate was 35600 (SD=29700, N=90). Sperm number was not found to be significantly correlated to spermatophore size. The mean standard error of sperm number, incurred from taking sub-samples from the whole was 15.8% (N=79) (each error expressed as a percentage of its mean).

Comparison of male mating investment between 1st and 2nd matings: Are males sperm limited?

Proportion mated

A larger proportion of mating trials resulted in a mating for second mated males in comparison to first-mating males (76.2% compared to 71.4%). A direct comparison of these two proportions is complicated by the fact that all those males taking part in the second mating trials have already proved capable and ready to mate since they have showed a 100% rate of mating in the first trials. Therefore the information lacking to interpret these data is whether the mating performance in the first trial is a consistent feature of individuals ie. non-maters will never mate and maters will always mate. If this was true then beetles became less likely to mate in a trial if they have recently been mated (76.2% success compared to 100% success), however, if there is no consistency for each individual between trials then beetles became more likely to mate during a second mating trial (76.2% success compared to 71.4% success).

Time before mating

A frequency histogram of time before mating for first and second matings pooled for all treatments is shown in fig. 7.4.3g. Time before mating was numerically

lower for first matings compared to second matings, but this difference is not significant in this sample: median for first mating 7.5 minutes (N=84); median for second mating 9.5 minutes (N=42); Wilcoxon signed-ranks test for replicates where the male mated twice, z=1.07, N=32, p=>0.1.

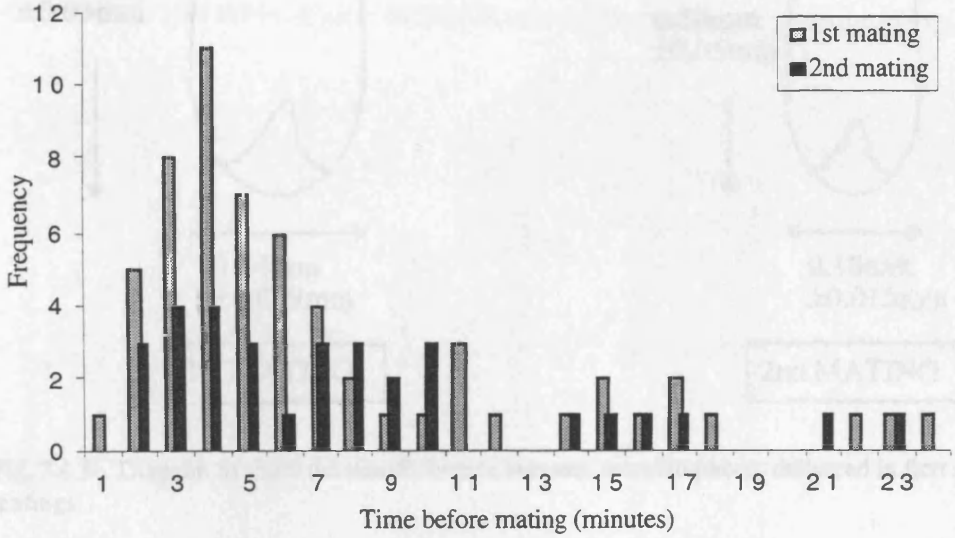


Fig. 7.4.3g: Distribution of time elapsed before mating for first and second matings. First matings, N=60; second matings, N=32. Wilcoxon signed-ranks test for replicates where the male mated twice, z=1.07, N=32, p=>0.1.

Weight changes in males and females

In 1st mating trials where the pair copulated, males lost a mean of 0.052mg more than their unmated counterparts and females gained 0.052mg more than their unmated counterparts (N=15). In 2nd mating trials where the pair copulated, males lost a mean of 0.019mg and females gained a mean of 0.028mg more than their unmated counterparts (N=11). A two tailed t-test comparing male weight loss for first mated males and second mated males found that males lost significantly less weight during second matings (t=-2.89, df=23.5, p<0.01). Likewise, females gained significantly less weight (in fact there was a net loss of weight) when mated with males who had been mated previously with another female compared to when mated with first mating males (t=-3.26, d.f.=23.5, p<0.01).

Spermatophore size

Spermatophores produced during the 2nd matings were significantly smaller than those produced during the 1st matings: t-test on widths of 1st *vs.* 2nd matings p=0.0012 (2 tailed), t-test on lengths of 1st *vs.* 2nd matings p=0.025 (2 tailed) (see fig. 7.4.3h).

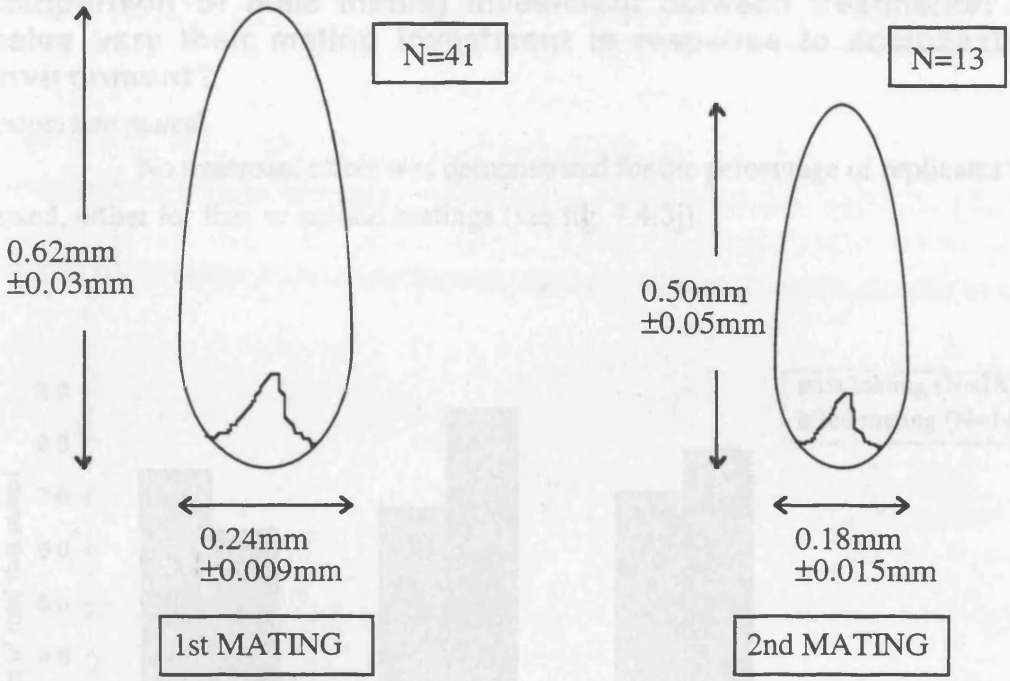


Fig. 7.4.3h. Diagram to show the size difference between spermatophores delivered in first and second matings.

Sperm number per ejaculate

Considering only replicates where males mated twice, the mean number of sperm delivered in second ejaculates is less than half that delivered in first ejaculates (see fig. 7.4.3i). Significantly more sperm per ejaculate are delivered in the first mating compared with the second (Wilcoxon signed-ranks test: $z=-3.693$, $N=32$, $p<0.001$).

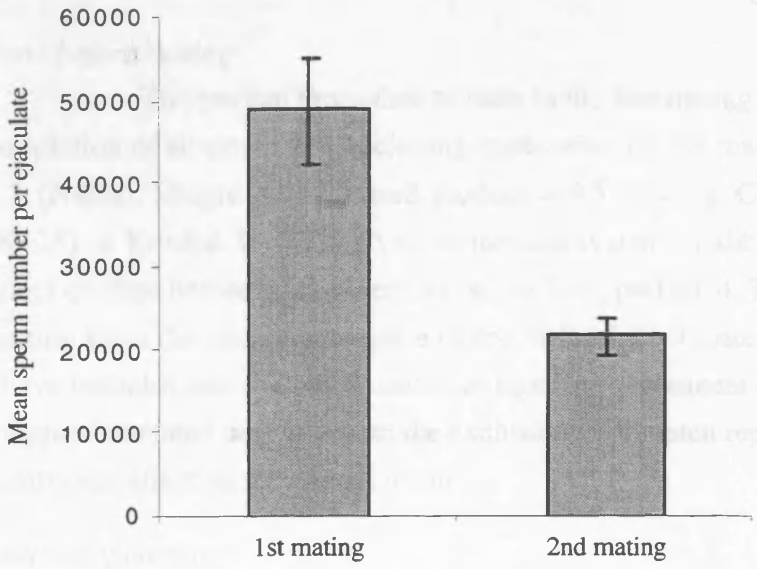


Fig. 7.4.3i: Mean sperm number per ejaculate for first and second matings (only replicates where males mated twice). N=32 for each column.

Comparison of male mating investment between treatments: Do males vary their mating investment in response to sociosexual environment?

Proportion mated

No treatment effect was demonstrated for the percentage of replicates that mated, either for first or second matings (see fig. 7.4.3j).

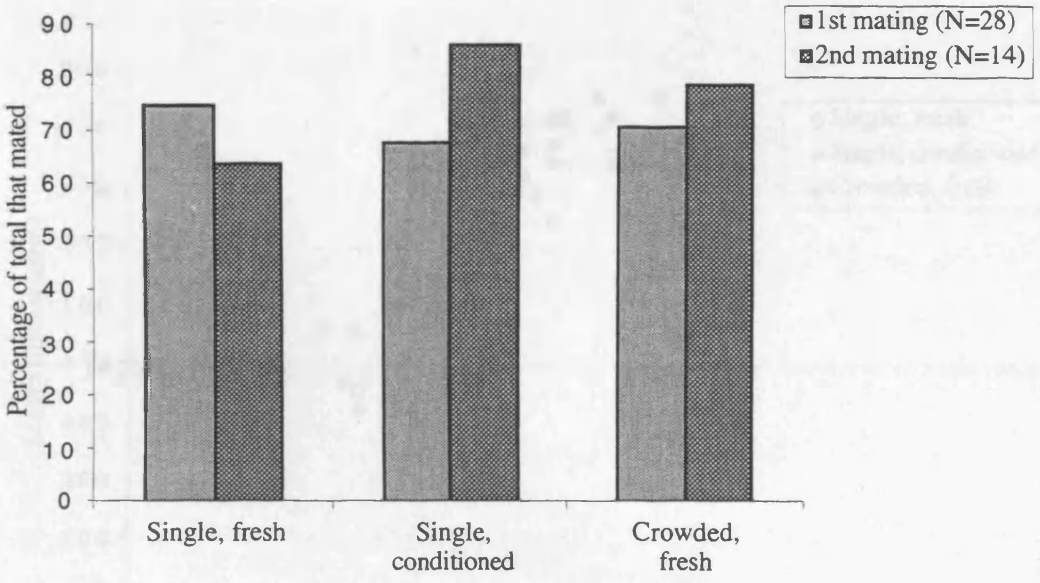


Fig. 7.4.3j: Bar chart to show the percentage of replicates that mated for all three treatments and for first and second matings. N=28 for 1st matings and N=14 for 2nd matings for each treatment.

Time before mating

The median time taken to mate in the first mating (when considering the total population of all treatments including males who did not mate) is: **Single fresh** median = 6.5 (N=28), **Single conditioned** median = 9.5 (N=28), **Crowded fresh** median = 6.5 (N=28). A Kruskal-Wallis analysis on the data available indicates that there is a treatment effect on time before mating: test statistic = 7.43, p=0.0024. This must be interpreted with caution since the data is incomplete (some males did not mate within the observation time). I have included this analysis because no significant treatment effect on proportion of the sample that mated was found, so the exclusion of unmated replicates may not have a significant effect on the overall result.

Spermatophore size

Only 13 intact spermatophores were recovered from second matings across all treatments so these were not used for analysis of treatment effect. The size and shape of spermatophores produced by males from each of the treatments is shown in fig. 7.4.3k. No

treatment effect on shape is apparent. A size index (width x width x length), was used to compare overall spermatophore sizes between treatments. Considering data from first matings only, **Crowded fresh** males produced the largest spermatophores (mean size index = 0.047±SE 0.008, N=15), and **Single fresh** males produced the smallest spermatophores (mean size index = 0.036±SE 0.006, N=16). The differences between spermatophore sizes for each treatment for 1st matings in this sample are not significant: single factor ANOVA in Excel 5, F=0.8, p=0.47.

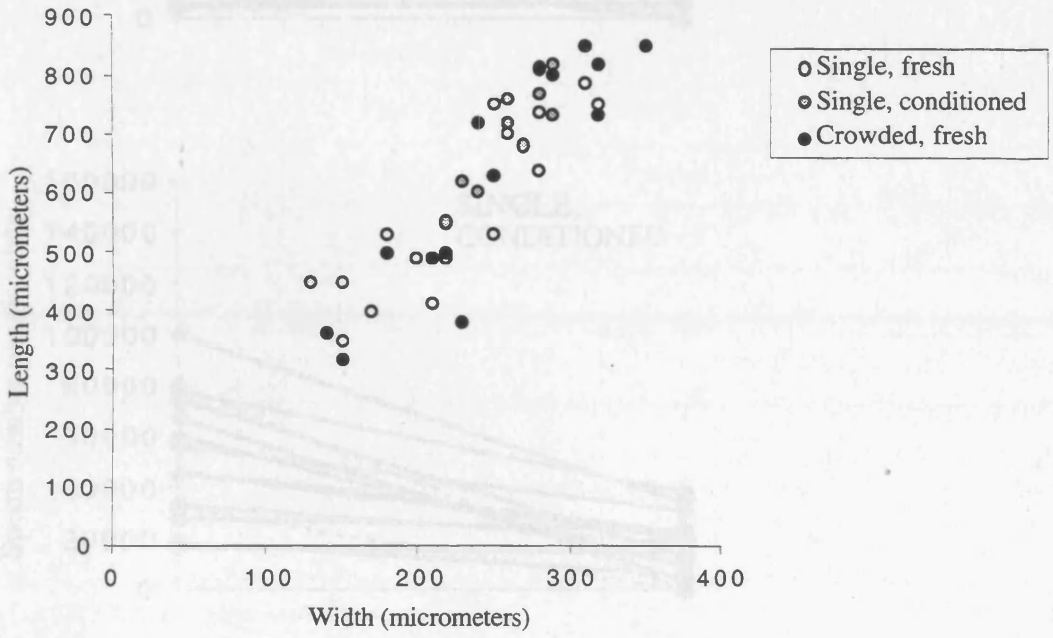


Fig. 7.4.3k: Scatter graph to show spermatophore length *vs.* width for first matings only (points separated by male treatment). Single, fresh, N=16; single conditioned, N=10; crowded, fresh, N=15.

Sperm number per ejaculate

No treatment effect has been demonstrated for sperm number per ejaculate. The raw data are shown graphically in fig.7.4.3l. There is a tendency for males kept in isolation on clean maize (single, fresh) to deliver relatively small first ejaculates. Sperm number in the first ejaculate is not a clear predictor of sperm number in the second ejaculate.

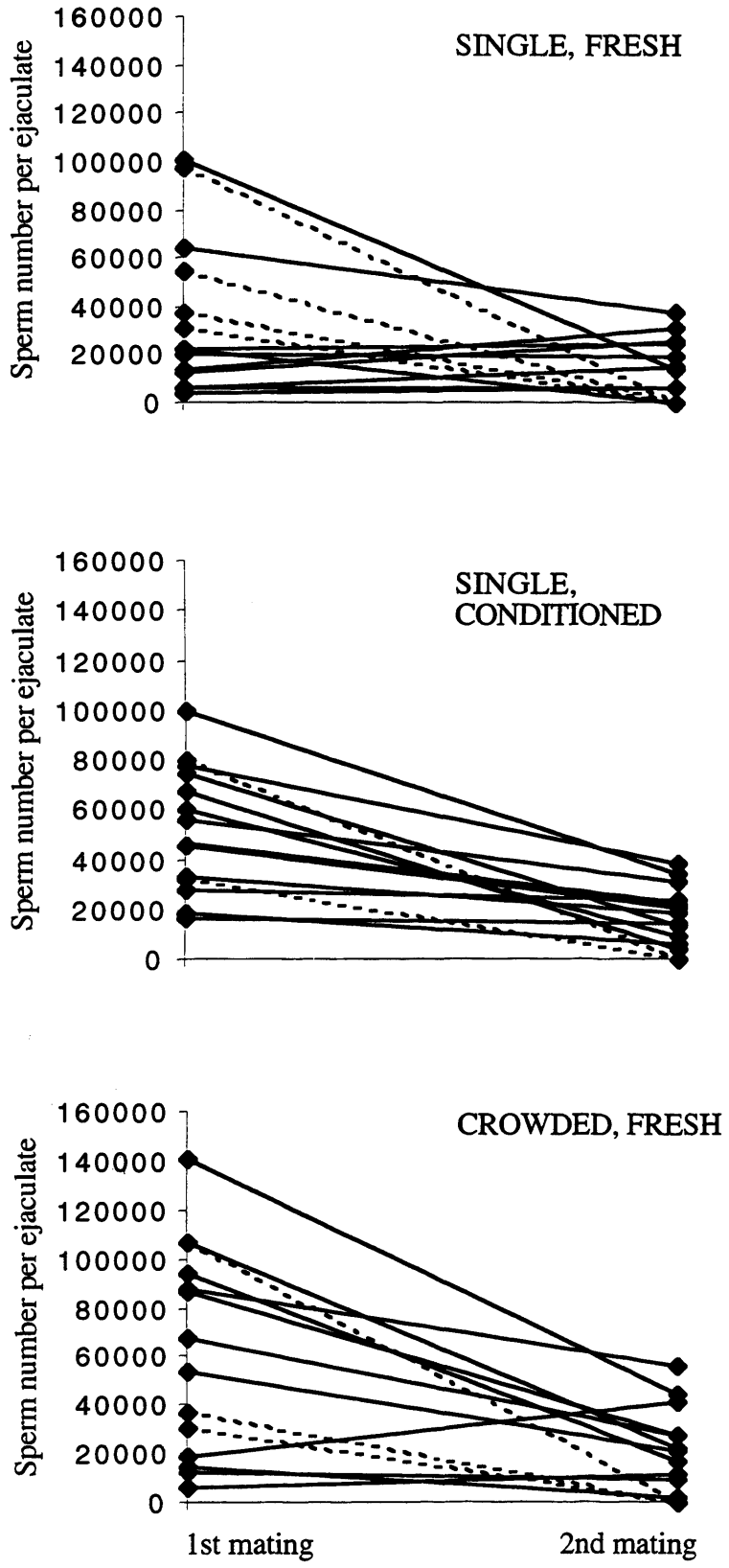


Fig. 7.4.3l: Three charts to show sperm number estimates for ejaculates of first and second matings of males in three treatments: single, fresh; single, conditioned; crowded, fresh. Dotted lines indicate replicates where the male did not mate with the second female within the time given.

Means and standard deviations of sperm number across treatments and associated statistical analysis are given below in table 7.4.3m. The distribution of sperm number per ejaculate for first matings was transformed by square-rooting all values to make the data approximate to a normal distribution. A one way ANOVA (Excel 5) of the transformed data showed that there was no significant difference between the treatments: $F=1.46$, $p=0.24$).

Table 7.4.3m: The mean number of sperm per ejaculate delivered by males exposed to three different sociosexual environments on their **first** mating.

	Mean sperm number per ejaculate	SD	N
Single, fresh	32400	27800	21
Single, conditioned	47600	28600	17
Crowded, fresh	51000	40900	20

The mean number of sperm per ejaculate delivered during second matings for the three treatments are shown in table 7.4.3n. No treatment difference for second matings is suggested by the data. No statistical analysis for a difference was conducted to confirm this because of the low sample sizes.

Table 7.4.3n: The mean number of sperm per ejaculate delivered by males exposed to three different sociosexual environments on their **second** mating.

	Mean sperm number per ejaculate	N
Single, fresh	18000	8
Single, conditioned	13500	8
Crowded, fresh	15900	5

Controls

Time of day control

Males allowed to mate for the first time in the afternoon gave a mean sperm number per ejaculate that fell between the values obtained for first mating males in the morning and second mating males in the afternoon (see fig. 7.4.3o). The data were normalised by square rooting all values. Comparisons between categories were tested for significance using a t-test (Excel 5), and no significant differences were demonstrated (1st mating a.m. *vs.* 1st mating p.m., $p=0.14$; 2nd mating p.m. *vs.* 1st mating p.m., $p=0.30$). The standard deviation bars in fig. 7.4.3o illustrate that the 1st mating (pm) sample has a

standard deviation more comparable to that of the 1st mating (am) sample than the 2nd mating (pm) sample.

Residual sperm number per female

The range of sperm number estimates for five females not used in mating trials was 250 - 3500 sperm, mean = 1775 sperm (see fig. 7.4.3o).

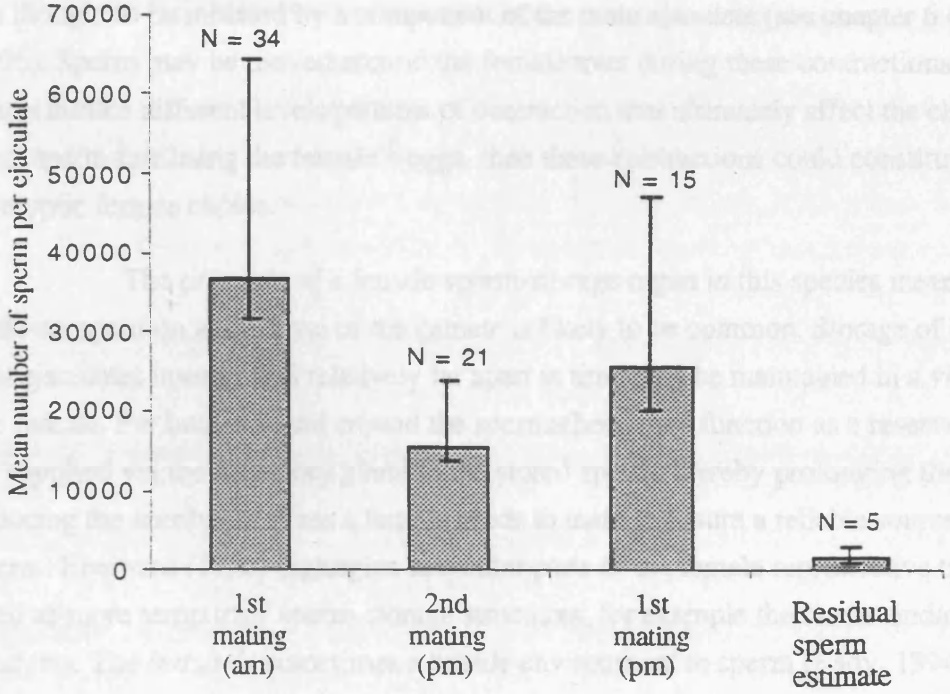


Fig. 7.4.3o: The mean sperm number estimates for time of day control and estimate of residual sperm in females are compared to previously obtained standards. Error bars above the bar are standard deviations and on the bar are standard errors.

7.5 DISCUSSION

7.5.1

Female reproductive organs

The number of ovarioles is very variable between Families of beetles, Scarabaeinae have just one yet 200 are found in Meloe (Cucujidae) (Wigglesworth 1972). The five per ovary found in LGB is a relatively low number. This is to be predicted by the known life-history characters of this species. LGB is a relatively long-lived species with a long laying period that starts about five days after eclosion and continues until death, 10-24 weeks later (Li, 1988). This gives rise to an average lifetime fecundity of 320 eggs per female (Li, 1988). More ovarioles may be expected in species that have a short burst of reproduction and require many eggs to be mature at once. Li found the size of clutch to vary

between 3 and 14 eggs per blind-ending tunnel in maize. It is perhaps surprising that the upper limit of clutch size is not 10, i.e. the number of ovarioles.

The *bursa* is of comparable size to one egg or one spermatophore. It is muscular in nature and violent contractions have been observed following mating and dissection of the reproductive tract. It is unknown whether the contractions are an artifact of the dissection procedure, though similar contractions have been noted in other studies and are thought to be initiated by a component of the male ejaculate (see chapter 6 of Eberhard, 1996). Sperm may be moved around the female tract during these contractions. If different males initiate different levels/patterns of contraction that ultimately affect the chances of their sperm fertilising the female's eggs, then these contractions could constitute one form of cryptic female choice.

The presence of a female sperm-storage organ in this species means that inter-male competition at the level of the gamete is likely to be common. Storage of sperm means that ejaculates inseminated relatively far apart in time may be maintained in a viable state in the female. Fat bodies found around the spermatheca may function as a reservoir of food to be supplied via the accessory gland to the stored sperm, thereby prolonging their life and reducing the number of times a female needs to mate to ensure a reliable source of viable sperm. Eberhard (1996) highlights that other parts of the female reproductive tract could be used as more temporary sperm-storage structures, for example the *bursa* and/or the oviducts. The *bursa* is sometimes a hostile environment to sperm (Eady, 1994). Sperm reaching these areas may, however, gain easier access to eggs as they are laid. Therefore, in situations of high population density and relatively high incidence of mating, spermathecal sperm may not play a major role in the 'mating game'. However, if females refrain from mating, perhaps during dispersal or because they are effectively isolated in a tunnel, then spermathecal sperm, which is of potentially extended lifespan from nutrients supplied by the spermathecal gland, may play a larger role.

Villavaso (1975), quoted in Eady (1994), has shown that contractions of muscular tissue in the spermatheca in the boll weevil *Anthonomus grandis* expel sperm from the spermatheca. Eady (1994) envisages that a similar process may occur in *Callosobruchus maculatus*. In *C. maculatus* the tip of the comma-shaped sperm-storage chamber may be pulled round by the contraction of such a muscle stimulated by the straightening of the comma as it fills with sperm. The function of the blind-ending duct in LGB is unknown. If there is a physical connection between the blind-ending duct and the *receptum seminalis* then perhaps sperm from previous matings can be flushed out down this duct. In some species the duct leading to the spermatheca is vastly elongated and often convoluted in form (see examples cited in chapter 7 of Eberhard, 1996). Eberhard proposes that these structures have evolved since they allow cryptic female choice for ejaculates that can overcome such a barrier. In contrast the duct leading from the spermatheca to the point

of fertilization is often much shorter (Eberhard, 1996) so presumably the female is better able to manipulate fertilisation prior to storage. No such convolutions or elongation of ducts leading to or from the spermatheca were found in LGB.

Male reproductive organs

LGB transfer sperm using a spermatophore. It is not known exactly how sperm are transferred from this spermatophore to the spermatheca, but it is likely that both the long thin tube folded inside the spermatophore and the inverted region of the spermatophore are everted for this purpose. It seems unlikely that the intromittent organ in LGB can be used to scoop out previous male's ejaculates from the spermatheca since it is too short and wide. The hairs at the end of the parameres could, however, serve this function for any sperm/spermatophore material that may remain in the *bursa*. Removal of material may be particularly important if the remains of previous spermatophores act as a barrier to later ejaculates. The length of the parameres is approximately half that of the *bursa*, but it is feasible that the intromittent organ is articulated deeper into the female by structures at its base. It is not known how the spermatophore is delivered from the male to the female in LGB. It is likely that the spermatophore is at least partly formed within the female tract and in some species spermatophore shape is determined by the shape of the female *bursa* (Gerber, 1970). I propose that this is not the case in LGB since although the shape of the spermatophore is similar to that of the *bursa*, two normal shaped spermatophores have been dissected from the *bursa* of a female allowed to mate with two males in quick succession (pers. obs.).

LGB deliver an oversized ejaculate in the sense that many more sperm are delivered than can feasibly be stored in the female's spermatheca. A popular theory to explain oversized ejaculates is that they are more effective at diluting down competitor's sperm either where ejaculates are able to mix in a so called 'raffle contest', or if previously inseminated sperm can be displaced out of a sperm storage organ. This idea is supported by data collected by Eady (1995) in his study of *Callosobruchus maculatus*. In this study male ejaculates were manipulated by allowing males prior matings. Males delivering smaller ejaculates were found to fertilise a smaller portion of offspring when they were the second male to mate in twice-mated females. Cases where males transfer more sperm when competition between ejaculates is likely to be higher also support this idea that increasing sperm number can increase paternity in multiply-mated females.

Paternity of offspring from doubly mated females:

Technical difficulties prevented direct determination of whether males can secure paternity of eggs laid by females who have already mated. The indirect evidence that this is the case is strong. A review of all insect studies currently available shows that the proportion of offspring sired by the second male to mate in a doubly mated female (P_2 value) is variable and often such that the last male fathers more than half the offspring (last-

male sperm precedence). The fact that LGB males invest highly in ejaculates and spend much time associated with non-virgin females also hints that total first-male sperm precedence is unlikely. Indeed, one mating is not enough to supply enough sperm for a female's entire reproductive lifetime (assuming females do not die for other reasons). Mating frequency of beetles observed within plant hosts in this study (chapter 6) in LGB has been found to be higher than that required for fertilisation alone. Ridley (1989) found that high mating frequency is associated with high P_2 values. One measure of P_2, although interesting, is not really enough to quantify the relative costs and benefits of multiple mating, mate guarding and investment in ejaculate. P_2 is determined by many factors such as time between matings, previous mating history, availability of a suitable host as well as individual differences between males and females and interactions between these factors. Indeed, increasing the number of male mates from two to three can greatly complicate patterns of paternity (Zeh and Zeh, 1994). Efficient methods of tracing paternity (for instance using molecular markers) will help scientists to obtain the kind of direct measurements of reproductive success needed to test hypotheses about mating tactics.

Ejaculate investment:

It was possible for an estimate of sperm number per ejaculate to be determined for LGB. The mean standard error associated with sampling was fairly large (15.8% of the mean). Possible contamination of ejaculate samples with sperm already stored in the female spermatheca was a maximum of about 10% of total recorded, though it must be stressed that stored sperm tended to remain in the sperm reservoir whilst the spermatophore was removed from the female tract, so this error is likely to be much less than the maximum. It is impossible to quantify the error associated with imperfect removal of the full ejaculate from the female although containment of sperm within spermatophores made full removal easier. Of course these errors were the same on average for all treatments. The large variation in sperm number per ejaculate for all treatments makes the errors associated with the methods less crucial. That this variation is not an artifact of the methods used is demonstrated by the consistent difference detected between first and second ejaculates.

This study did not find any change in some measures of ejaculate investment for males placed in three different sociosexual environments (single male on fresh grain, single male on conditioned grain and crowded males on fresh grain). Ejaculate investment was, however found to be extremely variable (standard deviation of sperm number in first ejaculates was, on average 76% of the mean). Neither male size nor female size were found to account for this variation. Male age was not strictly controlled in these experiments and could be a main determinant of ejaculate size. Fox *et al.*, (1995) propose that male body size does often correlate with ejaculate size, but that this relationship is only detectable when age and other factors are controlled for. Female size has also been correlated with ejaculate size in the cricket, *Acheta domesticus* (Gage and Barnard, 1996). Larger females

are more fecund and males could therefore preferentially invest more ejaculate resources in these females if such resources are limited.

Male LGB are sperm limited. Both sperm number, spermatophore size and male-weight loss were found to decrease for the second matings compared with the first. They delivered smaller ejaculates if allowed to mate twice within five hours instead of being more reluctant to mate. Unfortunately, it was not conclusively demonstrated that these smaller ejaculates were a feature of second matings as opposed to ejaculates delivered in the afternoon. Males lost a detectable proportion of their body weight (an average of 1.6% of body weight) after the delivery of ejaculates, which is less than the 5% body weight loss found by Fox *et al.*, (1995) in another stored product beetle, *Callosobruchus maculatus.*

There is not enough information currently known about other species similar to LGB for a full appraisal of the significance of the results obtained in this study. The results, however, suggest that the large numbers of sperm delivered per ejaculate are an adaptation to maximise paternity in the face of sperm competition in this polyandrous species. Ejaculate size is extremely variable in this species and alternative mating tactics could possibly maintain this variation. Female Factor and male crowding are apparently not used as cues by males to alter their investment in ejaculates.

CHAPTER 8: DISCUSSION

8.1 Evolution of the aggregation pheromone signal/ response:

Sex-specific ornaments could arise from a variety of non-exclusive mechanisms of which sexual selection is just one possibility. Current ideas are summarised by Andersson (1994) and are given below. These ideas can be used to address the question, 'why is it only LGB **males** who produce aggregation pheromone?'. Each of these possible mechanisms will be discussed in reference to the system found in LGB in order to review the evidence to date. Although only mate acquisition benefits of signalling are tested in this thesis, possible collective feeding benefits derived from the initiation of aggregations will also be discussed.

A. Male ornaments may have evolved because of:

1. Pleiotropic gene effects
2. Selection of ecological sex differences
3. Males being unprofitable prey for predators
4. Male contests
5. Female choice and mating preferences

B. Female preferences for male ornaments may have evolved because of:

1. Fisherian self-reinforcing selection
2. Indicator mechanisms
3. Selection for species recognition
4. Direct phenotypic benefits to choosy females
5. Selection of the sensory system in other contexts (sensory bias)
6. Advantages in the timing of reproduction (mating synchronization)

Taken from chapter 1 in Andersson (1994).

A1 Pleiotropic gene effects: It is possible that male ornaments could be by-products of other sex-linked genes that serve a different function. In this case the pheromone signal could be a biochemical waste product of, for instance, sperm production. Alternatively the genes for pheromone production could be linked to those for sperm production and could be expressed since those for sperm production are maintained in the population through natural/sexual selection. Aggregation pheromone signalling in LGB

does however, 'enhance mating and reproductive success', and it is therefore unlikely that this trait is a selectively neutral by-product of pleiotropy.

A2 Selection of ecological sex differences: Andersson proposes feeding differences as an example of ecological sex differences. I take the phrase, 'ecological sex differences' to encompass any sex-specific difference in traits favoured by natural selection other than those that are sexually selected. Such ecological sex differences need to be identified if the primary benefit of aggregation pheromone signalling is host conditioning, since otherwise, why do females never signal?

If the main benefit of signalling is host conditioning then we would predict females to signal until the host is suitable as a site for oviposition. Perhaps female *Prostephanus truncatus* do signal when they arrive on hosts that require conditioning and we have not detected such a signal because so far we have looked for signalling on stored products which may not require conditioning. Its a long shot, and there is no evidence for this. We may still need to infer a mate-acquisition benefit for males to explain why they signal on stored products.

Males and females may differ in their propensity to disperse. Perhaps females are never the first to arrive at a new host. Sex differences in the costs of dispersing, for instance the ability to fly, or ability to store food for the journey, may have created a dichotomy. The benefits of dispersing may also be different between the sexes. The evidence we have of current populations is that there is no sex difference in flight duration or propensity to fly (Fadamiro, 1995). Indeed, a female bias has been found in the dispersing population (Scholz, 1997 and chapter 5 this thesis). Therefore the evidence to date does not support this hypothesis.

The key point may be that eggs and larvae are more vulnerable to predation than adults. Natural selection may tend to reduce the use of signalling in the presence of one's offspring. Therefore ovipositing females should not signal, and this might also explain why males decrease their signal in the presence of females.

A3 Males being unprofitable prey for predators: Baker and Parker (1979) proposed that male signals (in this case in the form of conspicuous plumage in birds) may be a signal to predators. The 'unprofitable prey hypothesis' proposes that certain individuals of a population (perhaps males) are better able to escape if predators pursue them or are perhaps smaller. The predator will be more efficient if it ignores these individuals and only pursues the prey items that provide more resources per effort spent catching them. Predators may learn/evolve to avoid unprofitable prey if they can be distinguished from profitable prey by a reliable signal.

I propose that the costs of being conspicuous and therefore attracting a greater number of predators far outweigh any possible deflection of predation onto other conspecifics in LGB. LGB adults tend to decrease their signalling effort as an aggregation grows in direct opposition to the predictions of this theory (Prof.D.R.Hall pers. com.).

A4 Male contests: It is proposed that male signals such as plumage characters and song in birds may serve to ward off male competitors (see Fisher, 1930 referenced in Andersson, 1994). However, *Prostephanus truncatus* males 'prefer' to follow the same signals as females and therefore 'strong' signals do not result in avoidance of other males. Males may tunnel a short distance away from male competitors and there is the possibility that males will space themselves further away from 'strong' signals perhaps after using the strongest signal to locate higher quality hosts (see effect of host on signal discussion in chapter 3). Male avoidance of strong signals has so far only been demonstrated for the signal produced by the equivalent of many males signalling in the same patch of food (Schlyter *et al.*, 1987).

A5 Female choice and mating preferences: Sex-specific signalling could have arisen and be maintained if females preferentially mate with signalling males. This is the hypothesis that I have been testing in this thesis. Previous evidence showed that the aggregation pheromone signal is a determinant of conspecific distribution which, in turn, is obviously a crucial determinant of mating opportunity. Work presented in this thesis shows that variation in the aggregation pheromone signal is perceived by responding beetles and does determine the relative numbers of beetles arriving at each signaller. It has been shown that this is where female choice between signals ends in LGB, and other cues take over as determinants of mating success and probably fertilization success.

B1 Fisherian self-reinforcing selection: Fisherian self-reinforcing selection is a mechanism proposed to explain female preferences for male traits that do not necessarily confer fitness benefits directly, or indeed indicate good quality. It is proposed that male traits can be selected for because they confer a mating advantage because females preferentially choose such males. Females choosing such traits benefit by producing 'sexy sons'. Thus the alleles for the female preference of the male trait favoured by Fisherian self-reinforcing selection will be genetically linked to the alleles that produce the trait. If the preference is for the most extreme form of a trait, for instance the longest tail, then the trait may become exaggerated in what is called a runaway process, until it confers enough of a disadvantage to the bearer to be arrested by natural selection. This hypothesis has not been tested here.

B2 Indicator mechanisms: Honest indicating of general quality is likely if production of the signal is energetically costly and therefore disproportionately so to poorer quality males. There is some evidence that this is the case in LGB (see discussion in chapter

3). Favoured aggregation-pheromone signals in LGB may be both an indicator of a physiologically healthy male and also one who is able to locate a good host. If variation in these aspects of male quality are partly heritable then females would benefit from acquiring these 'good genes' for their offspring.

B3 Selection for species recognition: Species recognition through sex and aggregation-pheromone signals in general is well supported since many of these signals are species specific. Even the blend of chemical components and enantiomers have been shown to be species specific, and have been inferred to be an isolating mechanism between some sympatric species of bark beetles (Wood, 1982). The pheromone from another Bostrichid beetle, *Rhyzopertha dominica* is attractive to LGB (Hodges *et al.,* (1983). However, there is no evidence to date that the reverse is true (Rick Hodges pers. comm.) and only LGB and its predator *Teretriosoma nigrescens* are consistently caught in traps baited with LGB aggregation pheromone (T1 and T2). Aggregation pheromone in LGB may be related to species recognition, though there is no evidence that this is a primary function.

B4 Direct phenotypic benefits to choosy females: Direct phenotypic benefits to choosy females could include the selection of superior hosts if host quality influences the pheromone signal (see effect of host type on signal in LGB, chapter 3). This is broadly analogous to the correlation between female attraction through male bird song and food abundance in the territory found in red-winged Blackbirds (Ewald and Roher 1982, quoted in Andersson, 1994). Increasing the food available to male blackbirds led to them attracting more females to their territory. Females may also benefit from selecting a 'good phenotype' (which need not be heritable) in males since LGB males possibly assist during oviposition, by reducing the entry of predators into the tunnel system, and by constructing the main tunnel into the host. Females may assess these particular benefits more efficiently by the pushing behaviour observed prior to mating (see chapter 6). Data presented here are consistent with, but do not test this hypothesis.

B5 Selection of the sensory system in other contexts (sensory bias): Female response to male stimulants may have evolved in another context. Aggregation pheromones may be a mimic of plant volatiles that conspecifics follow to locate a host ('sensory trap' tactic). An alternative view is that volatiles released by beetle feeding were exploited by other beetles for host location. However, LGB is not known for its specific reactions to host volatiles (Pike *et al.,* 1994), and the chemical structure of aggregation pheromone in LGB is not particularly characteristic of that of plant volatiles (David Hall pers. comm.), so this hypothesis is not supported by the data available at the moment.

B6 Advantages in the timing of reproduction (mating synchronisation): Andersson points out that, 'sex ornaments and courtship may function after pair formation and are not involved in competition over mates'. Sex-specific ornaments may initiate appropriate behaviour/physiological changes in readiness for copulation and fertilization. This does not appear to be the case in LGB since manipulation of the pheromone signal did not detectably alter either precopulatory courtship behaviour or ability to transfer sperm. The possibility that aggregation pheromone might determine some aspects of cryptic female choice have not been tested. Processes such as sperm uptake to the spermatheca could conceivably require the pheromone signal as a stimulant, but it is more likely that any chemical stimulants required are contained within the ejaculate (see Eberhard, 1996).

8.2 Alternative mating tactics:

Features of mate acquisition/ fertilization however, tend to be very plastic within individuals and males may switch strategies in response to features of the plant host, conspecific signals and behaviours, and male physiological state. The possibility that males may be polymorphic in respect to mate acquisition behaviour has not been indicated by the results obtained in this study. Although variation in key traits (such as signalling, body size, sperm investment, pushing behaviour) that may influence reproductive success in males have been shown to be extensive in LGB, discontinuous variation was not found when males were placed in the same conditions.

If we assume that LGB males are behaving optimally then we can infer that maximum reproductive success of males should gained by: locating a suitable host, constructing a tunnel system into which males can lure females; mating the female many times a day with oversized ejaculates; and guarding her during oviposition. By-passing the construction of a tunnel system altogether and permanently specialising in acquiring mates as a roving male does not appear to have evolved in LGB. The absence of a permanently roving male strategy might be a consequence of the difficulty in gaining access to a paired female once she has entered another male's tunnel system. Males will mate with females that they encounter away from a tunnel system. However, if an uncolonised suitable host is available, males will always start to construct a tunnel system, even though this effort is far beyond that required for feeding alone. Males will follow other male's signals when dispersing themselves, presumably as an efficient way of locating a high quality host. Observations of beetles arriving at a large host would show whether males and females differ in the precision to which they locate the signaller and the degree to which they stop searching once contact is made on the host. I would predict that females are more likely to search over the surface of a host for the male signaller and his tunnel system than males, who may actively space themselves a short distance from competing males.

8.3 The limitations of an evolutionary approach:

The discussion above is riddled with words like 'maybe, possible and likely' which are indicative of the inherently low predictive power of an evolutionary approach. Few, definite conclusions can be made regarding the evolution of male-produced aggregation pheromone in LGB, but the process of evaluating the possibilities does highlight important gaps in our knowledge of this pest. An appreciation of the benefits and costs of pest behaviours is crucial for the prediction of the effect that any man-made manipulation of such behaviours may have on the population dynamics of the pest.

8.4 Practical applications of this work:

This study has for the first time developed a bioassay for detecting differences in the aggregation-pheromone signalling of individual beetles. Use of single beetles has not only helped to understand the evolutionary function of aggregation pheromone in LGB, but has also shed some light on its potential use in pest control.

Monitoring

Single insects have been shown to be an effective bait for LGB when loaded into very simple funnel traps. This paves the way for a host of studies that could investigate natural signalling and response in LGB. Live LGB adult males could also be used in the construction of a very low cost trap when artificial pheromone vials are unavailable or inappropriate. Fears of increasing the infestation rate of stores by placing traps near them could be reduced if single-male-baited trap are used instead of artificial pheromone lures in cases where the trap cannot be placed further from a food store.

Courtship disruption

The aggregation pheromone signal is not used by *Prostephanus truncatus* to elicit courtship behaviour on contact and therefore the chance that mating behaviour can be disrupted at this stage by interfering with this signal is minimal.

Mass trapping/ manipulation of dispersal patterns

Beetle perception and choice between the natural variation in aggregation pheromone signal is an important consideration for any proposed programme of mass trapping or mating disruption (through influence on dispersal) where artificial sources are presented as point sources. The greater the extent to which beetles invest in choosing between signals, the more efficient artificial trapping could be, provided the artificial lure is engineered to be more attractive than the natural signal. Care should be taken not simply to use high dose lures without considering that beetles may land some distance from high concentrations of pheromone to avoid the high levels of intra specific competition that this would naturally indicate.

LGB have been shown to become habituated to aggregation pheromone. Saturating stores with pheromone to disorientate LGB would be too risky and more likely to promote infestation than reduce it. There could be pheromone analogues, however, which do not elicit the orientation response, but yet lead to desensitisation of insects to the pheromone. Such analogues could be identified and deployed in stores.

Host selection

There is some evidence that host characteristics can influence LGB's aggregation signal (chapter 3). Identification of the factors that limit the male signal could lead to a method of suppressing the signal for beetles feeding in stores, thereby making these colonising beetles less attractive than those signalling on other, as yet unidentified hosts in the environment away from stores.

8.5 Suggestions for future work:

- Identify the spatial distribution of LGB away from stores and identify the important hosts used by LGB in the natural environment (field based).

- Confirm that host identity influences the attractiveness of aggregation pheromone signal in LGB (field or laboratory based) and identify which aspects of the host are important (laboratory based).

- Use hosts baited with single male signallers to investigate the behaviour of arriving beetles (field based).

- Determine the nature of ejaculates delivered by males during multiple mating of females within a tunnel system to assess the function of multiple mating behaviour (promote success in sperm competition and/or provide nutrients to the female) (laboratory based).

- Establish whether male presence in the entrance of tunnel systems prevents entry of predators such as *Teretriosoma nigrescens* (laboratory based).

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