ENTOMOPATHOGEN BASED AUTODISSEMINATION FOR THE CONTROL OF *PLODIA INTERPUNCTELLA* (Hübner) – an examination of the critical components

IAN H. BAXTER

Thesis submitted for the degree of Doctor of Philosophy

ECOLOGY AND EVOLUTIONARY BIOLOGY
SCHOOL OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF SOUTHAMPTON

JULY 2008

Aims and objectives

The objectives of this research are to evaluate the potential of using the entomopathogenic fungus *Beauveria bassiana* (Balsamo), formulated with electrostatic carrier powders as the basis of an autodissemination approach to control the Indianmeal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) a major pest of raw and processed stored food products.

The project is broken into sections as follows:

The delivery system:

1. To quantify the electrostatic attraction forces associated with the test insects and carrier powders after surface contact with corrugated polypropylene (a type of plastic typically used in insect bait station construction).
2. To model the relative distances over which a carrier particle will levitate to an Indianmeal moth by virtue of electrostatic forces.
3. To investigate which of two candidate electrostatic carrier powders (Entostat® or Entomag™) is the most effective at being horizontally transferred between conspecifics of *P. interpunctella*.
4. To investigate whether or not contamination with candidate carrier powders impacts on the mating frequency and ability of male *P. interpunctella* to successfully locate a synthetic pheromone attractant source.
5. To evaluate the regions on the body of the moth where most powder contamination occurs (a) after visiting a dispenser and (b) during mating with treated conspecifics.

The entomopathogenic insecticide:

1. Investigate the effect of the EU91/414 registered fungal entomopathogen, *B. bassiana* (strain ATCC74040 – 'naturalis') on the mortality of *P. interpunctella*.
2. To evaluate the sub-lethal effects of *B. bassiana* inoculation on fecundity and anemotactic flight responses to determine factors which may impact on the suitability of *B. bassiana* being used in an autodissemination approach.
3. Establish whether the uptake of *B. bassiana* by *P. interpunctella* is synergised through mixing conidia with an electrostatic carrier powder.

Evaluating the concept of biological autodissemination as a control system:

1. Develop individual-based models based on the laboratory data to evaluate the most important parameters required for an autodissemination system to successfully impact on a pest population.

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Acknowledgments

I wish to acknowledge the assistance of the following persons –

Dr. C. Jackson, Southampton University – supervisor.

Dr. A. Page, Southampton University Hospital Bio-imaging Unit – for teaching me how to use the scanning electron microscope.

Dr. C. Nansen – for statistical help in chapter 2, experimental methodology chapter 4.

Dr. J. Egger - for statistical help in chapter 2.

Prof. D. Clarke – University of Hertfordshire – assistance with electrostatic equations.

Dr. K. Baxter – DSTL Porton Down, UK – assistance with electrostatic equations and electrostatic measurements.

Mr. D. Webster, Exosect – general technical assistance.

Mr. J. Chandler, Former MD Exosect, for encouragement and opportunity to complete this study.

Dr. H. Schuppe – assistance with confocal microscopy, contribution to chapter 4.

Intrachem Bio Italia – for supplying the 'Naturalis' isolate.

Dr. D. Chandler, University of Warwick – information on commercial uses of entomopathogens.

Mr. P. Abbott, Statfold Seed Oil Ltd. – for allowing me to observe a commercial infestation of *Plodia interpunctella*.

Dr. J. Knight, Imperial College, Silwwod Park, University of London – Individual based modelling and Crystal Ball®.

Dr Lex Kraaijeveld, University of Southampton, for statistical advice and comments on an early manuscript.

Male *Plodia interpunctella* (Hübner) flying to pheromone lure

1. Introduction

1.1 Indianmeal moth life-history and biology

1.1.1 Indianmeal moth

The Lepidopteran family of Pyralidae, contains some of the world's most destructive pests of raw and stored products (Hinton, 1943). Pyralidae members of the genera *Plodia, Cadra, Corcyra* and *Ephestia*, feed on a variety of dried and growing plant material in a wide range of environments and are considered pests of major economic significance (Hains, 1991; Rees, 2004). The Indianmeal moth, *Plodia interpunctella* (Hübner) (see fig. 1.1) from the family Pyralidae and subfamily of Phycitines, is a major pest of raw and processed stored food products (Cuperus et al., 1990; Doud & Phillips, 2000; Hinton, 1943; Lecato, 1976; Nansen et al., 2004; Nansen & Phillips, 2004; Sedlacek et al., 1996; Storey et al., 1983; Vick et al., 1986). Indeed, their broad diet have even led to clinical presentations of *P. interpunctella* infestation in live feline and avian subjects (Pinckney et al., 2001). The name, 'Indianmeal moth' was given by the renowned American entomologist, Asa Fitch, after observations that it was a common pest of cornmeal, or 'Indian meal' as it is also known. The origins of this moth are unknown, with many conflicting reports citing Europe, the Americas and South Asia as the original habitat for this species. The moth is distributed wherever there is permanent human habitation, but is most prolific in temperate to sub-tropical regions (Rees , 2004).

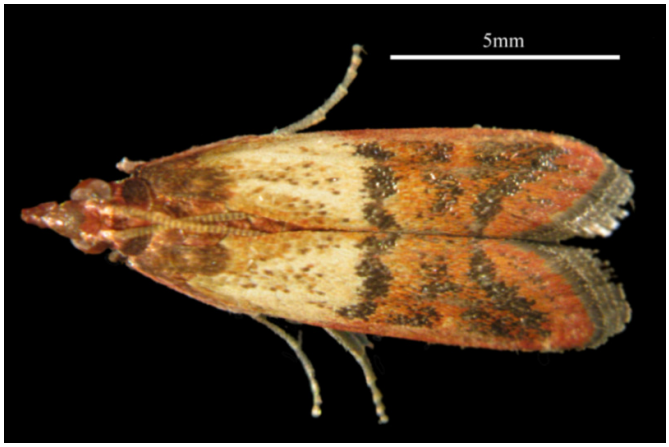


Figure 1.1. An adult Indianmeal moth (*Plodia interpunctella* (Hübner))

Adult moths average 12-14mm in length with the male and female moths easily distinguished by the external genital structures (see fig.1.2). When freshly emerged from the pupae, Indianmeal

moths have bi-coloured wings with the thoracic portion largely cream in colour and the abdominal portion a reddish-brown – this colour rapidly fades as the moths get older.

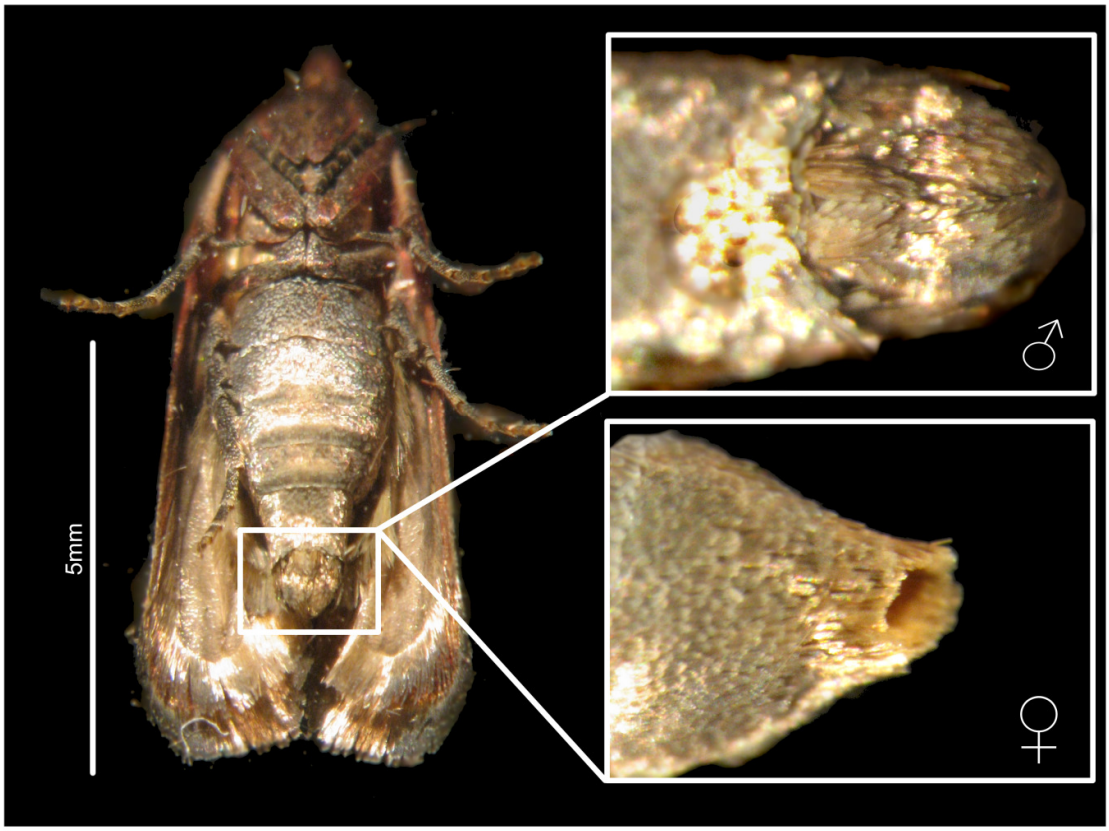


Figure 1.2. Adult Indianmeal moth, showing easily distinguishable differences between male and female external genital structures

1.1.2 Mating behaviour and reproduction

The entire *P. interpunctella* lifecycle may be completed, under optimal conditions, in 30-days (Rees, 2004). Adult *P. interpunctella* do not feed and have, under laboratory conditions, been shown to live for up to 12-days (Huang & Subramanyam, 2003). During the adult life-stage, *P. interpunctella* females release a sex pheromone attractant (see fig. 1.3); many of the components of this pheromone have been identified with the major constituent being (Z,E)-9,12-tetradecadienyl acetate (abbreviated to ZETA) (Brady et al., 1971; Kuwahara et al., 1971; Zhu et al., 1999). Several other species of Phycitines also share significant cross-attraction to ZETA including the genera *Cadra* and *Ephestia* (Brady et al., 1971; Brady & Daley, 1972; Brady & Nordlund, 1971; Ganyard & Brady, 1972; Kuwahara et al., 1971; Phelan & Baker, 1990; Struble & Richards, 1983). The mating behaviour of *P. interpunctella* has been well researched (Grant

et al., 1975; Grant & Brady, 1975; Phelan & Baker, 1990; Trematerra & Pavan, 1995) and the respective authors have identified a complex coordinated pattern of courtship with wing-fanning, abdominal raises, head-butting and ultrasonic communication included in the ritual.

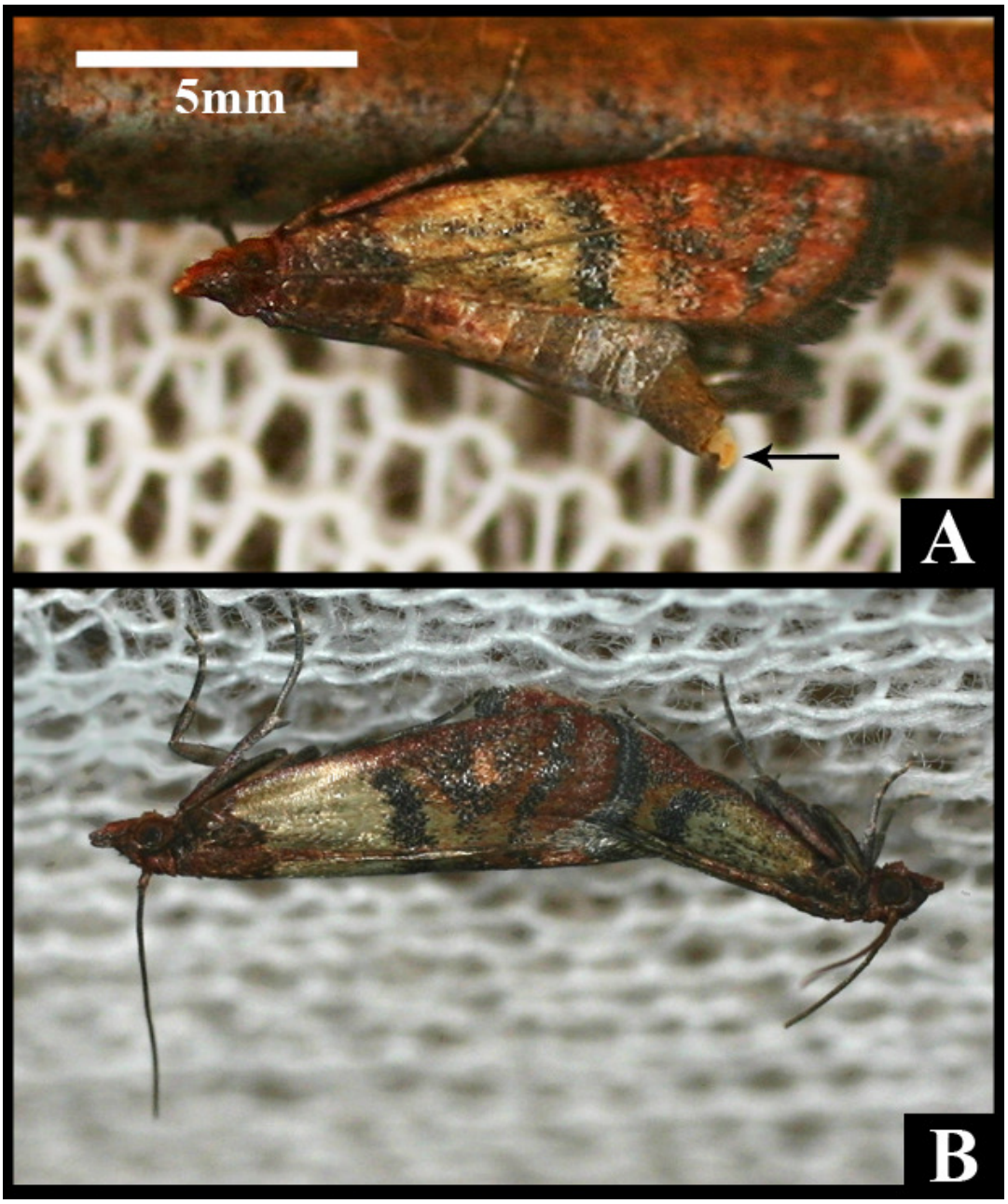


Figure 1.3. (A). Female Indianmeal moth calling, note raised abdomen and extruding distal abdominal segments (arrow) releasing pheromone; (B). Indianmeal moths mating, note larger female to left of image.

Phelan and Baker (1990) undertook an analysis of the mating behaviours of twelve different Phycitine moths, including *P. interpunctella,* and suggest that the complicated courtship displays by Phycitines may be linked to the fact that many of them share at least one pheromone component and that different courtship displays could prevent interspecific mating mistakes. Several of the male Phycitids, including the Indianmeal moth, also produce a sex pheromone (Grant, 1971; Grant, 1974), which are known from only a small number of other Lepidoptera species and are more correctly referred to as 'aphrodisiacs' (Birch, 1974). The aphrodisiac which, at the present time is uncharacterised, is released from a gland in the membraneous flap located at the base of the forewing in the costal fold (Grant, 1974; Grant & Brady, 1975). When a male *P. interpunctella* detects a female he immediately starts wing fanning and searching behaviour until he gets close enough for visual cues to allow him to orientate to the rear of the female – it is during this close-quarters phase that the wing glands open and the male is believed to be releasing his scent over the female (Grant & Brady, 1975).

Both male and female *P. interpunctella* are polygamous with evidence to suggest that males' can detect whether females have been mated, assess how many competing sperm they contain and increase the number of fertilising eupyrene sperm that they transfer accordingly (Cook & Gage, 1995; Cook, 1999). Presence of multiple spermatophores (which is not necessarily an indication of polygamy) in the *bursa copulatrix* of the female has been shown by some authors to increase the overall fecundity (Huang & Subramanyam, 2003). However, they hypothesise that it is not simply more sperm that is the key to increased fecundity, moreover that the paternal investment of nutrients within the spermatophores which may be absorbed by the females, which is increasing vigour. However, Cook (1999) found that the presence of multiple spermatophores neither increased fecundity or longevity. Like other Lepidotera, *P. interpunctella* males produce significant amounts of two distinct types of sperm, eupyrene and apyrene (Cook & Gage, 1995; Cook, 1999). Eupyrene sperm is the 'normal' fertile type, whereas apyrene are smaller and more highly mobile at ejaculation than eupyrene, yet lack any functional genetic material (Cook & Gage, 1995). The function of apyrene sperm are, as yet, unknown, but various authors have suggested that they may represent a nutritional investment from the male to the female or they may simply assist eupyrene sperm transport (Silberglied et al., 1984). Studies on the cotton bollworm (*Heliothis virescens* (Fabricius)) (Park et al., 1998) demonstrated that when the male transfers his sperm to a female he also provides her with a quantity of juvenile hormone. The juvenile hormone stimulates egg production and triggers the female's *corpora allata* to produce her very own juvenile hormone; Huang and Subramanyam, (2003) propose that this is a potential mechanism to explain the increased fecundity observed in female Indianmeal moths that contain multiple-spermatophores. Fecundity is reduced with the older that both male and female *P. interpunctella* become, which for each delayed day of mating males transfer 0.32-

0.45 fewer spermatophores and females produce 24-26 fewer eggs (Huang & Subramanyam, 2003).

Adult moths are able to detect food odours which act as oviposition stimulants (Nansen & Phillips, 2003; Phillips & Strand, 1994). Additionally, secretions from the mandibular glands of larval Indianmeal moths also stimulate oviposition (Mossadegh, 1978; Mossadegh, 1980; Phillips & Strand, 1994). Under optimal conditions a female *P. interpunctella* may produce 150-200 individual eggs (see fig. 1.4) which are laid randomly over the food substrate during the crepuscular periods (Rees, 2004). The eggs hatch in around 3-days at 30°C and the larvae wander until they find food, often entering through minor imperfections in food packaging (Hains, 1991). The larvae burrow into food and create silk-lined tunnels (webbing) as they travel which commonly bind food items together and become unsightly around warehouse machinery, shelving (Rees, 2004). *Plodia interpunctella* larvae also produce significant amounts of allergens (Bernton & Brown, 1967; Binder et al., 2001; Wittich, 1940), which can cause asthma or dermatitis to hypersensitive individuals either working in an infested warehouse or exposed to contaminated food.

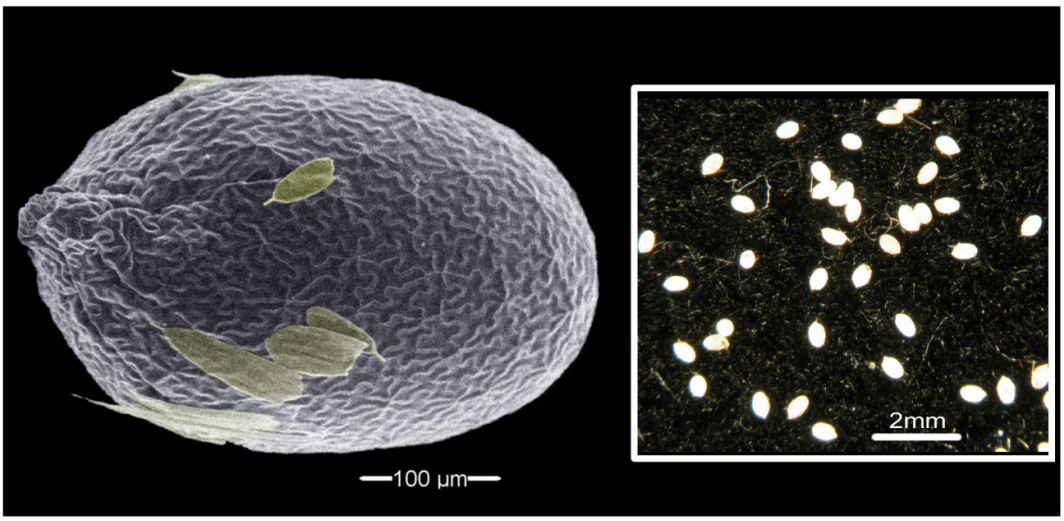


Figure 1.4 Scanning electron micrograph of *Plodia interpunctella* egg, showing adhesion of maternal scales to outer surface. Inset shows white eggs as normally observed when inspecting food products for infestation under low power magnification, such as a hand-lens.

A total of five larval instar (see fig. 1.5) stages take, on average, 20-days to complete (Bjornstad et al., 1998). In temperate regions, decreasing day length and a drop in temperature may stimulate diapause of the larvae, whereby it can maintain a quiescent state for many months until such time as conditions improve (Rees , 2004). During the fifth instar, the mature larvae will seek a pupation spot of a small crevice or retreat to pupate and spin itself a cocoon (Hains, 1991)

(see fig. 1.6). The pupation lasts for approximately 8-days (Bjornstad et al., 1998) after which the adult moths will eclose. The sex of pupae can be easily distinguished through the more rounded appearance of the female terminal segments, whilst the male has a distinctive raised nodule and is somewhat slimmer in appearance than the female.

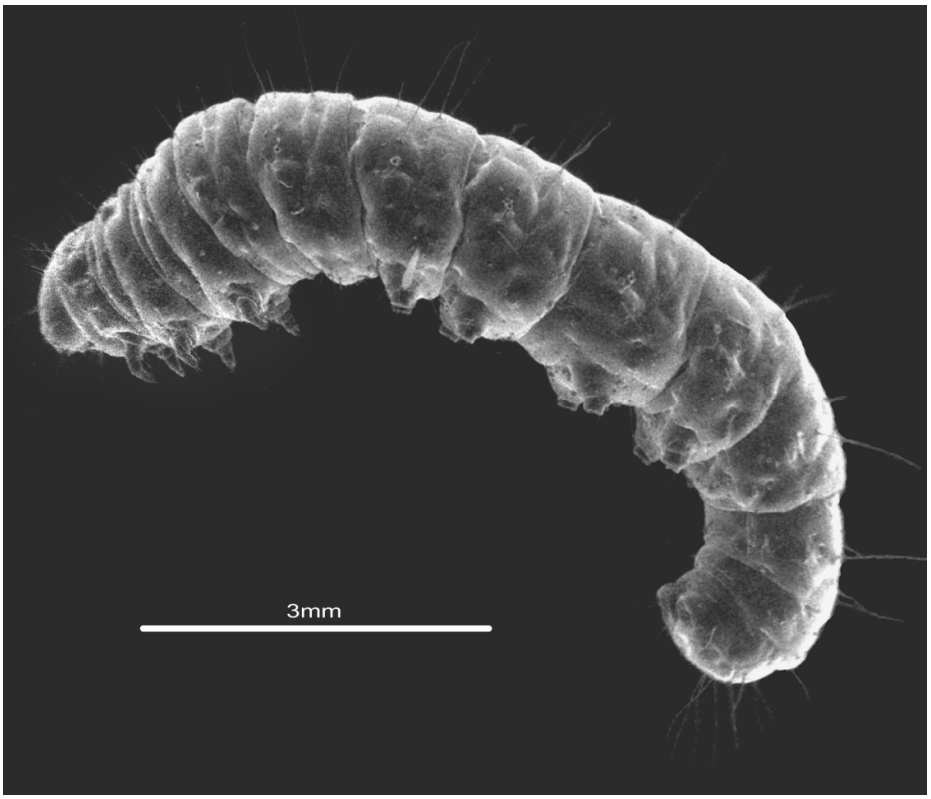


Figure 1.5. Indianmeal moth larvae

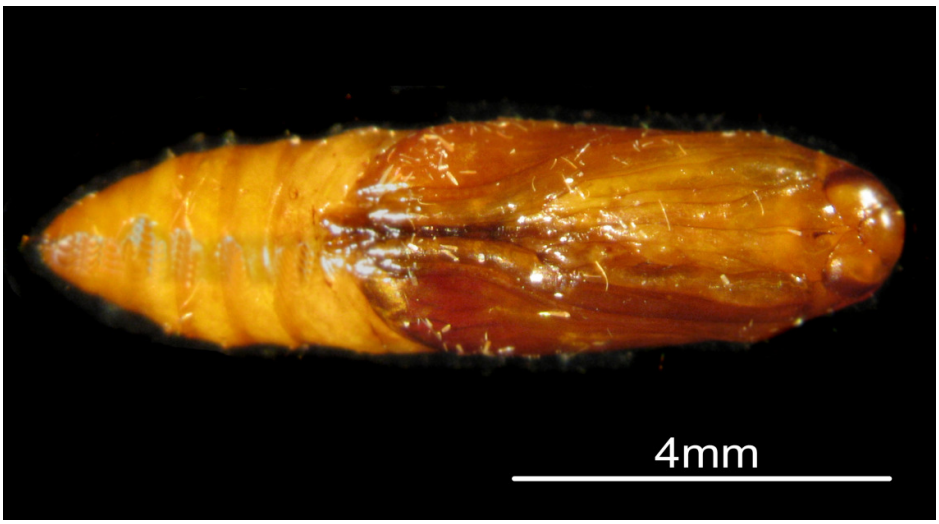


Figure 1.6. Indianmeal moth pupae

1.2 Economic impact

Larvae of *P. interpunctella* have been observed as destructive pests and, thus, causing significant economic losses in grain (Storey et al., 1983), dried fruits and nuts (Johnson et al., 2002), flour and other milled products (Lecato, 1976), dried preserved flowers (Sauer & Shelton, 2002) and even *Bombus terrestris* (L.) pollinator mass rearing facilities (Kwon et al., 2003). The larvae are particularly adept at penetrating a wide range of packaging materials (Cline, 1978) and the large amounts of webbing they produce blocks machinery and binds product together (Rees, 2004) (see fig. 1.7). The economic damage is not simply losses due to infested food, as packaging in unaffected product may also be ruined by larvae attempting to burrow in or by cocoons sequestered into folds in around seam lines, or by the webbing trails left by wandering larvae. Additional economic losses may be incurred where machinery, particularly in a continuous operation automated factory set-up, needs to be temporarily decommissioned for cleaning, where webbing has clogged conveyor belts, gearing, etc. (*P. Abbott, Statfold Seed Oil Ltd., pers. corr.).*

Figure 1.7. Indianmeal moth larvae feeding on pumpkin seeds. Note quantity of webbing covering seeds.

In the UK the dried fruits importation industry is particularly at risk from Indianmeal moth infestation, largely because of the length of time product spends in containers during transport (Faustini, 2006). The value of dried fruit imports were estimated at £80-90 million in 1996 (Wakefield, 2004) and raisins and sultanas form the largest volume of these imports with over 100,000 metric tonnes arriving annually to UK ports, at an average cost of $1100/tonne (Meadows, 2006). When a shipment may take several months to reach its destination a combination of warm temperatures, a nutritious food source and a sealed container may lead to extraordinarily high levels of Indiameal moth infestation. In addition to loss of product, foods showing obvious signs of contamination may be subjected to mandatory fumigation by customs officials before release to the purchaser. This is particularly costly when the product has left the origin as a certified organic product, for which a premium has been paid, as fumigation is likely to invalidate the organic status of that entire shipment (*P. Abbott, Statfold Seed Oil Ltd., pers. corr.).*

1.3 Current Indianmeal moth control methods adopted in the UK

In recent years the most widely used control method for bulk treatment of raw and processed agricultural foodstuffs was methyl-bromide (bromomethane, CH_3Br) (Hemminger, 2005; Roberts, 1995), a potent broad-spectrum insecticide, rodenticide herbicide, fungicide and nematicide. However, methyl-bromide was discovered to be an ozone-depleting substance (ODS) and in 1987, under an international treaty known as the 'Montreal Protocol', it was agreed to eliminate the production and consumption of all ODS (including methyl-bromide) world-wide by 2015 (Anon, 2002). In 2007, methyl-bromide is still used in the UK, under restricted license as a fumigant in public hygiene and vertebrate control in agreed critical use situations or for quarantine, pre-shipment and emergency use (PSD, 2008). The withdrawal of methyl-bromide for routine treatments have left growers and warehouse managers with few options to treat their products effectively against stored product pests such as *P. interpunctella.*

The structure of the UK regulatory system is complex in that two Government agencies regulate the approval and use of pesticides in different situations. The Pesticides Safety Directorate (PSD) have authority over agricultural and raw food products, whereas the Health and Safety Directorate (HSE) preside over processed food materials and storage areas. This creates problems particularly in the stored product area where a treatment may be approved for use in specific circumstances by one body and not the other. For example, in a chocolate processing factory raw cocoa beans may enter the factory where it is stored and treated against pests such as *P. interpuctella* under the Governance of PSD, but as soon as the cocoa is processed pest control treatments are regulated by HSE. As the pesticide approvals process is different for each

authority, many of the active ingredients licensed for use in one part of the factory cannot, legally, be used in another and it is not often clear what products are legal to use and in what circumstances. Table 1.1 shows the entire list of legally registered insecticides potentially used against *P. interpunctella* in raw food and raw food handling areas (PSD, 2008). Many of the insecticides here are not recognised by HSE and consequently could be used inadvertently in the processed food area of a factory.

Table 1.1. Active ingredients approved for stored product use in the UK (January 2008)

Product MAPP No	Marketing Company	Crop(s) Approved	Active(s)
Degesch Fumigation Pellets 11436	Rentokil Initial UK Ltd	cereal, millet, rice, sorghum	aluminium phosphide
Detia Gas Ex-P 09802	Igrox Chemicals Limited	cereal, millet, rice, sorghum	aluminium phosphide
Degesch Fumigation Tablets 09313	Rentokil Initial UK Ltd	processed consumable products	aluminium phosphide
Detia Gas Ex-T 03792	Igrox Ltd	space fumigation (gas-tight situation), space fumigation (non-gas-tight situation)	aluminium phosphide
Detia Gas-Ex-B 06927	Igrox Ltd	crop handling and storage structure	aluminium phosphide
Phostoxin I 05694	Rentokil Initial UK Ltd	space fumigation (gas-tight situation), space fumigation (non-gas-tight situation)	aluminium phosphide
Greencrop Storeclean 225 11098	Greencrop Technology Ltd	grain	chlorpyrifos-methyl
Reldan 22 08191	Dow AgroSciences Ltd	grain	chlorpyrifos-methyl
Greencrop Storeclean 225 11098	Greencrop Technology Ltd	crop handling and storage structure	chlorpyrifos-methyl
Prostore 157 Ul 12017	Nickersons Seeds Ltd	barley (grain), oats (grain), rye (grain), triticale (grain), wheat (grain)	bifenthrin and malathion
Profume 12035	Dow AgroSciences Ltd	For use in emptied cereal grain facilities crop handling and storage structure	sulfuryl fluoride
Actellic D 10509	Syngenta Crop Protection UK Limited	equipment, grain store (empty)	pirimiphos-methyl
Actellic Smoke Generator No 1010448	Sorex Ltd	grain store (empty)	pirimiphos-methyl
Actellic Smoke Generator No 2006627	Zeneca Crop Protection	grain store (empty)	pirimiphos-methyl
Actellic Smoke Generator No 2010540	Syngenta Crop Protection UK Limited	grain store (empty)	pirimiphos-methyl
Detia Gas-EX-B Forte 10661	Igrox Ltd	crop handling and storage structure	magnesium phosphide
Degesch Plates 07603	Rentokil Initial UK Ltd	processed consumable products	magnesium phosphide
Prostore 420EC 12210	Nickersons Seeds Ltd	grain store (empty)	bifenthrin and malathion
Mebrom 100 04869	Mebrom NV	food storage area	methyl bromide
Methyl Bromide 100% 01336	Bromine & Chemicals Ltd	food storage	methyl bromide
Methyl Bromide 98% 01335	Bromine & Chemicals Ltd	food storage (commodity treatment), food storage (space treatment)	methyl bromide

*Data from Pesticides Safety Directorate, 2008.

In the UK there are currently a number of chemical treatments that may be used to control a *P. interpunctella* infestation, all of which are broad-spectrum insecticides. The insecticide products fall into three broad categories – fumigants, aerosols and admixtures.

Fumigants: Even though an amount of 1,608kg of methyl-bromide was applied to raw and processed stored products in the UK during 2004 (CSL, 2006), this is set to fall dramatically since the label restrictions have all but withdrawn this active substance from the market. The only fumigant currently licensed for use on food material is aluminium phosphide of which there was 2,648kg applied to UK food materials during 1994 (CSL, 2006). Other fumigant products such as sulfuryl fluoride and pirimiphos-methyl may only be used in empty warehouses.

Aerosols: There are a wide variety of insecticide-based aerosols that are licensed for use around stored processed products, these include deltamethrin, pyrethrum, cypermetrhin and allethrin (HSE, 2007). None of these are licensed for use directly onto stored raw product and, given the difficulty of treating a large area effectively with an aerosol canister, are most likely used in small shop/warehouses where *P. interpunctella* can be a major pest.

Admixtures: These are materials which are added directly to the stored product as either a dry granule or as a low volume spray. Dry products include desiccant dusts such as silicon dioxide, chitosan and diatomaceous earth (which are exempt from UK registration by virtue that they kill target pests by way of a physical rather than chemical action). Wet sprays, such as the broad spectrum organophosphorous insecticide chlorpyrifos, of which there was 239kg applied to UK foods during 2004 (CSL, 2006) are more frequently applied at the grain storage stage rather than to processed food materials.

Additional non-chemical control treatments used:

Controlled Atmospheres: The use of controlled atmospheres potentially offers a safe and environmentally benign means of protecting stored food products without the need for chemical spray/fumigation. Hermetic sealing of product, followed by flushing with either carbon dioxide or nitrogen, particularly when combined with an elevated temperature (Sauer & Shelton, 2002) can be an effective means of controlling *P. interpunctella*. This is, however, an expensive programme to establish and is not widely in use within the UK with only 1 company, Rentokil™, holding a license for a small portable CO_2 unit (Navarro, 2006).

Gamma Irradiation: Under experimental conditions gamma radiation has been shown to be successful in controling *P. interpunctella* (Ashrafi et al., 1972; Azelmat et al., 2005; Ozyardimci et al., 2006). However, to date, only certain labelled herbs and spices are irradiated in the UK

(Anon, 2007), so this is not currently considered to be a widely adopted method of *P. interpunctella* control.

Desiccant Dusts: Products based on the desiccant dust, diatomaceous earth, have been shown to have strong activity on early instar P. *interpunctella* (Mewis & Ulrichs, 2001), but little effective impact in older larvae. The use of such dusts is localised in the UK, with just 7% of stored grain being treated with 389 tonnes of product being applied during 2004 (Dawson et al., 2004). The use of such dusts are largely as an adjuvant to improve penetration of conventional insecticides rather than a stand-alone control treatment.

1.4 The 'Lure and Kill' Approach to Pest Control

Using a semiochemical to attract target pests to an insecticide is an appealing proposition as this may reduce the need to broad scale apply chemicals to crops (Suckling & Brockerhoff, 1999), food materials (Nansen & Phillips, 2004), surfaces, packing equipment or any other place where contamination of the food chain is likely to occur. It also provides a species-specific means of attracting the pest of interest with less impact upon the fauna of non-target species present in that particular ecosystem (Howse et al., 1998). Unlike the stations used for pest monitoring or mass-trapping, once the pest is attracted to the semiochemical dispenser it is not subjected to physical trapping in glue or surfactant and water, but to either a lethal dose of insecticide or pathogen, a fatal electric shock or even chemosterilisation. The technique is frequently referred to as 'lure and kill', 'attract and kill', 'attracticide' or attraction-annihilation' (Lanier, 1990).

The lure and kill technique is generally presented as either a small deposit of gel containing a mixture of semiochemical and insecticide (such as Sirene® from Syngenta, or Last Call™ from IPM Tech Inc.) or as an insecticide/semiochemical impregnated board (e.g. Magnet™Med from Agrisense) which has visual cues to enhance the attractiveness of the system to the pest. The semiochemical may be a sex pheromone or kairomone (e.g. a food odour) with other attractants including an ultra-violet light source, specific colours (Mitchell et al., 1989) and even objects such as large dark areas that are thought to mimic the shape of their cattle hosts in the case of Tabanidae (Vale et al., 1986). The pest killing or sterilising agent, more correctly referred to as the 'affector' (Lanier, 1990), is also contained within or on the gel deposit/dispenser and may promote an affect upon the pest by either topical contact toxicity (e.g. Last Call™), oral toxicity (Goliath®Gel manufactured by BASF), vapour action (dichlorvos impregnated strips such as those manufactured by Vapona), desiccation (e.g. Cluster Buster™) or infection from pathogens (Winstanley et al., 2005). The lure and kill concept has been used with a wide range of pests including Diptera (Hanley et al., 2004; Vargas et al., 2001; Vargas et al., 2002; Vargas et al.,

2005), Coleoptera (Mckibben et al., 1990), Isoptera (Almeida & Alves, 1996) and especially, considering the vast number of semiochemicals that have been identified and synthesised for them, Lepidoptera (Brockerhoff & Suckling, 1999; Charmillot et al., 2000; Curkovic & Brunner, 2005; Downham et al., 1995; Losel et al., 2002; Maxwell et al., 2006; Trematerra & Capizzi, 1991).

For a lure and kill strategy to be successful a number of considerations must be made; (1) the responsiveness of the pest to the attractant is critical, if the attraction is merely long-range and pests cannot orientate to the affector then the system will fail; and the same *vice versa* should the attractant only function as a short-range orientation pests may not be lured in from a sufficient distance (Lanier, 1990); (2) if the lure is only attracting males, one has to consider to what extent are they polygamous and what proportion of available males is required to be removed from the population before an impact on the overall population is measured. If, as in the case the Indianmeal moth, the male may mate with at least 10 different females (Brower, 1975) then one can reasonably assume that over 90% of the males need to be removed before there will be an impact on the overall population; (3) the distance between stations and overall station density should be enough to inoculate a large proportion of the population but not too dense so as to cause pheromone confusion due to false trail following (Cork, 2004); (4) the affector should not be a repellent to the target pest as they may not become drawn to the point-source (Silverman & Ross, 1994; Verkerk & Bravery, 2001); (5) in the case of short-lived pest species the affector will need to act very quickly to either kill or sterilise the pest otherwise the system will not register an appreciable effect; (6) the sublethal effects of the affector should be considered as levels of exposure are likely to vary considerably between the pests – not properly managed this could become a source of insecticide-resistant strains of pests. Additionally, the phenomenon of autotomy (the shedding of limbs) has been observed in several species of Lepidoptera where they have been in sublethal contact with insecticides (Krupke et al., 2002), authors have suggested that this is an adaptive trait to avoid the uptake of toxins (Moore et al., 1989; Moore & Tabashnik, 1989). Therefore the lure and kill system should be evaluated to assess bait avoidance behaviours such as autotomy.

Nansen and Phillips (2004) examined the efficacy of the 'Attracticide' Last Call™, a non-drying gel preparation dispensed as a droplet, which contains permethrin and in this experimental case, the pheromone component ZETA, in laboratory assays on *P. interpunctella*. In their studies they evaluated both the amount of permethrin (3, 6, 12 and 18% (wt:wt)) and the amount of ZETA (either 0.16 and 0.32% (wt:wt)) in a forced contact test and wind tunnel evaluation. They concluded that a <3 second contact with either concentration of permethrin affected male survivorship and ability to mate. In wind tunnel tests the higher dose of pheromone resulted in

fewer contacts with the point source suggesting that the moths were repelled/disorientated by large doses of the single pheromone component. In contrast the amount of permethrin contained within the gel had no impact on the number contacts and did not appear to repel the moths.

1.5 Autodissemination

A modification on the lure and kill technique is 'autodissemination' (see fig. 1.8) where biological agents, in particular fungal entomopathogens, would establish a foci of infection in a population of insects and the living insects themselves would transmit the pathogen throughout the population (Soper, 1978) (see table 1.2 for a range of the materials and situations in which insect vectoring of biological agents have been investigated). Subsequent authors have evaluated autodissemination as a modification upon the lure and kill system by which the affector is an entomopathogen instead of a synthetic insecticide or similar and that conspecifics themselves are vectors. Examples of autodissemination include the diamondback moth (*Plutella xylostella* (L.)) (Vickers et al., 2004; Furlong et al., 1995) to deliver fungal condia within brassica crops; the sweet potato weevil in Japan, using *B. bassiana* conidia (Yasuda, 1999); the Japanese beetle with the the fungal entomopathogen, *Metarhizium anisopliae* (Metchnikoff) Sorokin (Klein & Lacey, 1999) and enomopathogenic nematodes (Lacey et al., 1993; Lacey et al., 1995a); the tobacco budworm (*Heliothis virescens* (Fabricius)) with an entomopathogenic virus (Jackson et al., 1992); and more pertinent to this programme, the *P. interpunctella* (Vail et al., 1993).

In examining autodissemination with Indianmeal moth, Vail et al., (1993) used a synthetic source of the major pheromone component, ZETA, to entice male moths onto a station containing the particles of granulosis virus. Male moths were introduced into the room containing the autodissemination station and cups containing bran diet which acted as an oviposition site. They were left undisturbed for 24-h prior to the introduction to an identical number of females. The exposure section of the study was left to run for 2-weeks, or until all the adult moths were dead. The cups of bran diet were frozen to kill any pre-existing eggs or larvae and 25 neonatal larvae from a culture added to the cups. The efficacy of these treatments was determined by adult emergence from the cups when compared to an untreated control. Vail et al., (1993) obtained 52-60% mortality where the male moths had been released first, however only 16% was recorded in a subsequent experiment whereby the males and females had been simultaneously released. This led the researchers to conclude that autodissemination used in this manner would be only moderately successful as a stand-alone treatment, but may have potential when used in conjunction with other control programmes in, presumably, a reduced conventional insecticidal treatment regimen. Further work by Vickers et al., (2004), with the diamondback

moth, *P. xylostella*, demonstrated that the autodissemination approach could cause epizootics of fungal entomopathogen in field

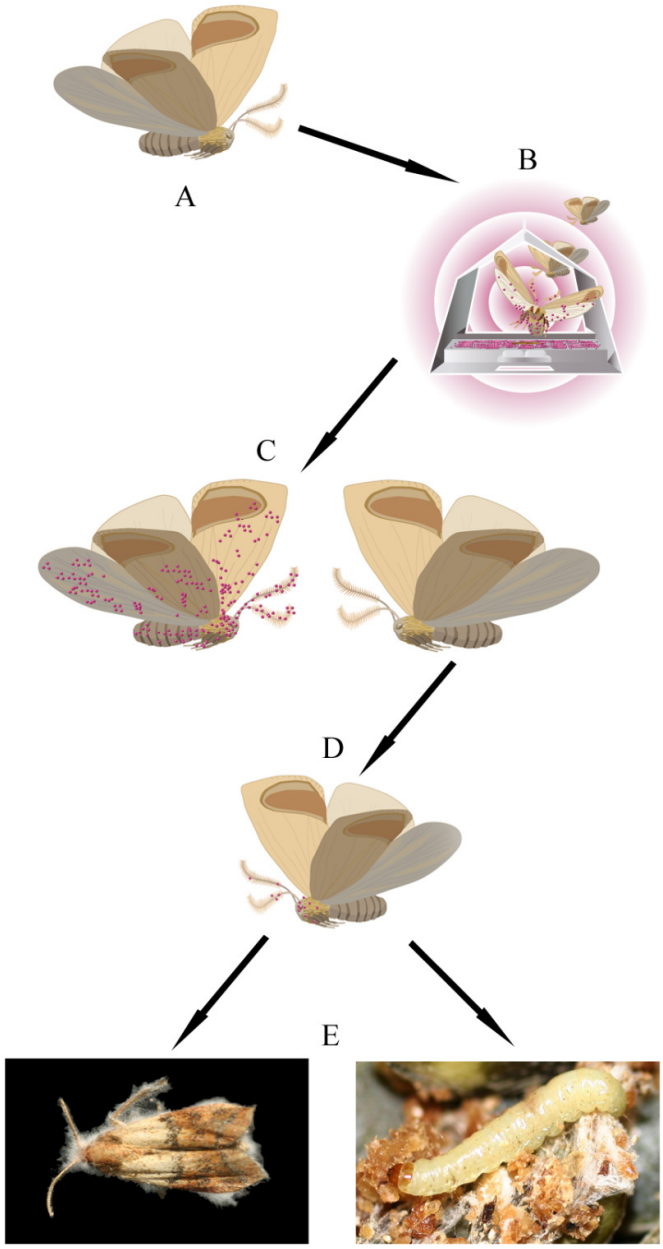


Figure 1.8. The Concept of Autodissemination.

(A) Target insects (usually males) are lured to a dispenser using pheromone or food baits (B) where they come into contact with active ingredient. (C) The contaminated pest passes on active ingredient to conspecifics during normal social activity such as mating or lekking. (D) Depending on the active ingredient, the contaminated conspecific either dies (E) and, in the case of a pathogen such as *B. bassiana*, may sporulate and become a foci of infection to contaminate other pests, or, in the case of certain synthetic active ingredients the pest or its offspring may be rendered sterile/premature death.

Table 1.2. Examples of insects being used as disseminators of biological agents

Author	Insect	Agent	Target	Laboratory/Field	Comments
Lacey et al, 1995b	Japanese Beetle: *Popillia japonica*	Nematode: *Steinernema glasiri*	Conspecifics	Laboratory and Field	Mass-reared adult beetles were exposed to infected larvae/adults. Mark-release-recapture was then used to determine levels of inoculated individuals. Only 1% of treated beetles were recaptured, but 33% of those were infected. Authors conclude that this technique would NOT be successful in field due to low rate of transfer. Possible sub-lethal effects of infection on flight ability discussed.
Scholte et al, 2004	Mosquito: *Anopheles gambiae*	Fungus: *Metarhizium anisopliae*	Conspecifics	Laboratory	Conidia-only formulation placed on filter papers. Females exposed to conidia for unspecified period of time. Females then coupled with males in either 1:1 or 1:10 ratio. Authors conclude that between 11 and 33% of males horizontally received lethal dose and that autodissemination has potential as a control strategy.
Kovach et al, 2002	Honeybee and bumblebees: *Apis mellifera* & *Bombus impatiens*	Beneficial fungus: *Trichoderma harzianum*	Plant pathogenic fungus *Botrytis cinerea*	Field	One of several highly successful studies showing that a device on the front of a hive can deliver material to a bee which, in turn, can be horizontally transferred to a plant. This field example, showing good levels of replication over four seasons, demonstrated that Botrytis control was equal and often better than conventional treatments, with strawberries from the dissemination treatment containing 22% more seeds and 26-40% more average weight.
Dedej et al, 2004	Honeybee: *Apis mellifera*	Beneficial bacteria: *Bacillus atrophaeus* (formerly *B. subtilis*)	Blueberry pathogens	Field	Device placed on front of hive to augment *B. atrophaeus* into blueberry crop. Bees, whilst needed for pollination, also vector plant pathogenic fungus, *Monilinia vacciniicorymbosi*. Incidence of blueberry infection with pathogen was shown to be reduced by up to 14.1%.

cage experiments. Using the fungal pathogens *Zoophthora radicans* Brefeld and *B. bassiana*, inoculated adult male moths were released into field cages (16 x 16 x 2m) containing larvae infested brassicas. They found that conidia inoculated male moths were able to passively transfer infection to larvae and that clusters of infection could develop as a result. The autodissemination concept, they determined, may be effective under conditions of low pest pressure as the isolates are unlikely to impact on the pest population rapidly enough for commercial growers.

An alternative and highly novel form of entomopathogen dissemination has been investigated using honeybees as vectors of *B. bassiana* (Mohammad, et al., 2007). Hive mounted dispensers served to inoculate bees with *B. bassiana* conidia as they exited the hive to forage. The conidia were mixed with various inexpensive powder diluents, such as corn flour and potato flakes, in order to determine which 'carrier' powder maximised conidia uptake. The authors found that conidia uptake was largely influenced by carrier particle size, with the smaller particles facilitating increased uptake. A number of studies have used bees as vectors of control agents and, whilst this is a more simple approach than the present example for *P. interpunctella*, the authors' conclusions that honeybee vectoring shows potential justifies the approach being taken here.

1.5.1 Potential issues with using Autodissemination

In the present example, the attractant for the *P. interpunctella* autodissemination system is a synthetic analogue of the female sex pheromone, ZETA (see section 1.1.2), the same lure that is used extensively in monitoring traps (Plarre, 1998). Pheromone from the autodissemination system would, therefore, compete with that of the monitoring traps and the pest control operative (PCO) may find that monitoring trap catches will become unreliable due to the high ambient levels of pheromone (Thomson, et al., 1999). A potential way of overcoming this issue would be to use a different attractant, e.g. water traps (Chow, et al., 1977, Ryne, et al., 2002) or a female *P. interpunctella* lure (Konemann, et al., 2008). However, as PCO's have developed a good understanding of when to treat based on historical trap catch data, the industry would be reluctant to change to a new, largely untested system of monitoring based on captures of the female moth alone.

Autodissemination is regarded as an effective way of limiting the effects of pest control on non-target organisms (Lanier, 1990). This is largely down to the attractant, which being a pheromone, is seen as species-specific. However, ZETA is attractive to a number of other Pyralidae stored-product moths (see section 1.1.2), which may even be seen as an advantage as this gives the PCO the potential to control a pest-complex with one product. Additionally,

several other UK indigenous Lepidoptera, which would not be considered pests, are also attracted to Zeta, these includes the rustic (*Hoplodrina blanda* (Denis & Schiffermüller), the uncertain (*Hoplodrina alsines* (Brahm)) and *Anania verbascalis* (Denis & Schiffermüller) (www.pherobase.co.uk). Whilst none of these species appear to be threatened or subject to conservation legislation, it is important to consider that the amount of data on endangered species chemical ecology will be very limited and there may, indeed, be endangered moths attracted to the station. In all probability the location of a typical storage facility is likely to be in an urbanised area and, therefore, outside the sphere attraction of most 'at risk' species. Besides Lepidoptera, a number of Hymenopteran parasitoids also are attracted to ZETA (Schöller and Prozell, 2002), including the genus *Trichogramma* which often parasitizes *P. interpunctella* eggs. Again, for the reasons highlighted above, this is unlikely to impact majorly on wild populations of wasps, although it may be considered an issue where biological control augmenting parasitoids is occurring. To date, in the UK, this practice is not commercially available.

It is not unknown to catch *P. interpunctella* considerable distances away from food processing plants (Doud and Phillips, 2000). Therefore, by placing pheromone sources within the factory one wishes to protect, the PCO is potentially attracting further males from the wider environment. Whilst attracting males may not matter too much if females are not present (with the exception of foreign objects presenting themselves in food), Yamanaka, et al's., (2003) modelling of a pheromone plume suggests that a station may cause moth clusters and, indeed, overall mating could increase as a result of only a proportion of the male moths actually getting trapped. Whilst it is difficult to determine how much of a risk this actually is, it highlights the bigger issue that most control programmes will work more effectively if area-wide treatments are synchronised. In mills/packing houses/food distribution plants, *etc*. this means treating the whole building and not just 'hotspots'. Where similar industries lie adjacent to each other, a benefit may be observed from co-ordinating treatment programmes to prevent immigration.

1.6 Entomopathogenic fungi : background information

Approximately 750 species of fungi in 56 genera are reported as pathogens of arthropods (Hawksworth, 1991). Although entomopathogens are represented in all four phyla of the true fungi, most species reside in phyla Zygomycota and Ascomycota and a number are commercialised and registered as biological control agents (Copping, 2004). The Entomophthorales (Zygomycota) contain some key species that are obligate pathogens and cause natural epizootics in a range of agricultural pests, although a number of these species cannot be grown readily *in vitro* (McCoy et al., 1988; Pell et al., 2001). Members of the phyla Ascomycetes - especially anamorphic species in the order Hypocreales - are associated less

commonly with natural epizootics, but they are popular choices for biopesticides because they can be mass-produced and formulated easily (Evans, 2003; Jackson et al., 2003; Shah & Pell, 2003; Inglis et al., 2001; Jenkins & Goettel, 1997; Wraight et al., 2001). These commercialised isolates of Ascomycota have also demonstrated viability under field-conditions, allowing them to be applied to the target using similar approaches and methods for chemical pesticides both for aerial sprays and application to soil (Bateman & Chapple, 2001; Chapple et al., 2000).

Infection occurs by the attachment, germination and growth of spores through the host cuticle. The fungus then invades the haemocoel before ramifying throughout the host (Tanada & Kaya, 1993). Tissue necrosis is caused by mechanical disruption, water loss, and the action of secondary metabolites (Tanada & Kaya, 1993). Death occurs within 3 – 10 days of infection, often followed by prolific sporulation on the surface of the cadaver. Both infection and sporulation require the presence of free water or high humidity, the lower limit for the germination of spores *in vitro* being c. 93 % r.h. (relative humidity) (Gillespie & Crawford, 1986). In some cases, entomopathogenic fungi are able to cause infections at seemingly lower humidity than those required for germination *in vitro*, because the microclimate % r.h. of the host is higher than ambient (Milner et al., 1997). Many isolates of entomopathogenic fungi require moderate temperatures (15 – 27ºC) for optimal infection, although the maximum temperatures for growth vary extensively with isolate, e.g. 33 – 36ºC for *Lecanicillium* spp., 33 – 40ºC for *Conidiobolus coronatus* (Cost.) and 37ºC for *Beauveria* spp. (Davidson et al., 2003; Yeo et al., 2003). Viability of isolates at ca. 37ºC is of concern from a mammalian safety standpoint as, although exceptionally rare, *B. bassiana* infection in immuno-compromised individuals has been observed (Tucker et al., 2004).

1.6.1 The use of entomopathogenic fungi in biocontrol today

Commercial agents for insect pest control are available based on the Ascomycetes order of hypocrealean genera *Beauveria*, *Lecanicillium*, *Metarhizium*, and *Paecilomyces*. A summary description of entomopathogenic fungal products registered in OECD countries is given in Table 1. 3. A total of 20 products are registered in OECD (Organisation for Economic Co-Operation & Development) countries based on 14 different strains of six fungal species: *Beauveria bassiana* (4 strains); *Beauveria brongniartii* (2 strains); *Lecanicillium longisporum* (Petch) Zare & Gams (previously known as *Verticillium lecanii* but has recently undergone taxonomic revision) (1 strain); *Lecanicillium muscarium* (Petch) (also previously known as *V. lecanii*) (1 strain); *Metarhizium anisopliae* (5 strains); *Paecilomyces fumosoroseus* (Wise) Brown & Smith (1 strain) (see Table 1.2). In Europe, they are intended for use against pests of protected crops, particularly sap-feeding species, whereas in the USA the target is pests of both glasshouse and field crops. An important point to note is that species in these genera are pleiomorphic with

respect to pathogenicity-related characteristics and different isolates from the same species often vary in host range, pathogenicity and response to the external environment (McCoy et al., 1988).

Table 1.3. Fungal entomopathogens registered for use in OECD countries

Species	Strain	Product name	Manufacturer	OECD registration	Label crops	Target pests
Beauveria bassiana	GHA	BotaniGard ES	Laverlam International USA (prev. rights held by Emerald Bioagriculture Corp./	Denmark, Italy, Japan, Mexico, Spain, Sweden, USA	Vegetables, fruit, herbs, ornamentals	Aphids, whitefly, thrips, mealybug, weevils, chinch bug
	GHA	BotaniGard WP	ditto	Denmark, Italy, Sweden, USA	Vegetables, fruit, herbs, ornamentals	Aphids, whitefly, thrips, weevils, leafhoppers, psyllids, crickets.
	GHA	Mycotrol 22WP	ditto	Denmark, Italy, Sweden, USA	Various fruits & vegetables, ornamentals	Aphids, whitefly, thrips, weevils, leafhoppers, psyllids, crickets.
	GHA	Mycotrol ES	ditto	Denmark, Italy, Japan, Mexico, Spain, Sweden, USA	Various fruits & vegetables, ornamentals	Aphids, whitefly, thrips, weevils & beetles, leafhoppers, psyllids.
	GHA	Mycotrol-O	ditto	USA	Various organic fruits, vegetables, & ornamentals	Aphids, whitefly, thrips, weevils & beetles, leafhoppers, psyllids, crickets.
	ATCC 74040	Naturalis-L*	Troy Biosciences Inc. USA	Greece, Italy, Mexico, Spain, Switzerland, USA	Fruit, vegetables, ornamentals, cereals, cotton,	Thrips, mites, Coleoptera, whitefly, aphids, caterpillars, grasshoppers, crickets,
	-	Ostrinil	Natural Plant Protection France	France	Maize	Corn borer (*Ostrinia nubilalis*)
	-	Trichobass-L & P	AMC Chemical / Trichodex, Spain	Spain	Cotton, sugarcane, vegetables, fruit, ornamentals	Whitefly, aphids, thrips, beetles, mites.
Beauveria brongniartii	-	Engerlingspilz	Andermatt Biocontrol AG Switzerland	Switzerland	Meadow, Turf	Cockchafer (*Melolontha melolontha*)
	-	Betel	Natural Plant Protection France	France	Sugar cane	Sugar cane beetle (*Hoplocheus marginalis*) : may be confined to use in French overseas protectorates?
Lecanicillium longisporum (*Verticillium lecanii*)	-	Vertalec	Koppert, Netherlands	Finland, Japan, Norway, Switzerland, UK	Tomato, Cucumber, beans, Aubergine, Lettuce, Pepper, Ornamentals	aphids
Lecanicillium muscarium (*Verticillium lecanii*)	-	Mycotal	ditto	Finland, Italy, Japan, Netherlands, Norway, Switzerland, Turkey, UK. Registration pending in Denmark.	Tomato, Cucumber, beans, Aubergine, Lettuce, Pepper, Ornamentals	Whitefly, Thrip
Metarhizium anisopliae	FI-1045	Bio-Cane Granules	Becker-Underwood Inc. Australia	Australia	New plant cane crops	Greyback canegrub (*Dermolepida albohirtum*)
	F001	Bio-Green Granules	ditto	Australia	Pasture, Turf, Lawns	Redheaded cockchafer (*Adoryphorus couloni*)
	F52	TAE-001, Taenure	Earth Biosciences Inc. USA	USA	Ornamentals	Thrips, weevils, sciarids, shore fly,
	F52	Tick-Ex EC & G	Earth Biosciences Inc. USA	USA	Lawns, dwellings	ticks
	ESF1	BioBlast	EcoSciences Corp., USA	USA	Lawns, dwellings	termites
Metarhizium anisopliae subsp. *acridum*	FI-985	Green Guard SC	Becker-Underwood Inc. Australia	Australia	unspecified crops	Locust, wingless grasshopper
Paecilomyces fumosoroseus	apopka strain 97	PFR-97 20% WDG	Certis USA,	USA, unspecified countries	Ornamental plants	Spider mite, thrip spp., whitefly spp., aphids
	apopka strain 97	Preferal WG	BioBest Biological Systems, Belgium	Belgium, Finland, France, Luxemburg, Netherlands, Norway, Poland, Sweden.	Tomato, cucumber, ornamental crops, glasshouse crops	greenhouse white fly (*Trialeurodes vaporariorum*), some countries *Bemisia tabaci* on tomato

*Shaded area highlights isolate used in this research
Data from Kabaluk & Gazdik, 2005, (http://www.agr.gc.ca/env/pdf/cat_e.pdf); the US Environmental Protection Agency biopesticide database (http://www.epa.gov/pesticides/biopesticides); and *D. Chandler pers. comm*.

There has been a steady trend of increasing numbers of micro-organisms being adopted as agents of biological control which is, perhaps, best illustrated by the increasing number of inclusions appearing in subsequent editions of 'The Biopesticide Manual', a compendium of OECD registered biological-based products produced by the British Crop Protection Council. In the 1998 edition, just 59 micro-organism-based products (including fungi, bacterial toxins, viruses and nematodes) were listed, but this increased to 96 in 2001 and 110 in 2004. Overall data for market value varies considerably between sources, however well quoted figures suggest that during 2001 the world biopesticide sales were thought to approximate $300million, a little over 1% of the estimated $25billion agrochemical market (Anon, 2001).

1.6.2 The Genus Beauveria

According to Li et al., (2001) there are five species within the genus *Beauveria – B. bassiana* (Balsamo) Vuillemin, *B. brongniartii* (Sacc.) Petch, *B. araneara* (Petch) von Arx, *B. felina* (Carmicheal et al.,) and *B. amorpha* Samson & Evans. Two of these species, *B. bassiana* and *B. brongniartii*, are widespread in nature and have been investigated extensively as biocontrol agents.

***Beauveria bassiana*:** The entomopathogenic fungus, *B. bassiana*, is also known as the 'white muscardine fungus', because of the characteristic white un-pigmented fungus that appears on infected cadavers. It was the first recognised entomopathogen after it was demonstrated by Bassi in 1834, that it could be used to infect the larvae of the silkworm, *Bombyx mori* L. (Rehner, 2005). This species is perhaps the most widely studied of all the entomopathogenic fungi (Tanada & Kaya, 1993). There are three main commercial biopesticide products based on it: the BotaniGard and Mycotrol family of products (sold and produced by Laverlam in the US, and licensed and sold by Certis in Europe) and Naturalis (developed by Troy Biosciences in the US, licensed and sold by Intrachem Bio in Europe) and a product for corn borer control, Ostrinil, from NPP (formerly Calliope) in France (see Table 1.2). BotaniGard / Mycotrol and Naturalis are labelled for control of sap feeding pests (whitefly, thrips, aphids, psyllids) plus some Coleoptera (mainly weevils), Lepidoptera and grasshoppers for use on protected crops and field vegetables. *Beauveria bassiana* is also used widely in China for the control of forest Lepidoptera (Mcfadden et al., 1981). Well organised systems exist for its mass production, which is largely based around the production of aerial conidia and there is a great deal of expertise that has been accrued over the knowledge of formulations and application within the companies producing it (D. Chandler, *pers comm.*). Overall, *B. bassiana* has been reported to infect a wide range of insects and mites, but significant variation in host range exists between different fungal strains (McCoy et al., 1988).

1.6.3 Mode of action of B. bassiana

Registered commercial preparations of *B. bassiana* are exclusively supplied as conidia, which is a robust spore life-stage. The conidia are small spherical structures (ca. 3.5 µm diameter) which may be easily dispersed by air currents (Hodge, 2003). The precise abiotic and biotic factors which determine optimum growth and pathogenicity of the fungus vary considerably between isolates. However, Ekesi et al., (1999), found that optimal growth of their isolate occurred at 23-25^{o}C and Ferron (1981) determined that conidia required a relative humidity of at least 92% to germinate. Once attached to the host, the conidia form germ tubes that penetrate the host's cuticle and circulate as blastospores throughout the haemocoel (Pekrul & Grula, 1979). Death of the host is caused by a combination of dehydration, destruction of bodily organs and, possibly,

the release of secondary metabolites such as beauvericin and bassianolide (Inglis et al., 2001; Vey et al., 2001). The infected insect may take 3-10 days to die from the infection, after which, if conditions are optimal, conidiophores will break through thinner parts of cuticle such as intersegmental membranes and secondary cycling of the fungus may occur in new hosts.

1.6.4 The activity of fungal entomopathogens in a stored product setting

The key attributes that are required by fungal entomopathogens used in a stored product environment are virulence against the target pest, ability to infect the pest under typical ambient storage conditions (low-medium humidity 5-50% r.h., moderate temperatures <30^{o}C, *I Baxter pers. obs.* at seed oil production silos, UK), ability to persist within the storage facility for a reasonable period of time after application, no negative effects of the pest control agent to operators/warehouse production staff and certainly no negative effects on the consumer should the pathogen enter the food chain. In addition to this, the isolate must be easily mass produced and sufficient and reliable shelf-life so as to offer a reasonable period of product storage stability.

A practical use for fungal pathogens on a large scale is yet to be implemented in a stored product environment (Schöller et al., 2006) and one reason for this may be the lack of available moisture. A high relative humidity (Ferron, 1981) is required for the conidia to germinate, the dry controlled atmosphere of a food storage depot may not seem like a viable setting from which to implement fungal pathogen control. Lord (2005) investigated the use of combinations of desiccant dust (diatomaceous earth and 10% silica gel) and *B. bassiana* to control the storage pest beetle *Rhyzopertha dominica* (F.) at different levels of relative humidity. Somewhat contradictory to common thought, the author found that efficacy of *B. bassiana* was at its highest when the humidity was at the lowest level tested (43%). The author hypothesises that the apparent increase in efficacy is attributable to moisture loss by the insect itself. An additional benefit from low humidity is shown by Smith et al., (2006), who found that mixing the wood ash remains from a fire with conidia of *B. bassiana* was an effective way of controlling the stored product pest, *Prostephanus truncatus* (Horn). The authors proposed that the dry microclimate within the ash 'dust' layer may be prolonging the viability of the conidia by preventing them from germinating. Whilst inoculation of pathogen with *B. bassiana* may indeed cause death, even at low humidity, the ability to cause secondary recycling of the pathogen (*i.e.* sporulation of the fungi from the infected host acting as a foci of infection for conspecifics) would most likely be reduced (Luz and Fargues, 1998).

1.7 Enhancing deposition of active ingredients to the target by electrostatic charging

According to Law (2001), the concept of using electrostatic technology to improve the targeting of pesticide applications was originally proposed by Bowen (1951), in which pesticide dusts were electrostatically charged to enhance their adherence to plant surfaces. Today, numerous companies produce commercial pesticide spray applicators incorporating electrostatic technology and laboratory and field trials have demonstrated that electrostatic spray systems increase target deposition of pesticides (Brown et al., 1997; Law & Lane, 1981). Herzog et al., (1983) treated cotton plants over a 3-year period and demonstrated that electrostatic spraying of half the dosage of permethrin and methyl parathion provided control of *Heliothis spp*. equivalent to conventional spraying. Similarly, Gaunt et al., (2003) demonstrated that through increasing the electrostatic charge on an insecticide aerosol, the deposition of insecticide onto houseflies may be increased. These electrostatically charged sprays of bioallethrin and bioresmethrin could be used in the domestic environment to increase the knockdown of houseflies compared to conventional sprays.

1.8 'Electrostatic' Powders

So-called 'electrostatic' powders, Entostat™ and Entomag™ manufactured by Exosect, Winchester, UK, are used extensively in this research to evaluate their potential as carriers of fungal conidia within an autodissemination bait station. Some of the earliest widely used insecticide preparations such as sulphur and rotenone were applied as dry dusts directly to the crop (Cremlyn, 1991). However, in recent years the use of powders in pest management has declined, largely due to the fact that small particles are often not crop-fast, particularly when applied in windy climates (Matthews, 1992). Concern about the adverse effects of pesticides on the environment and residues in food products has increased the need for improved delivery systems to reduce the quantities of pesticides used and to target their application more effectively (Matthews & Thomas, 2000). Recent research has focussed on the use of 'electrostatic' powders, which the manufacturers claim are constructed from materials that obtain a relatively high charge to mass ratio in order to aid adhesion to the cuticle of the pest (Howse & Underwood, 2000). These powders may be combined with insecticidal active ingredients and contained within a bait station to which the pest is attracted with pheromone or food baits. These powders, have been shown to adhere to insects and pass between conspecifics during normal social activity such as mating and lekking (Baxter et al., 2008; Armsworth et al., 2006; Barton et al., 2006). A summary of the research carried out with Entomag and Entostat is shown in table 1.4.

Table 1.4. A summary of published research carried out with Entostat™ and Entomag

Author	Pest Species	Active ingredient	Powder	Summary
Howse and Underwood (2000)	Medfly Olive fly Codling moth Diamondback moth Brown-tail moth	Chlorpyrifos *Metarhizium anisopliae*	Entostat	Speculative report with very few results, concentrating more on potential future opportunities
Barton et al., (2006)	Medfly	-	Entostat	Measured powder uptake by male and female medfly. Found that smaller Entostat diameter particles were more readily taken up and that powder was passed between conspecifics during mating.
Armsworth et al., (2006)	Medfly	-	Entostat Entomag	Effects of powder contamination on flight and mating behaviour. Temporary (1-h) arrestant effect of contamination to males with both powders, although Entostat effects more pronounced than Entomag.
Nansen et al., (2007a)	Grape Berry Moth	Pheromone	Entostat	Effects of powder (with and without pheromone) contamination on EAG responses, mating success, anemotactic mate-location in a wind tunnel. Marginal effects of blank powder observed, considerable effects when combined with pheromone.
Nansen et al., (2007b)	Stored-grain beetles	Aggregation pheromone	Entostat	Entostat was shown to be repellent to some beetles when present in quantities >5% in food. Beetles still became contaminated with powder when residue of dust placed in crack/crevice treatment.
Meikle et al., (2007)	*Varroa* mite	*Beauveria bassiana*	Entostat	Entostat used a diluent with conidia of *B. bassiana*. Entostat-*B. bassiana* used in a 10:1 ratio, although no rationale given for doing this. *B. bassiana* infection high in *Varroa*, but low with bees.
Baxter et al,. (2008)	*P. interpunctella*	-	Entostat/ Entomag	Uptake, retention and horizontal transfer of powder (see chapter 2).

1.9 Insects and electrostatics

It has been demonstrated that insects can acquire and retain a significant electrostatic charge (Gan-Mor et al., 1995). The charges may arise from friction between the wings and other body parts of the insect or from charge separation following contact with surfaces (Jackson & McGonigle, 2005; McGonigle et al., 2002; McGonigle & Jackson, 2002) or other bodies in airborne suspension (Cross, 1987). Frictional or, tribocharging, is the process of the transfer of electrons from one surface to another during contact and resulting in equal but opposite charges arising on each surface (Cross, 1987). Different materials require varying amounts of energy to lose electrons which is said to be their work function. A material which donates electrons more easily than the surface it is in contact with will become positively charged whereas the benefactor of the electrons will become more negatively charged. Materials may be ranked by work function into a triboelectric series with positive charging materials at the opposite end to negative charging ones (Cross, 1987). Gan-Mor et al., (1995) proposed that frictional charging (tribocharging) during flight and through wing-fanning behaviour is likely to generate a significant charge. Previous authors (Edwards, 1962; McGonigle et al., 2002; McGonigle & Jackson, 2002; Yes'Kov et al., 1976) have demonstrated that frictional charging by surface contact had a significant effect upon insect charge. Insects may also become electrostatically charged by other means including ionic absorption through the insect cuticle via dermal pores (Scheie & Smyth, 1967). Cross (1987) also describes how charge of a body may be influenced by induction charging and through the adhesion of charged particles to the exterior, both of these concepts are pertinent to the insect model.

Measurements of electrostatic charges on insects have been investigated on bees (Erickson, 1975; Yes'Kov & Sapozhnikov, 1976; Corbet, et al., 1982; Colin et al., 1991), hornets (Ishay, et al., 1991) houseflies (Edwards, 1962; McGonigle and Jackson 2002; McGonigle, et al., 2002) beetles (Edwards, 1962) and moths (Edwards, 1962). Several authors have hypothesised that electrostatic charges are important in the adhesion of pollen particles from flower to insect (Erickson, 1982, Erickson & Buchmann, 1983). Yes'Kov and Sapozhnikov (1976) calculated that the electrostatic potential of a foraging honeybee may reach hundreds of volts. When Corbet et al., (1982) replicated these potentials experimentally, pollen grains of oilseed rape were found to jump a distance of 0.5 mm across an air gap from flower to freshly-killed honeybee. Later, Gan-Mor et al., (1995) used a wind-tunnel to measure an average charge of 23.1 pC on a flying bee. They also calculated the forces required to detach pollen from a range of commercial crop plants including avocado and eucalyptus. Mathematical modelling suggested that non-contact pollen detachment from flower to bee was possible. Erickson (1982),

hypothesised that the reason a honeybee has higher electrostatic charges on the pore plates of the antennae relative to the rest of body is that there will be increased deposition of airborne particulate matter to these specialist odour detecting regions and that this could enhance their ability to detect floral scents.

1.10 Effect of electrical charges on insect behaviour

Some authors have found that certain weak electric fields may act as repellents to certain insect species. Perumperal et al., (1978), found that houseflies presented with a choice chamber were less likely to settle in retreats subjected to an electrical field while Orlov (1990) found that flying insects were repelled by the electric fields surrounding high voltage power cables. Maw (1964), observed that crawling insects were severely slowed in their progress when presented with a charged surface.

With the potential complication of electrostatic repellence to consider, the material from which the bait-station is constructed should be selected on the basis that it does not cause repellence of the target insects. Maw (1964), found that certain species of parasitic wasp (Hymenoptera) were repelled from plastics that had become tribocharged from air movement. The amount of charge that is accumulated on the trap will be dependent on the material that is used and the environment in which it is placed. As air currents increase the station will become tribocharged both from molecules in the air and by contact with the surface on which the station is attached. However, environmental conditions will affect the charge build up of a station considerably. The higher the humidity then the faster the charge is decayed through dissipation of electrons to the water vapour. It is, therefore, an important consideration that both the autodissemination station and powder itself are not constructed from materials that are repellent to the target insect.

2. The potential of two electrostatic powders as the basis for an autodissemination control method of *Plodia interpunctella* (Hübner)

Abstract

A comparison of the retention, horizontal transmission and effect on mating of two electrostatic powders (Entomag™ and Entostat™) was made to evaluate their potential as a component of an autodissemination method for the control of *P. interpunctella*. Both powders were shown to have some effects on mating behaviour and ability of treated males to locate a pheromone source when applied in high doses. However, no effects were observed at rates consistent with the amount of powder that was actually taken up when the moths visited a prototype bait station device. Male and females lost 69.9 and 64.3% by weight respectively of Entomag by 48h after exposure but lost more Entostat, 89.8% and 75.9%, over the same period. Critical to the efficacy of autodissemination is the transfer of powder from males to females and on average a 49% greater weight of Entostat was transferred than Entomag. Due to the different densities of the powders it was calculated that a 49% increase in transfer of powder equated to over three times more Entostat than Entomag particles being passed from male to female. It was concluded that Entostat would appear to be the carrier of choice for a prototype *P. interpunctella* autodissemination system.

2.1 Introduction

The Indianmeal moth is a widespread major pest of raw and processed stored food products (Hinton, 1943; Lecato, 1976; Storey et al., 1983). The larvae are particularly adept at penetrating a wide range of packaging materials (Cline, 1978) and the large amounts of webbing they produce blocks machinery and binds product together (Rees, 2004). Allergens have also been associated with the Indianmeal moth (Binder et al., 2001), which may lead to conditions such as asthma, rhinoconjunctivitis and dermatitis developing in sensitive individuals.

Since treatment of stored products with methyl bromide is to be phased out by 2015 (Anon, 2002), alternative treatments for stored product pest protection are being urgently sought. Research into biorational control measures for the Indianmeal moth have included controlled

low-oxygen environment (Johnson et al., 2002), high temperature controlled atmosphere (Sauer and Shelton, 2002), mating disruption (Fadamiro and Baker, 2002), gamma irradiation (Azelmat et al., 2005), desiccants (Mewis and Ulrichs, 2001), granulosis virus (Vail et al., 1993) and attracticides (Nansen and Phillips, 2004).

Adult male Indianmeal moths will positively orientate towards a source of (Z,E)-9,12-tetradecadienyl acetate (ZETA), a compound identified as being part of the sex pheromone secretion released by the female (Brady et al., 1971; Kuwahara et al., 1971). Traps using a lure containing ZETA and a sticky catch board are frequently used for monitoring for the presence of Indianmeal moth (and several other Pyralid species that also respond to ZETA). As it is possible to lure Indianmeal moths into monitoring traps containing ZETA, the concept of a lure and kill system, whereby an insecticidal active ingredients (or the 'affector', Lanier, 1990) are contained within a bait station, seems like a viable approach to suppress moth populations. Nansen and Phillips (2004) showed that a combination of ZETA and permethrin as an attracticide for Indianmeal moths resulted in reduced mating frequency when treated males were paired with virgin females. However, a male attractant in a lure and kill system containing a rapidly acting insecticide is only going to impact on the male population of moths. As both male and female Indianmeal moths are polygamous, with males capable of mating up to 10 times (Brower, 1975), one can reasonably assume that over 90% of the population of males need to be removed before there will be an impact on the overall population. A potentially more efficacious control could be achieved if the active ingredient also targeted the female moth.

A modification on the lure and kill technique is 'autodissemination' (Soper, 1978) whereby insecticidal active ingredients are horizontally transmitted between conspecifics. Soper (1978) refers to a technique by which biological agents, in particular fungal entomopathogens, would establish foci of infection in a population of insects and the living insects themselves would transmit the pathogen throughout the population. Using a similar approach in grain stores, Smith et al., (1999) examined the use of combining a pheromone attractant and entomopathogenic spores into a vegetable fat pellet formulation to act as an 'auto-inoculation' dispenser to control the larger grain borer (*Prostephanus truncatus* (Horn)). Most pertinent to this programme, Vail et al., (1993) examined the autodissemination of granulosis virus by the Indianmeal moth. In their experiments, Vail et al., (1993) demonstrated larval mortality of 52-60% from virus taken up by males and subsequently passed to females and oviposition sites.

The success of an autodissemination approach may be increased if the active ingredient (a.i.) can be colocalized with an adhesive carrier (Armsworth et al., 2006; Barton et al., 2006). For a powder to be used as a carrier of an a.i. in a bait station, it must have several important qualities

in that it must stick to the target pest in sufficient quantities and must stay in place long enough for the a.i. to have an effect. These qualities are desirable if the aim is to kill or sterilise the original recipient, but a better level of pest suppression may be obtained if the powder is readily passed between conspecifics.

In this study two proprietary electrostatic powders, Entostat™ and Entomag™, belonging to Exosect Ltd. (Winchester, UK) were evaluated for their retention, horizontal transmission, effects on mating frequency and effects on ability to locate a pheromone source. Previous studies (Armsworth et al., 2006; and Barton et al., 2006) have demonstrated that these powders adhere to Mediterranean fruit fly (*Ceratitis capitata* (Wiedemann)) and that a bait station system may be an effective method of disseminating a.i.'s throughout the pest population. However, Nansen et al., (2007a) working on the Lepidopteran vine pest, *Lobesia botrana* Dennis & Schiffermuller, found that inoculation with blank Entostat powder caused a reduction in male moths making contact with calling females. As it is essential for the autodissemination concept that treated moths can both locate and initiate contact with conspecifics the studies presented here aim to examine factors that might impact on the use of Entostat and Entomag as autodissemination carrier powders in an Indianmeal moth bait station approach to pest suppression.

2.2 Materials and Methods

2.2.1 Test insects and rearing conditions

See Appendix I for culturing information. For all tests virgin moths of 24-48 h old were used.

2.2.2 Carrier powders

See Appendix II for details of the carrier powders, Entostat™ and Entomag™.

2.2.3 Quantification of powder uptake

The amounts of Entomag and Entostat taken up by the test insects were quantified using a fluorescent assay, which incorporates a dye (Glo-Brite® AW Powder, Himar, Bradford, UK) into the powder (see Armsworth et al., 2006 and Barton et al., 2006 for further details). From hereon in "dyed powder" refers to Entostat or Entomag that has been mixed with Glo-Brite dye.

2.2.4 Powder dosing

A sample of powder was measured into a plastic general-purpose container with hinged lid (57 mm length x 17 mm diameter) (code 216-3136, from VWR International; www.vwr.com). The moths were exposed to four amounts of powder – 1-3 mg, 5-7 mg, 9-12 mg, 17-20 mg, except in experiment 4 where powder doses of up to 200 mg per tube were used; this was because earlier observations had revealed no discernable treatment effects between 1-3 mg rate and the 17-20 mg rate. The powder was applied to the sides of the container using a plastic (polypropylene) drinking straw in a circular movement so as to create a fine film of powder. A single virgin moth was held in a glass 5 ml sample tube and then tipped into the powder tube. Once in the treatment tube, the glass vial was quickly reversed so that the base formed a seal over the end of the tube. The tubes were held in place as still as possible, for 1 min, before the moth was allowed to climb back into the glass holding tube. To mitigate the risks of powder contamination, treatments were applied in a different room to the observational phase of the experiment and powder sample tubes were disposed of after a single replicate. Each powder sample tube was to treat only a single batch of moths, each batch being a single replicate for statistical purposes. For all treatments there were five such replicates.

2.2.5 Experiment 1. Sequential dosing from the same tube

To determine if there was a significant decline in powder uptake between the first and last test insect to be treated in a single tube, the lowest rate of powder (1-3 mg) was tested for both

Entostat and Entomag. Ten male moths were treated sequentially and their position in the treatment sequence noted. The amount of powder adhering to the moth was then calculated using the fluorometric assay described by Armsworth et al., 2006 and Barton et al., 2006. This experiment was repeated 5 times for each powder. The amount of powder taken up by moths treated first and tenth from the same tube was compared.

2.2.6 Experiment 2. Powder retention over time

For each treatment and treatment duration, five male or female moths were treated in a tube containing one of the four treatment rates of either dyed Entostat or Entomag. After treatment they were immediately placed individually into a holding arena (polypropylene food storage container, 26 cm x 26 cm x 26 cm, Asda, Chandlers Ford, UK) with a sheet of laboratory roll tissue paper held in place with sticking tape as a breathable lid. The entire cage was held at 24-27^{o}C, 60-80% r.h. and 16:8 h light/dark cycle. After the treatment time had elapsed, moths were immobilized by brief placement in a -18^{o}C freezer (<10 min) and removed individually using entomologists forceps (washed in ethanol between transfer of each individual to avoid powder contamination) into individual 1.5 ml microfuge vials (Eppendorf®) for fluorescent powder uptake analysis. Moths were analysed at 0, 1, 2, 4, 8, 12, 24 and 48 h after treatment. Five replicates were examined per treatment duration for each of the two powders.

2.2.7 Experiment 3. Powder transfer between conspecifics

Five female moths were placed into a holding cage as in Experiment 2. Five male moths were treated with powder as in Experiment 2 and placed into the holding cage with the females. After 24 h the female moths were immobilized and analysed for the amount of powder they had taken up. To correct the data for powder that might have been picked up by females walking over surfaces contaminated by males, five untreated males and five females were subsequently placed into the same holding cage for 24 h and also analysed for powder uptake. The observed mean value was used to correct the estimated uptake by females exposed to males. There were five replicates for each treatment (one replicate being 5 ♀).

2.2.8 Experiment 4. Effect of powder on mating behaviour

Five female moths were placed into holding arenas as in Experiment 2, except that in these tests the lid was constructed from thin cloth gauze so that the observer could see into the box. Male moths were treated with powder as in Experiment 2, except the amount of powder in the dosing tubes was either 20, 100 or 200 mg. Observations were made every 10 min to record the number

of moths mating. Mating was defined as 'coupling' and did not include courtship behaviour. Observations continued for 280 min. One replicate from each of the four treatments (control, 20, 100 and 200 mg) was sampled at the same time, so as to mitigate errors brought about by changes in mating propensity due to external factors such as time of day, ambient lighting conditions, *etc*. Although carried out on subsequent days all the replicates were started between 11.00 am and 13.30 pm and under laboratory lighting.

2.2.9 Experiment 5. Effect of powder on males' ability to locate a sex pheromone source

A 10% stock solution of the principle Indianmeal moth sex pheromone component (Z,E)-9,12-tetradecadienyl acetate (ZETA) (Bedoukian Research Inc., Danbury, CT, USA) was prepared in acetone (99% GC Grade, Sigma, UK). From this, a 1 mg quantity of ZETA was loaded onto a single rubber septum (Sigma, UK). The pheromone source was placed into the centre of a moth monitoring trap (Exosect Ltd, Winchester, UK), consisting of a delta-style outer section (157 (l) x 110 (w) x 110 (h) mm) and a sticky glue board along the base of the trap (152 x 106 mm). The monitoring trap was placed into a bioassay room (4.3 (l) x 2.3 (w) x 2.3 (h) m, 24-27^{o}C, 60-80 % r.h. and 16:8 h light/dark cycle). A second monitoring trap, minus the pheromone source, was placed at the other side of the room – this was to determine if moths were attracted to a separate stimuli such as humidity from the sticky board, rather than locating the pheromone lure. All male moths, including the untreated controls, were lightly anaesthetised using humidified CO_2 gas and marked on the apex of their wings using a non-toxic marker paint (Uni Posca, Bullet tip, medium line, Mitsubishi Pencil Co., Ltd, Japan) with different coloured dots (ca. <2 mm diameter) depending upon their assigned treatment which was control (untreated), 20, 100 or 200 mg of either Entomag or Entostat. The marked male moths were exposed to their respective treatments and placed into individual holding tubes (details as per the dosing tubes) and released from the centre of the bioassay room by placing the tubes on the floor on their sides, opening up the lids and allowing the moths to walk out. Previous studies had shown that the treated moths were often unable to exit the tube unless it was placed on a side as the powder acted like a pitfall trap. The monitoring traps were examined for numbers of moths caught after 24 h and the treatments identified by examining the wing tips under a dissection microscope. There were five replicates of five moths per treatment and for each of the two powders.

2.2.10 Experiment 6. Uptake of powder from a dispenser

A standard Exosect Exosex™ dispenser (the delta trap as described in Exp. 5, except that the monitoring board is replaced by an Exosect powder dispensing tray - 152 x 106 mm plastic base with sixty 4 x 4 x 4 mm tapered depressions) (Exosect Ltd, Winchester, UK). This tray was either empty (control), or contained 2.5 g of either dyed Entostat or Entomag powder. The entire dispenser was placed into the centre base of a ventilated Perspex box (600 (l) x 600 (h) x 400 (w) mm). A pheromone source was added to the dispenser as per Experiment 5, except that it contained 100 ng ZETA rather than 1 mg loading due to the Perspex box being in a confined space and previous authors have used this quantity in wind tunnel tests (Zhu et al., 1999). Ten male moths were released into the arena and left for 30 min. After this time, all moths were individually caught and placed in holding tubes before powder analysis was carried out. The Perspex box was thoroughly washed between treatments to avoid contamination left by moths walking over surfaces. As moths may have become contaminated with powder but not necessarily visited the dispenser, only those determined as having two standard deviations or more powder on them compared to the control were considered as having visited the dispenser. This study was replicated five times.

2.2.11 Experiment 7. Scanning electron microscopy

Moths were treated with powder as per experiment 6, then left in holding cages for 24 h. The moths were killed by freezing at -18^{o}C for ca. 20 min, before being placed into individual 1.5 ml microfuge vials (Eppendorf®) for storage. Individual moths were glued onto aluminium stubs (Agar Scientific Ltd, Stanstead, UK) and viewed using a FEI Quanta 200 scanning electron microscope (FEI Company, Cambridge, UK) in variable pressure mode (10 Kv and 0.4 Torr). Moths were examined for the presence of powder on body surfaces.

2.2.12 Data analysis

All statistical analyses were carried out using MINITAB (MINITAB Inc, v14).

In Experiment 1, data were normalized using log10(x+1) transformations. One-way analysis of variance (ANOVA) were performed to determine whether or not sequential treatment from the same tube had any effect on the amount of powder taken up by the moth at the lowest rate used (1-3 mg).

In Experiment 2, data were normalized using log10(x+1) transformations. One-way ANOVA were performed for each time point to examine for any differences in powder uptake either by

sex or by powder type. Tukey's *post hoc* test was used to establish which treatments were significantly different.

In Experiment 3, data were log10(x+1) transformed and the mean of powder taken up for each of the five replicates were calculated. A one-way ANOVA was carried out to examine the effects of powder type on secondary transfer to females.

In Experiment 4, binary logistic regression analysis was carried out with the control group reference factor subtracted from the other treatments to identify significant differences in the number of pairs of moths mating from that of the control.

In Experiment 5, data were normalized using log10(x+1) transformations. One-way ANOVA were performed for each time point to examine for any differences in powder uptake either by sex or by powder type. Tukey's *post hoc* test was used to establish which treatments were significantly different.

In Experiment 6, not all moths entered the dispenser, yet still became contaminated by powder present on the surface of the arena. Therefore, moths were only judged to have entered the dispenser if they were determined as having taken up more than two standard deviations of powder from that of the control. A Mann-Whitney U test was conducted to compare the amount of powder taken up from the dispenser for each of the powders.

2.3 Results

2.3.1 Experiment 1. Sequential dosing from the same tube

There were no significant differences in powder uptake between moths placed into the tubes either first or tenth (Entostat ANOVA: $F_{1,8} = 0.02$, $P = 0.0891$; Entomag ANOVA: $F_{1,8} = 0.13$, $P = 0.0729$). Due to resource constraints experiments proceeded with a maximum of five moths being treated from a single tube.

2.3.2 Experiment 2. Powder retention over time

Dyed powder analysis showed that there was some variation in the amount of powder uptake by the moths within treatments (table 2.1). Sampling at T = 0 shows a lower powder uptake for the Entostat females (ANOVA: $F_{3,16} = 3.92$, $P = 0.028$) of 10.79 µg, compared to the other treatments which are similar at 17.69, 16.33 and 17.12 µg for Entostat male, Entomag male and Entomag female, respectively. Moths treated with Entostat appeared to lose powder at a faster

rate than moths treated with Entomag. Entostat males lost 89.8% (*SE* ± 0.8) of powder between T = 0 and T = 48 h after treatment, compared to Entostat females, 75.9% (*SE* ± 3.9), Entomag males, 69.9% (*SE* ± 8.9) and Entomag females, 64.3% (*SE* ± 7.0%). The ANOVA analysis (table 1) demonstrated that there were significant differences in the amount of powder retained by the moths for both Entostat and Entomag. Entostat females took up significantly less powder at T = 0 than Entostat males, but not for any of the Entomag treatments. At T = 1 h there were no differences between the treatments, but by T = 4 h, Entomag females had retained significantly more powder than any of the other treatments (ANOVA: $F_{3,16} = 13.50$, $P = 0.004$), a trend which continued for three of the remaining four sample points. At the final sample point, T = 48 h, Entomag males also retained more powder than Entostat males (ANOVA: $F_{3,16} = 11.79$, $P = <0.001$).

Table 2.1. Powder retention on Indianmeal moth. Moths were treated in a dosing tube containing 17-20 mg of either Entostat or Entomag. ANOVA analysis carried out for each time point with differences between treatments calculated by Tukey's test.

Time after treatment (h)	Mean amount of powder per moth (µg) (±*SE*)					
	Males		Females			
	Entostat	Entomag	Entostat	Entomag	F and DF	*P*-value
	a	b	c	d		
0	17.7 (±2.6), b	16.3 (±1.7), *NS*	10.8 (±1.4), a	17.1 (±1.4), *NS*	$F_{3,16} = 3.92$	0.028
1	17.6 (±2.6), *NS*	16.1 (±5.2), *NS*	10.6(±1.3), *NS*	15.5 (±1.1), *NS*	$F_{3,16} = 1.21$	0.338
2	14.0 (±1.6), *NS*	7.8 (±1.7), d	10.1 (±2.2), *NS*	15.3 (±0.9), b	$F_{3,16} = 3.80$	0.031
4	9.0 (±1.1), d	5.6 (±0.5), d	9.0 (±1.0), d	15.2 (±1.3), abc	$F_{3,16} = 13.50$	<0.001
8	6.6 (±0.9), d	5.4 (±0.3), d	8.1 (±1.2), *NS*	12.1 (±1.8), ab	$F_{3,16} = 6.18$	0.005
24	3.9 (±0.3), d	5.8 (±0.6), d	5.8 (±0.9), d	8.9 (±0.3), abc	$F_{3,16} = 9.79$	0.001
48	1.8 (±0.2), bd	4.9 (±0.7), ad	2.5 (±0.4), d	6.1 (±1.4), abc	$F_{3,16} = 11.79$	<0.001

'*NS*' Indicates no significant difference from any other treatment, otherwise values not followed by any of the same lower case letters are significantly different.

2.3.3 Experiment 3. Powder transfer between conspecifics

The amount of powder transferred from treated males to females (fig. 2.1) indicated that Entostat powder transferred to females in greater weights than Entomag with 59.4, 64.8, 42.7 and 30.3% more powder (by weight) being transferred in the 1-3, 5-7, 9-12 and 17-20 mg treatments respectively. Treated moths transferred significantly less Entomag than Entostat in the 1-3 mg treatment (ANOVA: $F_{1,48} = 9.09$, $P = 0.017$), 5-7 mg treatment ($F_{1,48} = 6.22$, $P = 0.037$) and 9-12 mg ($F_{1,48} = 7.43$, $P = 0.026$), but not at the 17-20 mg treatment ($F_{1,48} = 3.12$, $P = 0.115$).

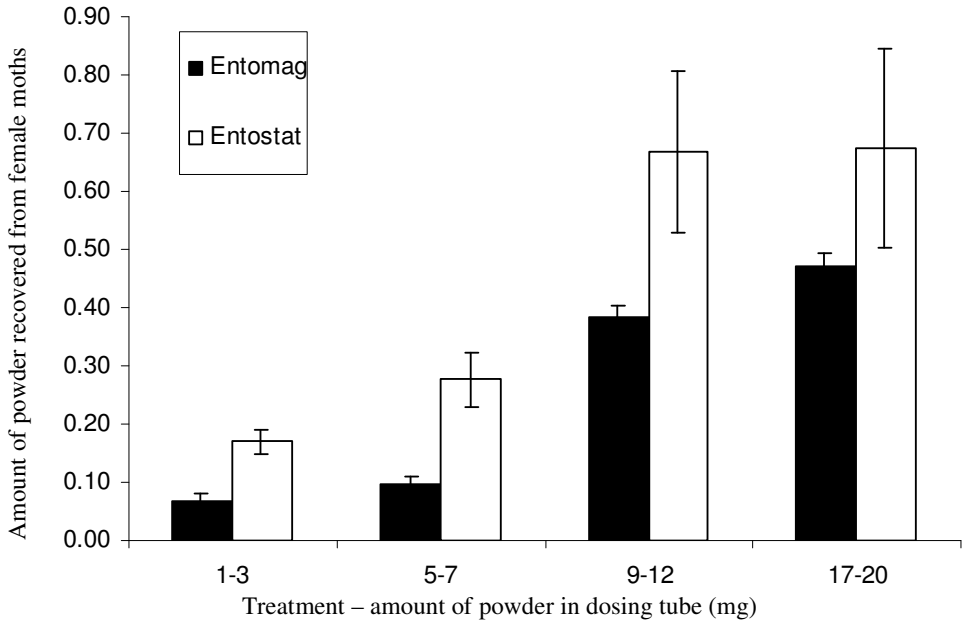


Figure 2.1. Mean weight (±*SE*) of Entomag™ and Entostat™ powders recovered from female *P. interpunctella* confined with treated males for 24h (weights corrected for powder transmission via male-contaminated surfaces – see methods section 2.2.7)

2.3.4 Experiment 4. Effect of powder on mating behaviour

All the powder treatments appeared to affect the proportion of moths mating (figs. 2.2A and 2.2B). However, in both Entostat and Entomag treatments, the difference was only significant at the 100 and 200 mg exposure level (Entostat - control vs 20 mg = *NS*; control vs 100 mg = $P = 0.006$; control vs 200 mg = $P < 0.001$; Entomag - control vs 20 mg = *NS*; control vs 100 mg = $P = 0.031$; control vs 200 mg = $P = 0.003$; $df = 3$).

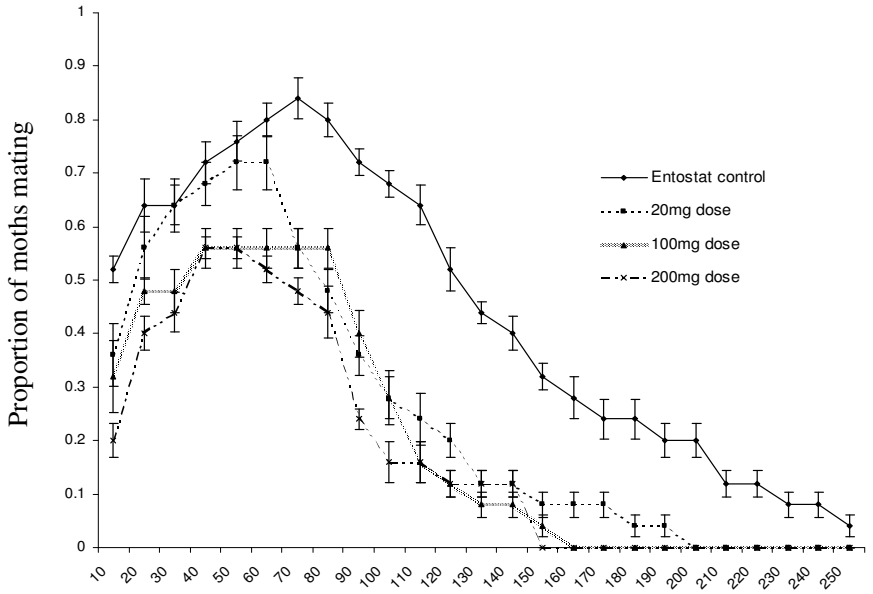


Figure 2.2A

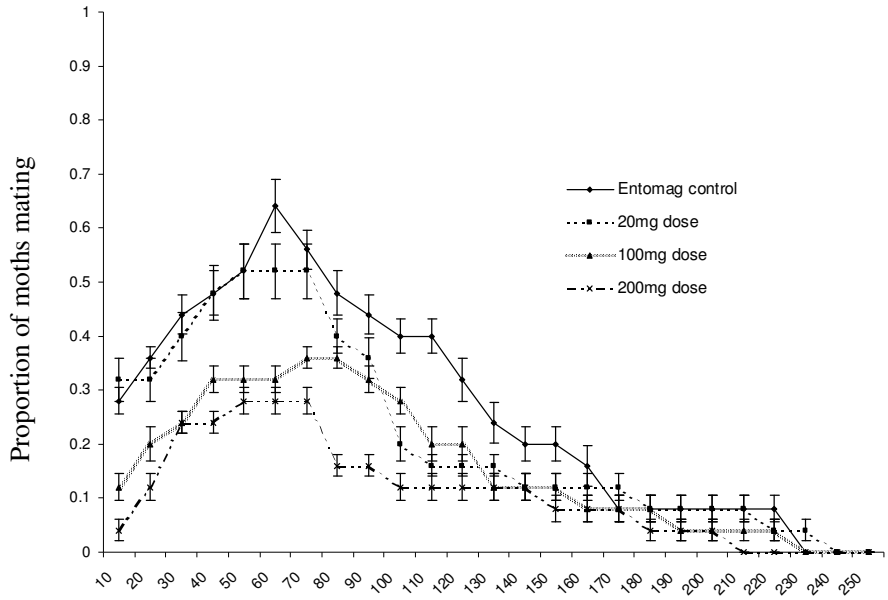


Figure 2.2B

Figures 2.2A and 2.2B. Effects of powder exposure to males on mating frequency. Males were exposed to different amounts of powders before being coupled with females. Observations on the numbers of moth pairs mating were made every 10 minutes (*N*=5).

2.3.5 Experiment 5. Effect of powder on males' ability to locate a sex pheromone source.

The analysis revealed that there were significant differences in the ability to locate a pheromone lure for both Entostat and Entomag treated moths (fig. 2.3) depending on the initial amount of powder they had been exposed to (Entostat - ANOVA: $F_{3,16} = 9.09$, $P < 0.001$; Entomag - $F_{3,16} = 9.33$, $P < 0.001$). With both Entostat and Entomag powder, the Tukey's *post hoc* tests showed that the 20 mg treatment of powder did not differ significantly from that of the control. No moths were captured on the un-baited monitoring trap.

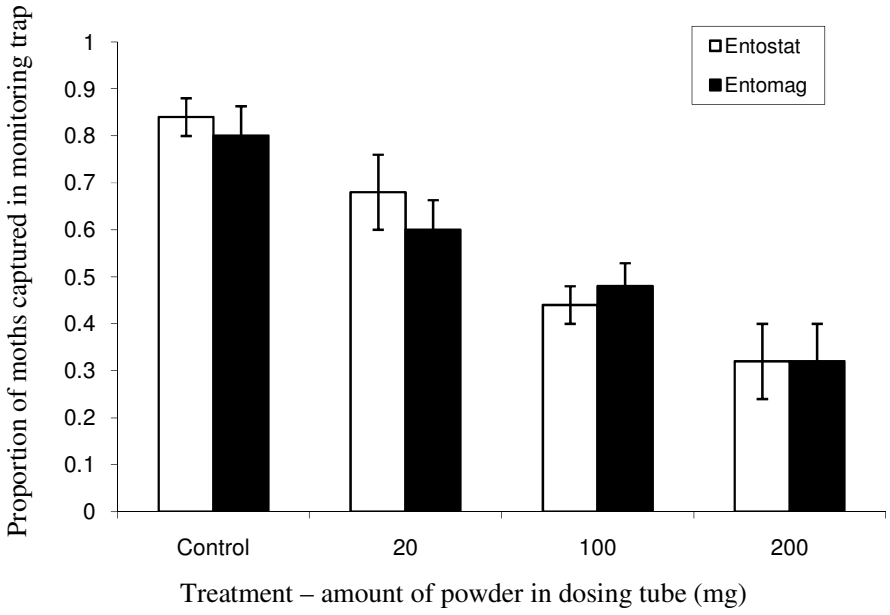


Figure 2.3. Effect of powder on male moths' ability to locate a monitoring trap. Male moths were exposed to different quantities of Entostat or Entomag powder and then released into a bioassay room containing a glue-board pheromone monitoring trap for 24-h.

2.3.6 Experiment 6. Uptake of powder from a dispenser

A total of 45 out of 50 moths were determined as having entered the Entostat station compared to only 17 for the Entomag station. A significantly greater amount of Entostat powder than Entomag powder was taken up by the moths (Mann-Whitney U test, $W = 1645.0$, $P = 0.0003$) (fig. 2.4).

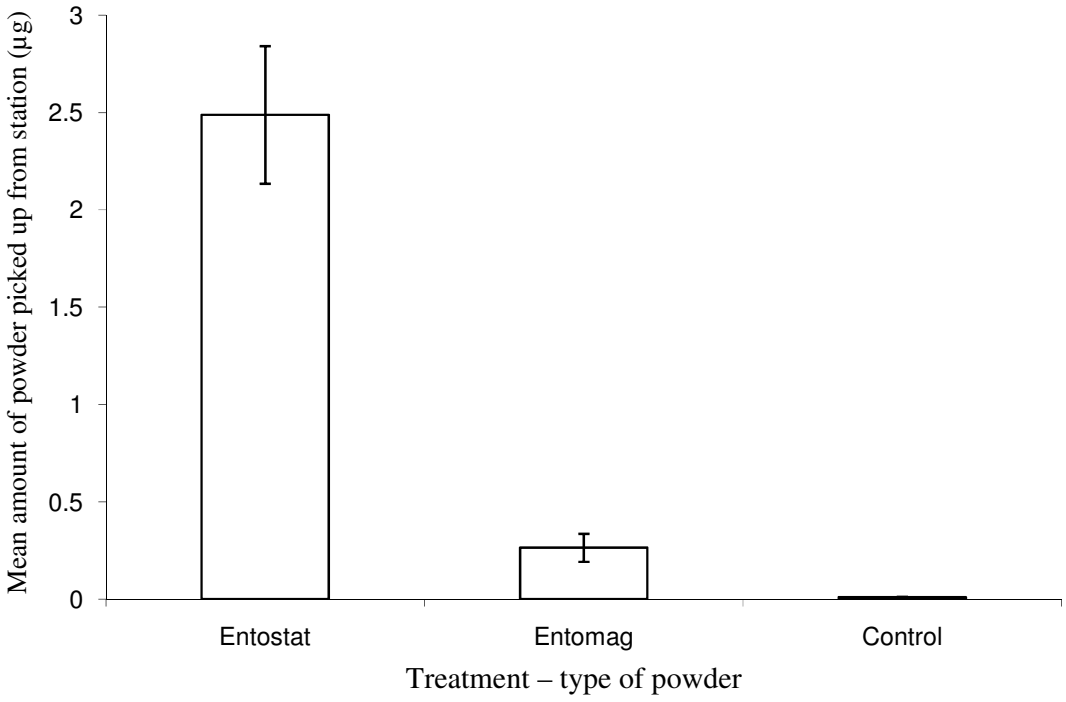


Figure 2.4. Powder uptake from a dispenser. Male moths were released into an arena containing an Exosect powder dispenser with a pheromone source. The amount of powder taken up by the moths was calculated.

2.3.7 Experiment 7. Scanning electron microscopy

Both Entomag and Entostat powders appeared to be retained after 24 h on most body parts (figs. 2.5A, B and C). Particular aggregations of both powders were observed on areas where scales were not present, such as the eyes and tarsi, although particle contamination was visible over the entire body.

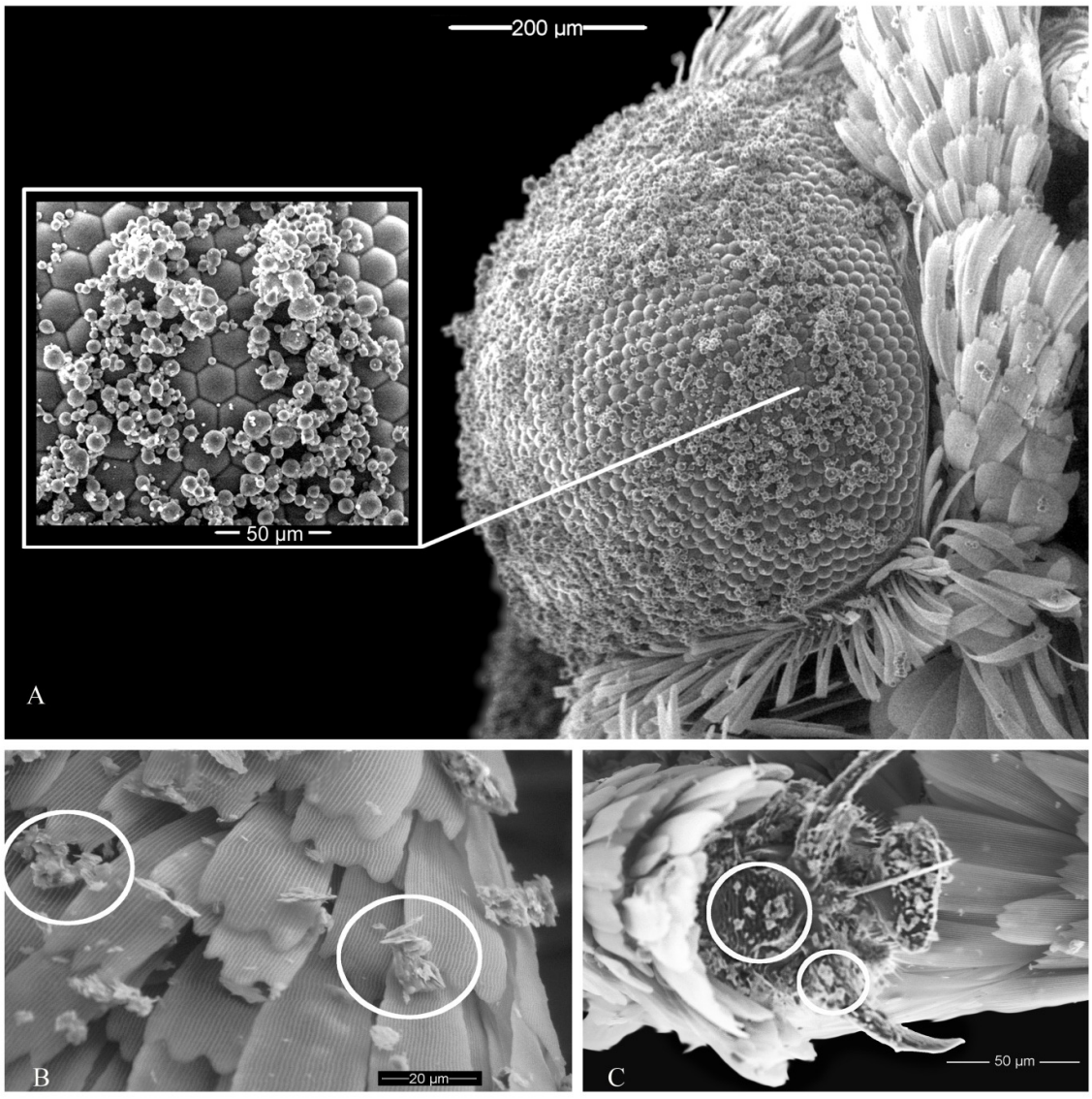


Figure 2.5A. Entostat contamination visible on Indianmeal moth eye.
Figure 2.5B. Entomag contamination on abdominal scales.
Figure 2.5C. Entomag contamination on tarsi.

2.4 Discussion

When a lepidopteran pest species elicits a strong orientation towards a synthetic pheromone lure, a bait station approach for auto-inoculation with an insecticidal active ingredient (a.i.) appears to be a viable option. Typically though, it is only the male of the species that can be attracted in any great numbers to the station and, when both sexes of the pest are highly polygamous, then male-annihilation (Lanier, 1990) would need to penetrate an often unrealistically large amount of the population before it could be successful. A variant of bait station-based 'lure and kill' is 'autodissemination', the use of conspecifics themselves to pass slow-acting insecticidal products throughout the population during normal social behaviour such as mating or aggregation. The horizontal transfer of pathogens from an auto-inoculation device has been investigated with varying degrees of success for a range of pests including *P. interpunctella* (Vail et al., 1993), mosquitoes (Scholt, et al., 2004) and Japanese beetle (Klein and Lacey, 1999).

The success of an auto-inoculation approach may be increased if the a.i. can be co-localised with an adhesive carrier, such as the powders used in this study (Armsworth et al., 2006; Barton et al., 2006). For a powder to be used as a carrier of an a.i. in a bait station, it must have several important qualities in that it must stick to the target pest in sufficient quantities and must stay in place long enough for the a.i. to have an effect. These qualities are desirable if the aim is to kill or sterilise the original recipient, but a better level of pest suppression may be obtained if the powder is readily passed between conspecifics. For example, attractants may be readily available for the male of the species but a greater impact on the overall pest population may be obtained if the a.i., such as a sterilant, can be targeted to the female. For optimal characteristics the powder should be transmitted horizontally between conspecifics during mating or other aggregating social behaviours, and must not affect the behaviour of the male moth to the point that it cannot mate or is not a desirable partner for mating.

The experiments in this study demonstrate that dosing with either Entostat or Entomag potentially have some undesirable effects on Indianmeal moth. Male moths were shown to be less likely to locate a monitoring trap when exposed to the 100 and 200 mg powder tubes and the proportion of moths mating was also reduced at the 100 to 200 mg rate. However, in reality these amounts of powder are unlikely to be encountered by a moth entering a dispensing station as demonstrated in this study and truly represent a worst-case scenario. It is therefore, interesting to note that there were no statistically significant effects on behaviour at the lowest exposure of powder (20 mg). Even with exposure at the lowest rate of 20 mg per tube, moths were taking up 10.8 and 17.6 µg of Entostat and Entomag, which contrasts considerably with the 2.49 and 0.26 µg of Entostat and Entomag respectively, that was taken up during the far more

realistic dosing regimen of moths entering a delta-style dispenser. Even though the dispenser design used in these experiments is far from optimised, it seems reasonable to assume an Indianmeal moth is unlikely to take up powder in excess of that found in the 20 mg tube exposure assays. Indeed, an appropriate dispenser would need to be designed to ensure that the moth does not take up such quantities of powder that could detrimentally alter behaviour. Given that *P. interpunctella* often thrive as pests of dusty environments such as flour mills (Lecato, 1976), it should not come as any surprise that they are only marginally affected by contamination with small amounts of powder.

Moths contaminated with Entomag powder appeared to retain powder more effectively over 48 h than Entostat, with the male and female Entomag moths losing 69.9 and 64.3% respectively, whilst the Entostat males and females lost 89.8 and 75.9% respectively. It is notable that male moths lost powder at a faster rate than female moths and this may reflect an increase in movement by the males as they search for mates.

Although not quantitative, the scanning electron micrographs appeared to reveal interesting tendencies for both Entomag and Entostat powder to adhere in considerable quantities on parts of the moths that are free from scales, including the eyes and the tarsal tips. These areas of the body, which are devoid of the heavy scaling so prevalent on other parts of the Indianmeal moth, potentially provide a route for the a.i. into the moth if the a.i. has transcuticular capabilities. It is unknown at this time if carrier powder can penetrate the moth's integument through spiracles and other natural openings, or indeed via oral ingestion. Deposition of powders on scale-less areas are likely to be enhanced due to direct contact with the waxy layer that coats typical insect cuticle. Additionally, these areas are geometrically complex with many reticulations allowing powder to become lodged. This contrasts heavily with the body's scales, which appear 'smooth' by comparison. Indianmeal moths have a precise courtship procedure that involves, amongst other behaviours, 'head-thumping' with the female (Phelan and Baker, 1990). The fact that powder appears to be accumulated around the eyes would suggest that during this phase of courtship secondary transmission from male to female is likely. A further part of this research programme will be to use fluorescent microscopy and image analysis to quantify the regions of the body on which the powder accumulates.

Indianmeal moths took up significantly more Entostat than Entomag from the dispenser with a mean 2.49 and 0.26 µg, respectively. There are several potential explanations for this. Firstly, as the moths were not attracted to the Exosex powder dispenser the assay had to be conducted in a Perspex box, where contact with the tray was largely forced-contact and does not represent the way inoculation would occur if the moth was to follow anemotactic behaviour towards a

dispenser. Secondly, moths may find the powder repellent and are not following such a strong orientation towards the pheromone source. Lastly, the same weight of powder was placed in each dispenser, yet the density of Entomag is some 7 x greater than that of Entostat. This means the Entostat dispenser had 7 x greater volume of powder in it than the Entomag. Perhaps a more revealing follow-up experiment would be to repeat this test but keep the volumes of powder the same rather than the weights.

In the horizontal transfer experiments, Entostat appeared to be more readily passed from male to female than Entomag with 59.4, 64.8, 42.7 and 30.3% more powder being transferred in the 1-3, 5-7, 9-12 and 17-20 mg treatments respectively. All treatments demonstrated a significant difference except the 17-20 mg exposure. An average single particle of Entostat powder weighs 0.22 ng, whereas a particle of Entomag weighs over twice as much, 0.49 ng. In horizontally transferring over 30% more weight of particles in the 17-20 mg exposure treatment, the Entostat treated male effectively contaminated a female with over three times more particles than the Entomag. These calculations are highly theoretical, but all the same provide a compelling reason to believe that Entostat would be the preferred choice of a.i. carrier over Entomag when the goal is to autodisseminate powder throughout the pest population. The chances of a.i. ending up not just on the target pest, but also in the target region will be greatly enhanced by having, on average, over 2000 more particles transferred from male to female.

3. The electrostatic attraction of two powders, Entostat™ and Entomag2, to the Indianmeal moth (*Plodia interpunctella*)

Abstract

The electrostatic charge attained by the Indianmeal moth was measured as being +2 x 10^{-11} Coulombs (C) (*SE* ±2 x 10^{-12} C) after 24 h placed in contact with corrugated polypropylene (Correx®). In addition to this, the electrostatic profile of two powders, Entostat™ and Entomag2 intended for use as carriers of insecticide, was measured using a Laser Doppler Velocimetry (LDV) device. Both powders were shown to be bipolar, with 86 and 55% of the Entostat and Entomag2 particles charging to the negative polarity. The average charge measured for the negative particles of each powder was -8 x 10^{-16} and -1.4 x 10^{-16} C for Entostat and Entomag2, respectively, whilst the average particle diameter was 3.4 µm for Entostat and 2.3 µm for Entomag2. Using these measurements a model was developed to predict the respective distance over which a particle of Entostat or Entomag2 could be levitated to the cuticle of an Indianmeal moth by virtue of electrostatics. Two scenarios were modelled - (1) a neutral particle is levitated as a result of the charge on the moth inducing a charge on the particle, (2) a charged particle of opposite polarity to the moth is levitated to the insect. The model predicted that Entostat would levitate over a greater distance to the moth than Entomag2 under both scenarios (neutral particle = 2.8 mm, compared to 1.9 mm; charged particle = 21.7 mm, compared to 6.8 mm). These differences are due to Entostat attaining a higher charge and being constructed from a lower density material than Entomag2. Therefore, Entostat was determined as being the most suitable material to use in an insect bait station where electrostatic levitation may be utilised to obtain better take up of powder to the target.

3.1 Introduction

Some of the earliest widely used insecticide preparations such as sulphur and rotenone were applied as dry dusts directly to the crop (Cremlyn, 1991). However, in recent years the use of powders in pest management has declined, largely due to the fact that small particles are often not crop-fast, particularly when applied in windy climates (Matthews, 1992). Concern about the

adverse effects of pesticides on the environment and residues in food products has increased the need for improved delivery systems to reduce the quantities of pesticides used and to target their application more effectively (Matthews & Thomas, 2000). Recent research has focussed on the use of powders constructed from materials that obtain a relatively high charge to mass ratio as carriers of insecticides in order to aid adhesion of the active ingredient to the cuticle of the pest (Howse & Underwood, 2000). These powders may be combined with insecticidal active ingredients and contained within a bait station to which the pest is attracted with pheromone or food baits. Using insecticides within such stations may help reduce the amount and frequency of pesticide sprays that are applied directly to the crop, thus reducing the effects on non-target organisms and the amount of pesticide residues (Lanier, 2000). These 'electrostatically charged' powders, have been shown to adhere to insects and pass between conspecifics during normal social activity such as mating and lekking (Baxter, et al., 2008; Armsworth et al., 2006; Barton et al., 2006).

It has been demonstrated that insects acquire and retain significant electrostatic charge (Gan-Mor et al., 1995). The charges may arise from friction between the wings and other body parts of the insect or from charge separation following contact with surfaces (Jackson & McGonigle, 2005; McGonigle et al., 2002; McGonigle & Jackson, 2002) or other bodies in airborne suspension (Cross, 1987). When an insect carries a net charge, charged particles and ions of the opposite polarity will be attracted to its surface and those of the same polarity will be repelled. For an insect to levitate particles from a surface by means of electrostatic attraction, the field that its charge produces must be sufficient to lift the particle from its resting place. Once levitated the particle will accelerate rapidly towards the insect, as the attractive force will increase exponentially as the separation distance diminishes.

The electric field on an insect can cause a particle to become attracted even if it is neutral or holds a small charge of the same polarity. Firstly, if the particle is a conductor or has a conducting surface, the field generated by the insect could induce a net charge on the particle. The field generated by the insect polarises the particle and the repelled charge leaks to earth and leaves a net charge on the particle of the opposite sign to that producing the field. Similarly, if the particle is not a conductor but has a conducting surface from ambient humidity, then the field generated by the insect could induce a dipole moment on the surface charge of the particle. These particles could then be attracted to the insect if the repelled charge leaked to ground, as with conducting particles. This would result in a particle of opposite polarity to that of that of the insect and would exert a level of electrostatic attraction. If the particles are already charged to the opposite polarity to the insect, then the attractive forces between the insect and the particle are even greater.

This chapter describes the measurement of the electrostatic charge present on *P. interpunctella* and two 'electrostatic' carrier powders, Entostat and Entomag2, produced by Exosect Ltd (Colden Common, UK. www.exosect.com), when placed in contact with corrugated polypropylene (Correx®), a material frequently used in the construction of insect traps. Using these charge measurements the distance over which the respective powders are able to levitate to the insect's cuticle as a result of both the electric field on the moth and the charge on the powder is modelled. The purpose of this work is to provide investigators with a tool to help determine the appropriate materials and particle dimensions to maximise electrostatic attraction of particulates to insects.

3.2 Materials and Methods

3.2.1 Test insect

See Appendix I for details of the culturing of insects.

3.2.2 Carrier powders

See Appendix II for details of the carrier powders Entostat ™and Entomag2.

3.2.3 Holding arenas

The moths (30 males and 30 females) were individually housed in 9cm plastic petri dishes lined with 4mm thickness of Correx (Exosect Ltd., Colden Common, UK). Correx is commonly used in the construction of insect traps as it is relatively cheap and durable, it has also been shown by previous authors working on houseflies to have the lowest work function of a range of materials tested (McGonigle & Jackson, 2002) and therefore, on both accounts, is an appropriate substrate for use in these experiments. The holding arenas were wiped with 100% ethyl alcohol prior to placement of the moths to dissipate any residual charge that may have been accumulated (McGonigle et al., 2002) and an individual moth was placed into the arena for 24 h. The arenas were held at 23-25^{o}C and 25-35% r.h.

3.2.4 Charge measurements of insects

Charge measurements were carried out within a Faraday cage, with both the cage and the investigator earthed so as not to affect recordings. Moths were anaesthetised by brief saturation of the arena with carbon dioxide. Once the moth was moribund, it was tipped into Faraday pail (model 231 Faraday Cup; Electro-Tech Systems Inc., Glenside, PA, USA), connected to an earthed battery-powered charge meter (model 230 Nanocoulombmeter, Electro-Tech Systems Inc., Glenside, PA, USA), set on 20 nanocoulombs scale and with a sensitivity of 0.01 nC. The Faraday pail was cleaned between samples with 100% ethyl alcohol. Prior to the initiation of the experiment this procedure was carried out without the moth present to determine if any electric field produced by the investigator, carbon dioxide or arena was creating a background charge that would need to be discounted. It was concluded that there was no interference of any of these factors (I. Baxter, *pers. obs*.).

3.2.5 Charge measurements of powders

A Laser Doppler Velocimetry instrument (LDV) (Foot et al., 2000; Withers et al., 1998; Hyde et al., 1993) was used to measure both the particle size and charge. This instrument completes optical particle size measurements by quantifying the amount of light scattered by an airborne particle, it also measures movement in a parallel electric field (30000 V/m) due to particle charge. The LDV instrument sampled from a 0.21 m^3 high efficiency particulate air (HEPA) filtered cabinet. A 0.1 g volume of powder was weighed out onto a Correx spatula, which had been previously cleaned with 100% ethyl alcohol to dissipate any accrued surface charge. The spatulas holding the powder remained undisturbed in the laboratory (21^{o}C, 40-45% r.h.) for 2 h prior to testing. The powders were propelled into the cabinet by a discharge of air from a regulated compressed airline, held approximately 2 cm away from the Correx spatula. Three consecutive puffs were measured with each powder material and the particle sizes were compared with Mann-Whitney tests (MINITAB for PC v14, MINITAB Inc.) to determine whether there were any significant differences between replicates. As no difference between replicates was found the data were combined. A total of 692 particles were measured for Entostat and 1198 for Entomag2, however not all the particles were included in the analysis as the particle charge distribution measured for both materials was bipolar (see fig.3.1) and consequently the positively charged particles were omitted from this analysis as these, generally, would be repelled by the positively charged moth. It is acknowledged that these measurements are calculating charge following powder dispersion rather than the in the bulk phase as would be the case with the majority of powder lining the base of a dispenser. However, these calculations show what charge could be attained at the point of separation when the particles became disturbed by movement of the dispenser in the wind or by physical contact of the substrate by the insect. Therefore this approach is considered appropriate for this exercise.

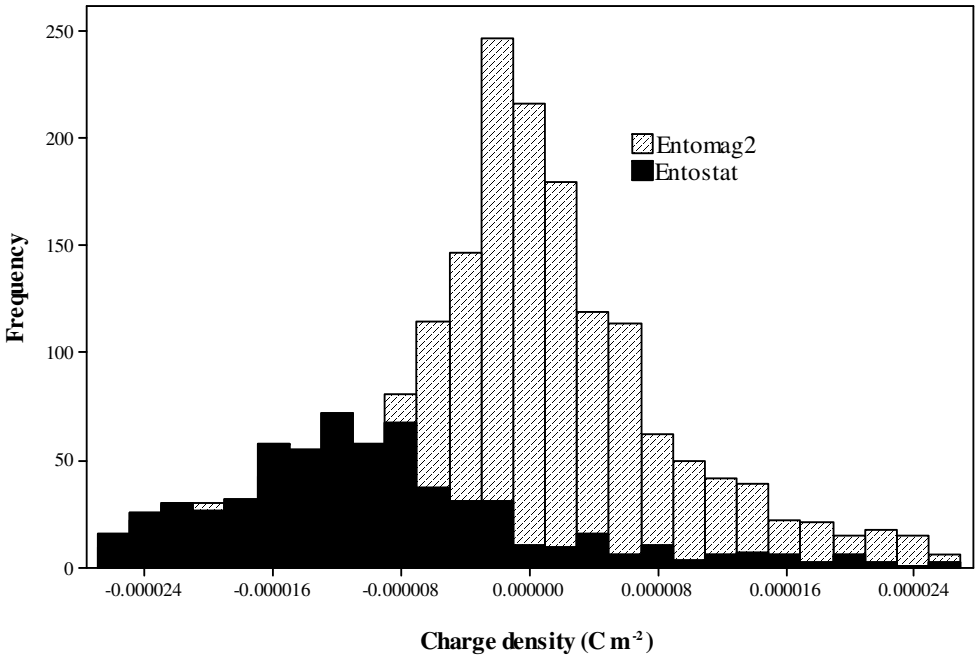


Figure 3.1. Frequency distribution of charge measured on individual particles

As the Entomag2 particles have a seven-fold increased density over Entostat the effect of this on the accuracy of the LDV was considered. This increased density has been calculated to delay the particle deflection in the electric field a small amount due to the particles' inertia. However, this effect takes place over a very short time period, calculated to equate to less that 30 µm of the particle trajectory in the 500 µm diameter measurement volume and so would not significantly affect the measured particle mobilities (K. Baxter, *pers. comm.*).

3.3 Results and Electrostatic Model

3.3.1 Charge on moths

The charges measured on the moths are shown in table 3.1. As with the houseflies measured by previous authors (McGonigle and Jackson, 2005) the Indianmeal moth generally charged to a positive polarity when housed in contact with Correx (mean= 2 x 10^{-11} C, *SE* ±2 x 10^{-12} C). There were no significant differences between the charges attained by either male or female moths (Mann-Whitney *U* test, *NS,* MINITAB for PC v14, MINITAB Inc.).

Table 3.1. Charge recorded from male and female *Plodia interpunctella* after being housed in corrugated polypropylene (Correx®) arenas for 24 h (n= 30).

Sex	*Mean charge (pC) (figures in brackets are standard error)*	*Max. charge Observed*	*Min. charge* Observed*
♂	+21 (±3)	+60	-20
♀	+19 (±3)	+50	-30
♂♀	+20 (±2)		

* Of the 60 samples taken, 54 were positive or neutral, whilst 6 charged negatively. The negative samples were included in the calculation of the mean and are shown here as the minimum charges recorded.

3.3.2 LDV measurements

The particle charge distribution measured for both materials was bipolar, with Entostat showing a significant skew towards the negative polarity (86% of Entostat were negatively charged compared to 55% of Entomag2 (see fig. 3.1) (Mann-Whitney U test, W = 455454.0, P = <0.0001). The mean overall charges measured were -6.6 x 10^{-16} (S.E. 4.17 x 10^{-17} C) and -1.02 x 10^{-16} C (S.E. 5.86 x 10^{-18} C) for Entostat and Entomag2 respectively. However, as the positively charged particles were ignored from these calculations, the mean charge on the negative particles were -7.4 x 10^{-16} C (S.E. 3.9 x 10^{-17} C) and -2.1 x 10^{-16} C (S.E. 1.37 x 10^{-17} C) for Entostat and Entomag2 respectively. The mean particle size was 3.44µm (*SE* ±0.09 µm) and 2.28 µm (*SE* ±0.04 µm) for the Entostat and Entomag2 particles respectively. The particle size distribution is shown in figure 3.2.

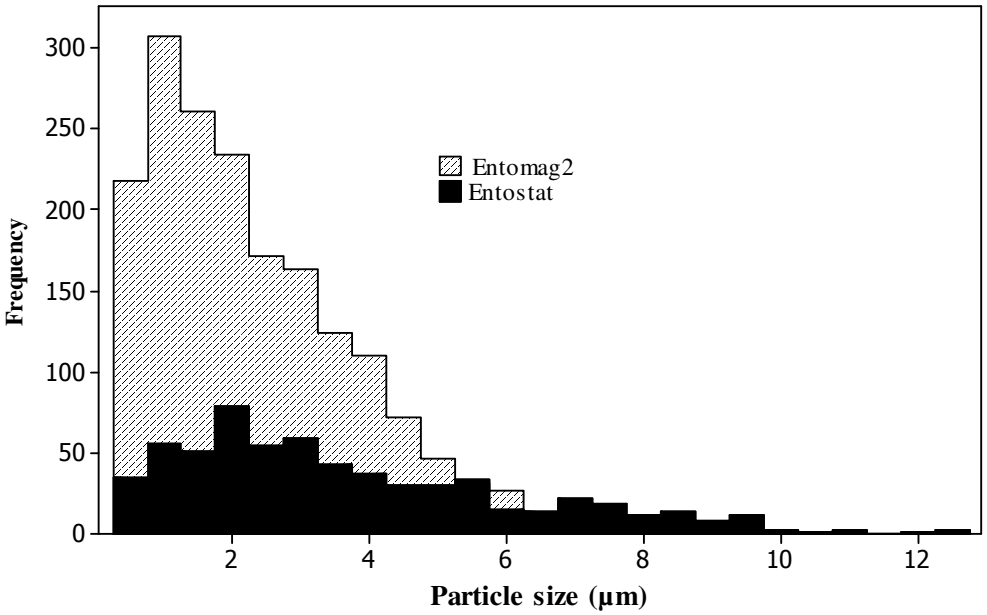


Figure 3.2. Particle diameter size distribution of Entomag2 and

3.3.3 Mass of an individual particle

The densities of Entostat and Entomag2 are 980kg/m^3 and 7300kg/m^3 respectively. Therefore, from the sizing data generated from the LDV and if it is assumed the particles are spherical, the mass of particle of mean size can be calculated to be 3.07 x 10^{-14} kg (Entostat) and 5.42 x 10^{-14} kg (Entomag2).

3.3.4 Electrostatic calculations.

The force needed to lift a body against the force of gravity is equal to the weight of the body.

$$F_w = mg \qquad (1)$$

where F_w is the force, m is the mass of the object and g is the acceleration of gravity 9.81 ms^{-1}. The electrostatic attractive forces between a particle and an insect would need to be equal to or greater than F_w in order to levitate a particle and adhere it to the surface of an insect. Using equation 1 and the mean particle size, F_w = 2.05 x 10^{-13} N (Entostat) and 4.44 x 10^{-13} N (Entomag2).

Levitation force due to a charged insect

An insect is a geometrically complex object and is likely to have regions with different electrical properties and thus would be difficult to realistically model in terms of the field it produces. The approach taken by those carrying out research in this field has generally been to model the insect as a sphere of approximately equivalent volume (V) (Gan-Mor et al., 1995). This approach has also been used in this example as it represents a "worst case" as, in reality, the legs, setae, antennae, *etc*. of insects will generate more intense electrical fields and will come into more intimate contact with the particles through which it is moving. Therefore, if it can be shown that particles can be levitated by the equivalent sphere, then in the real case the levitation forces will be greater even if not exactly quantifiable.

Charge on an insect

In their studies on bees Erikson and Buchmann (1984) , modelled them as equivalent spheres of 8.8mm diameter. The moths being considered here are smaller and so their 9 x 3 x 3 mm size (when at rest with wings folded in) has been modelled as a sphere of equivalent volume with a radius of 2.68 mm. The mean charge measurement for these moths was 2.0 x 10^{-11} C (see table 3.1) which is presented in these equations as Q_m. Therefore, the charge on the surface of the insect gives a charge density D_m = 1.5 x 10^{-7} C m^{-2}.

Field generated by a charged insect..

The field generated by an insect carrying a charge can be calculated by using Gauss's Law. The law states that if an imaginary surface is drawn around a uniform density of charge then the component of the electrical field at any point on the surface, and perpendicular to it, is proportional to the total charge enclosed (Cross, 1987). Therefore it can be treated as a point charge in further calculations. The field E generated by a charged insect is therefore given by:

$$E = \frac{\sum q}{S\varepsilon_0\varepsilon_r} \qquad (2)$$

Σq is the sum of the charges enclosed, S is the surface area, ε_0 is the permittivity of free space (8.84 x 10^{-12} F m^{-1}) and ε_r is the relative permittivity of the medium through which the field is propagated. In all the aspects under consideration here the field is being propagated through air where the value of ε_r is effectively 1 and so it may be ignored in the calculations.

If one considers a hypothetical sphere drawn an infinitesimal distance outside the real sphere representing the insect it will effectively have the same area. So then the field at a distance *d* from the surface will be:

$$E_d = \frac{Q_I}{4\pi\varepsilon_0 d^2} \quad (3)$$

where E_d is the field generated by an insect with a charge Q_I at a distance *d* from its surface

As it is known that the insects carry surface charge, there are two scenarios that are most likely to cause particle adhesion when an insect encounters powder in a bait station.

1) The powder is initially uncharged and the field generated by the insect induces an image charge that polarises the particle. The polarisation will in turn induce image charges on adjacent surfaces that will cause the particle to be attracted to those surfaces.
2) The powder is charged, so particles charged to the opposite polarity to the insect will be attracted by its electric field and adhere to the insect surface.

Example 1: A charged insect attracting uncharged Entostat and Entomag2 Particles

Entostat and Entomag2 are formed from carnauba wax and iron respectively, with the wax therefore being an insulator and the iron a conductor. These equations assume that the surface of the particles will have a layer of condensed moisture forming a conducting coating, which is a reasonable assumption given that the powders are likely to be deployed within the humid canopy of a crop. The charge per unit area induced by a uniform field on a conducting sphere (Q_t) can be shown, by the Laplace analysis (Rogers, 1954) to be:

$$Q_t = 3\varepsilon_0 E\cos\theta \quad (4)$$

integrating this over the surface area of the sphere gives the total charge Q_p as:

$$Q_p = 4\pi\varepsilon_0(1.64Ea^2) \quad (5)$$

where a is the radius of the sphere.

The field acts to lift the inductively charged particle, however for a particle resting on a surface there will be an image force due to the polarisation of the surface in the region of the particle. Lebedev and Skal'skaya (Lebedev N.N. & Skal'skaya I.P., 1962) and Felici (Felici N.J., 1966) (both cited by (Cross, 1987) both calculated the resultant force F_e for a sphere in contact with a surface to be:

$$F_e = 4\pi\varepsilon_0(1.37E^2a^2) \quad (6)$$

If the value for E_d is substituted into this equation in place of the generalised value E then one obtains:

$$F_e = \frac{1.37Q_m^2a^2}{4\pi\varepsilon_0 d^4} \quad (7)$$

To obtain the maximum distance at which the force of the electrical field can levitate the particles one can equate F_w, the force due to the weight of the particle with F_e and so:

$$mg = \frac{1.37Q_m^2a^2}{4\pi\varepsilon_0 d^4} \quad (8)$$

Solving this equation for *d* gives the distance at which a charged moth could levitate a neutral particle of the mean size measured here by virtue of electrostatic forces of 2.8 x 10^{-3} m (2.8 mm) for Entostat and 1.9 x10^{-3} m (1.9 mm) for Entomag2.

These values initially appear quite small. However, when it is considered that the insect is likely to be in close contact with the powder and these values in fact represent 844 and 854 particle diameters of Entostat and Entomag2 respectively, then it is clear that the electrical field from a charged insect is more than adequate to transfer particles to its body.

Example 2: A charged insect attracts an oppositely charged particle

Coulomb's Law describes the force between two charges Q_1 and Q_2 a distance *d* apart. If Q_1 is the insect charge (2.0 x 10^{-11}C) and the particle charge is Q_2 the force between the two charges can be calculated using equation 9 below.

$$F = \frac{Q_1 Q_2}{4\pi\varepsilon_0\varepsilon_r d^2} \qquad (9)$$

If the force is equal to or greater than the force due to gravity imposed on the particle (F_w), it will levitate and adhere to the insect. Therefore, solving equation 9 for *d* gives a distance at which a charged moth could attract a particle of the mean size measured here of 2.54 x 10^{-2} m (25.4 mm) for Entostat and 9.1 x 10^{-3} m (9.1 mm) for Entomag2. This is equivalent to 7384 and 3986 particle diameters of Entostat and Entomag2 respectively.

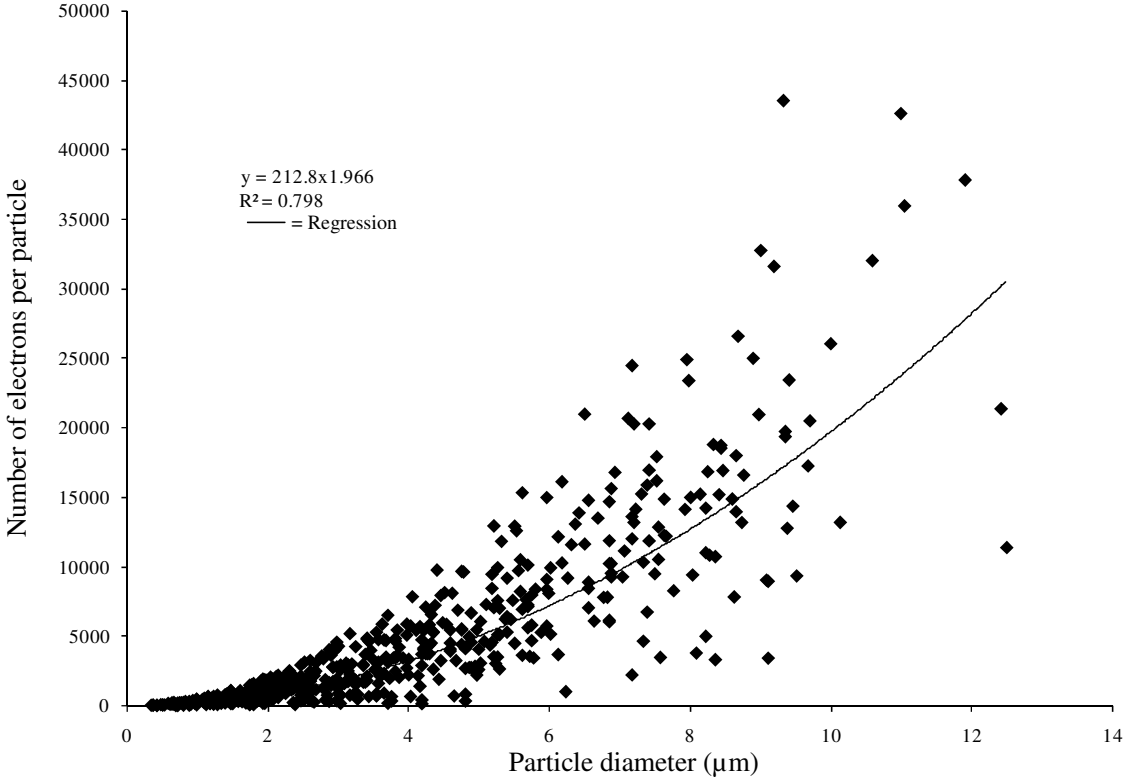


Figure 3.3. Entostat particle diameter plotted against the number of electrons present on the particle surface

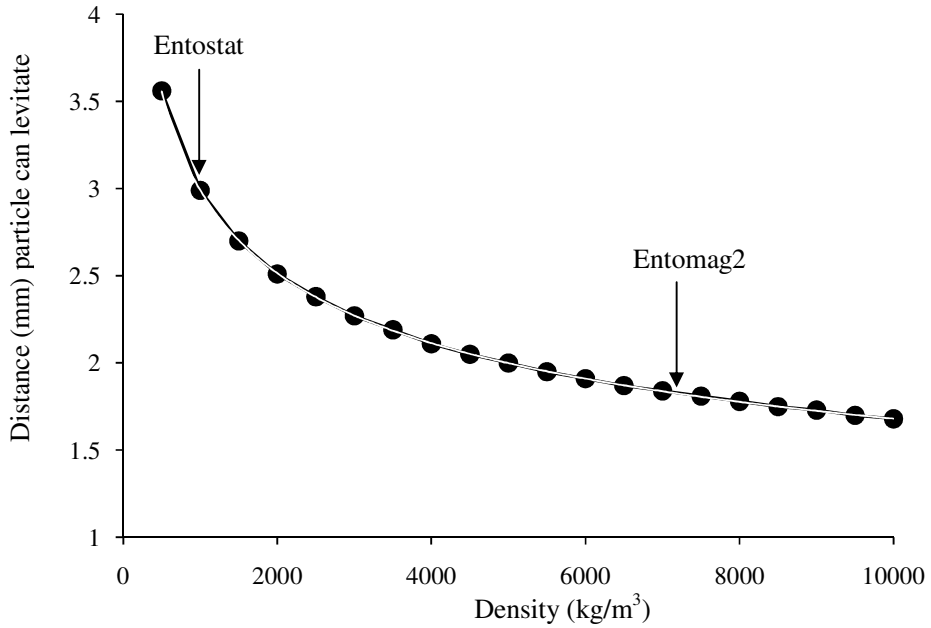


Figure 3.4. The effect of material density on the distance that a theoretical 3µm particle can levitate to an Indianmeal moth cuticle by virtue of the electrostatic charge on the moth

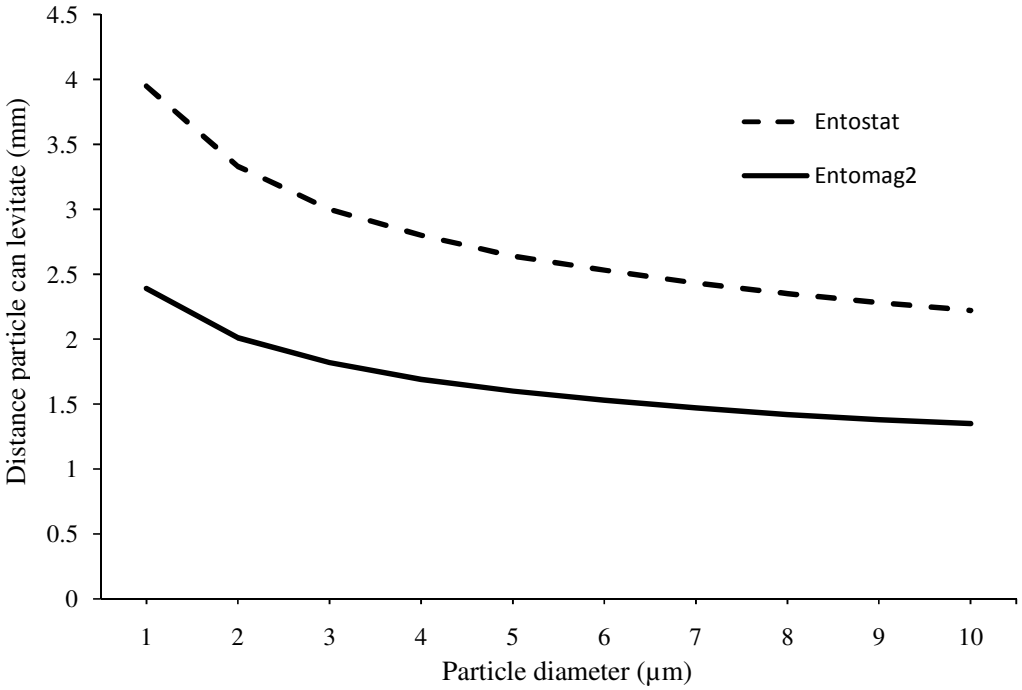


Figure 3.5. The effect particle size on the distance that a neutral Entostat and Entomag2 particle can levitate to Indianmeal moth cuticle by virtue of electrostatic forces

Table 3.2. Theoretical distance (mm) over which a particle of Entostat or Entomag2 can levitate to the cuticle of a charged Indianmeal moth

Particle type	*Distance over which particle may be levitated to Insect (mm)*	
	Neutral Particle	*Charged Particle*
Entostat†	2.9	25.4
Entomag2*	1.9	9.1

† = average Entostat particle size as measured by LDV = 3.4 µm
* = average Entomag2 particle size as measured by LDV = 2.3 µm

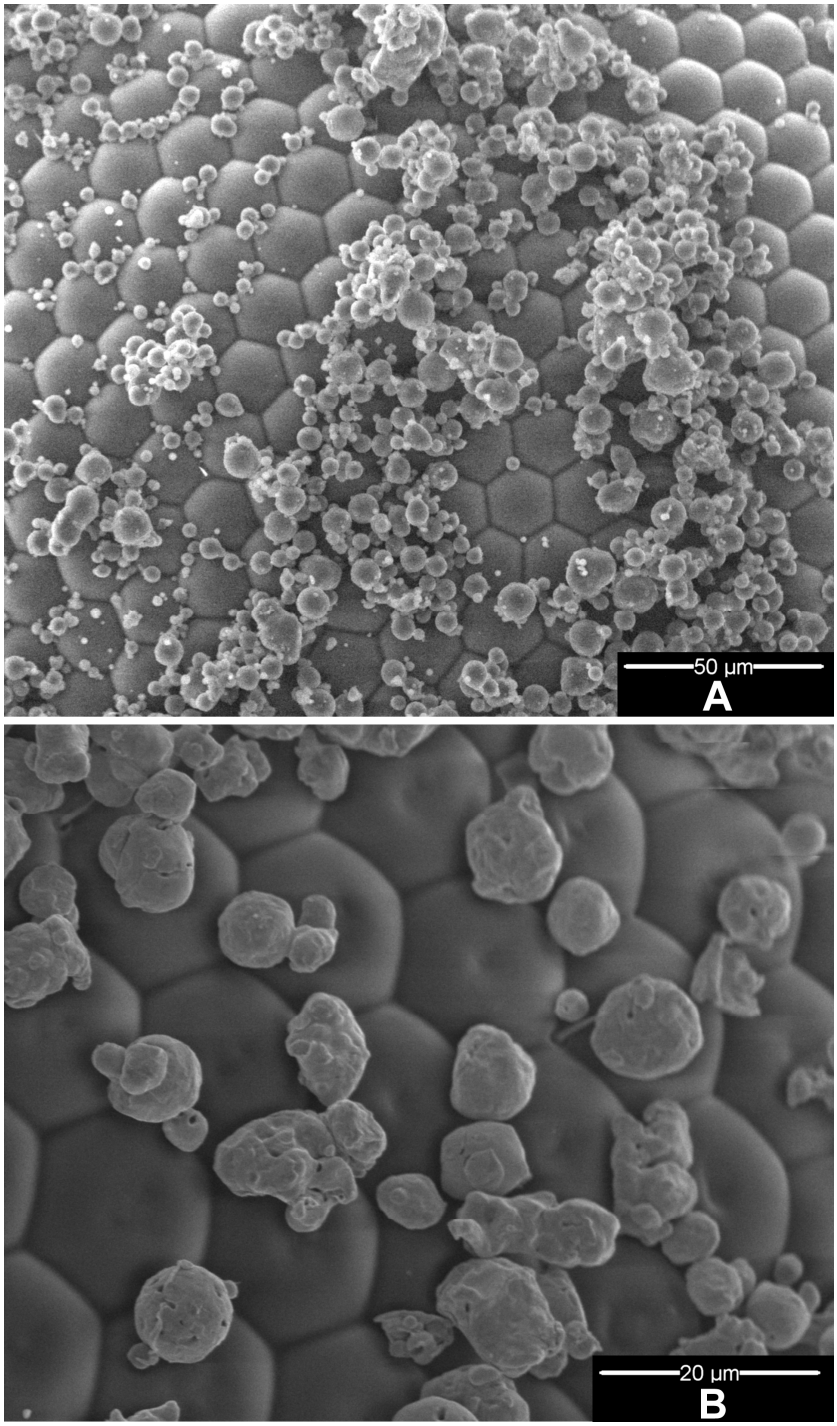


Figure 3.6. Scanning electron micrograph showing Entostat (A) and Entomag2 (B) adhering to a Indianmeal moth's eye

3.4 Discussion

The aims of this chapter were to carry out measurements of electrostatic charge on a pest insect (the Indianmeal moth) and two powders, Entostat™ and Entomag2 (both from Exosect Ltd, Winchester, UK), to enable a comparison of their respective electrostatic qualities for use in a delivery system to target insecticidal active ingredients to insects. Using these measurements, a theoretical calculation was made to determine the potential of both powders to be levitated by virtue of electrostatic forces onto the body of a pest insect. For an insect to pick up particles from a surface by means of electrostatic attraction, the field that its charge produces must be sufficient to lift the particle from its resting place, against the opposing force of gravity. Once levitated the particle will accelerate rapidly towards the insect, as the attractive force will increase exponentially as the separation distance diminishes and the particle will impact on the insect thus delivering insecticide to the target. The approaches taken here to calculate these forces are not exact, as the precise geometry and electrical characteristics of an insect would be highly complex to model. Therefore the approach adopted has been to approximate to the worst case wherever possible.

The moths themselves were found to carry an electrostatic charge of $+2 \times 10^{-11}$ C (*SE* $\pm 2 \times 10^{-12}$ C) after they had been held for 24 h in arenas lined Correx. These measurements were close to the limits of detection of the equipment used (10 pC ±1 pC) but broadly agree with the charges obtained by other researchers working in this area (Erickson, 1975; Erickson & Buchmann, 1984; Gan-Mor et al., 1995; McGonigle et al., 2002; McGonigle & Jackson, 2002) and thus are accepted as an appropriate basis for the theoretical calculations of particle/electric field interaction.

In this research, theoretical calculations were made to determine the potential levitation distances over which both of the experimental powders could span to make contact with the insect. This has been examined under two different scenarios whereby, in the first instance, the particles are assumed to be neutral and are levitating to the insect solely as a result of the electrical field from the moth. This is considered to be a worst-case scenario but is an important evaluation as powder-based insecticide stations are intended for use within multiple pest/environmental complexes and may be placed in an area of high humidity (e.g. within the canopy of an irrigated crop) where an electrostatic charge on the powders could rapidly dissipate. In the second scenario, the charge on the respective powders has been measured using the LDV instrument (Foot et al., 2000; Withers et al., 1998; Hyde et al., 1993) and the resultant levitation distances calculated as a result of measured charge on both the moth and powder. It is reasonable to assume that some charge will indeed be present on the powder as particle turbulence and,

therefore, tribo-charging may occur due to movements of the dispensing station in the wind and/or the entry of the moth into the station.

The force (F_w) needed to lift a particle of either Entostat or Entomag2 from its resting place was calculated, based on the mean particle size as measured by the LDV and density of the respective powders. This calculation assumes that the particle is entirely free to move and that there are no adhesion forces between the particle and other particles or the surface on which it is resting. In reality there will be some adhesive forces between particles and the fundamental force that causes adhesion at this level is the Van der Waals-London interaction (Corn, 1966; Erickson & Buchmann, 1984). However, for the purposes of these calculations where the objective was to compare the potential for electrostatic levitation between two different powders, these forces were ignored.

In the first scenario, a charged moth is modelled to be capable of attracting a neutral particle of mean size of either Entostat or Entomag2, distances of 2.8 $x10^{-3}$ metres (2.8 mm) for Entostat and 1.9 $x10^{-3}$ metres (1.9 mm) for Entomag2 (see table 3.2) over which a particle could be levitated by virtue of the moth's electrostatic field. This represents 844 and 854 particle diameters of Entostat and Entomag2 respectively. In the second scenario the attractive forces between the charged moth and the charge on the powder (as measured by the LDV device) was considered. The distances over which each powder could levitate towards the charged insect were calculated as being 2.54 x 10^{-2} m or 25.4 mm for Entostat and 9.1 x 10^{-3} or 9.1 mm for Entomag2 (see table 3. 2). This was equivalent to 7384 and 3986 particle diameters of Entostat and Entomag2. This increase in levitation distance due to the charge on the particles potentially enhances the amount of surface area of the pest that could become contaminated through contact with the bait station. However, the results from the neutral charged particle scenario also show that particle levitation, to a lesser extent, will occur by virtue of the moth's own electric field. Interestingly, Butt, et al., (1998) found that Styrofoam particles (well known for their electrostatic charging properties, e.g. packaging material sticking to polythene) were effective carriers of *M. anisopliae* conidia when examining vectoring of pathogens by honeybees. It is possible that the authors were, in fact, unwittingly utilising the electrostatic properties of these 'biobeads'.

The LDV device measures both particle size and movement of that particle within a parallel electrical field to calculate the charge. The particle size reading was taken as being the diameter of a perfect sphere, so it is acknowledged that Entostat and Entomag2 particles which are not spherical will potentially give misleading readings as their true shape may not be spherical. However, examination of these powders using a scanning electron microscope revealed that the

powders, in the majority, are spherical in shape (see fig. 3.6). The LDV readings also showed significant differences in the charge distribution for both powders (fig. 3.1) with 86% of the Entostat being negatively charged, compared to 55% of the Entomag2 particles. This is likely to be due to the materials' different positions on the tribo-series (Cross, 1987) with respect to Correx, resulting in Entostat particles obtaining a predominantly negative charge, where Entomag shows no clear preference for either polarity, giving a more symmetrical bipolar charge distribution.

The powders will be subjected to charge separation between particles where the distribution will be bipolar and the median zero where all the particles are made of the same material. Entostat, being formed from a naturally derived plant wax that varies considerably in components depending on a number of biotic and abiotic factors, could indeed show a skewed charge distribution as particles are likely to be constructed from different proportions of constituents. All the positively charged particles were omitted from these calculations as they would, in general, be repelled by the positively charged insects. Therefore, more Entostat particles would be attracted to the insect, indicating that it may be the more effective material to use as an insecticide carrier. Entostat also has a higher mean negative charge than Entomag, which would result in a greater attraction to the positively charged insect surface than Entomag.

As shown in Figure 3.3, there is a relationship between particle size and an increase in the charge (or number of electrons present per particle) for Entostat powder. The larger particles have a greater surface area, therefore, more electrons can be transferred to the surface during the frictional charging process that occurred when they were disseminated from the Correx surface during aerosolisation. However, the charge-to-size relationship for Entomag2 is far less pronounced and it is hypothesised that this may be due to the material being closer to the Correx on the Tribo-series than carnauba wax. This is implied by the measured symmetrical bipolar distribution of particle charge shown in Figure 3.1, indicating the Entomag2 powder has no strong bias to charge to either polarity against the Correx surface. This is also likely to be the case on an individual particle scale resulting in some interactions resulting in a gain and some in a loss of electrons. Therefore, it is not surprising that Entomag2 shows no correlation between an increase in particle surface area and negative charge is apparent.

The approximate 7x increase in density between the Entostat wax and the Entomag metal has a impact on the distance over which the particle can be levitated by the insect (see fig. 3.3). This is most noticeable in scenario 1, where both powders are assumed to be neutral, as Entostat, despite its bigger particle size is calculated as levitating almost 50% further than Entomag2. The effect of particle density on levitation distance of an uncharged particle is summarised in figure

3.4. The relationship between the levitation distance and the density is described by $D = \sqrt[4]{cD^{-1}}$ where c is a constant and D is the density in kg m^{-3}. This demonstrates that the material from which the insecticide carrying powder is produced should be from a low density matrix if transfer to the insect by electrostatic forces is desired. Figure 3.6 shows the effect of increasing the diameter size of Entostat and Entomag2 on levitation distance. As shown in equation 8, the influence of the particle size is $\sqrt[4]{a^{-1}}$ resulting in a reduction in levitation distance as particle size diameter increases.

Several authors have also observed that smaller particles generally adhere better than larger ones (Mohammad, et al., 2007; Barton et al., 2006; David & Gardiner, 1950). In work carried out by Barton et al., (2006), the take up of two different sizes of carnauba wax powder (milled = 7.59 µm; unmilled 9.17 µm) by medfly (*Ceratitis capitata*) was evaluated and found that 47% more of the smaller milled particles were taken up onto the insects. Based on the calculations in this paper for uncharged particles, the milled powders could potentially be levitated via electrostatic forces 2.38 mm towards the moth, compared to 2.27 mm with the unmilled. This difference of ca.5% could well result in more powder being taken up by the insect, but does not account for the 47% increase reported by the author. Moreover, the increased amount of particles being retained on the insect is likely to be linked to smaller size enabling mechanical retention of the particles into grooves, spiracles, *etc*. on the insect's body. The effect of Van der Waals-London interaction between particles and insect cuticle will also become more of a significant force when it comes to retaining smaller particles.

The work presented here indicates that Entostat has certain desirable characteristics that would make it a more efficacious insecticide carrier powder than Entomag2. In summary these advantages are (1) Entostat particles attained a higher negative charge than Entomag2, (2) more of the Entostat powders charged to the negative polarity when dispensed from Correx than Entomag2, (3) the relatively low density of Entostat compared to Entomag2 resulted in a lower F_w and therefore the charge on the moth could levitate the particle further, and (4) the high electrical impedance of Entostat may mean that charge is retained longer on the surface than in the conducting Entomag2.

This work aims to introduce some of the factors that are important in exploiting the electrostatic charge on pest insects as a means of treating it with a carrier of insecticide. Ensuring that the material from which the dispensing station is constructed functions to charge both the insect pest and the powder (at least in the majority) to opposite polarities from each other is critical if the electrostatic forces are going to be exploited to the full. Particle density and, to a lesser extent

size, would appear to be additional considerations for the manufacturer of such a system. Here, only the passage of particles from the dispenser to the insect is being considered and the insecticide carrying particles may need to be in contact with the insect for considerable periods of time before the biological active ingredients may cause an effect. For the system to be fully optimised it is likely that a number of considerations should be undertaken to not only examine electrostatic uptake of powder to the insect, but also factors which increase the retention of particles over time – this of course may, like the electrostatic properties of both powders, be substantially altered by the addition of an insecticide and would need to be separately evaluated. Particle shape should also be considered as, for example, a flake may provide a better surface area to weight ratio than a sphere and could enhance adherence by hydrophobic forces at the insect cuticle if the material from which the particle is manufactured is similar to the wax used here. The work presented here is intended as a guide for researchers in this field when selecting new and novel particulate carriers of active ingredients for insect pest control.

4. Using image analysis of powder deposition to evaluate two prototype autodissemination devices intended for the biorational control of *Plodia interpunctella*

Abstract

The extent to which Entostat™ carrier powder was taken up and horizontally transferred by contaminated Indianmeal moth (*Plodia interpunctella* (Hübner)) (IMM) individuals to conspecifics was evaluated. Using a marker dye which, through use of confocal microscopy, was shown to be colocalized with the carrier powder, the amount of powder on the IMM, as determined by spectrophotometry, was correlated with quantification based on a novel image analysis approach which enabled differentiation of powder uptake by body region. Over a 48 h period, more powder was retained on the ventral surface than the dorsal side, with the head region showing the greatest amount of powder uptake and retention. During courtship, powder was horizontally transferred by treated males to 1.6% of the head and 0.06% of the untreated females' body. To inoculate moths, a pit-fall style autodissemination station was determined as being more effective than a powder tray, as significantly more material was taken up to the key areas of the moth's body where powder is more effectively retained and more likely to be horizontally transferred. Additionally, a pit-fall station prevented moths from exhibiting avoidance behavior from the powder, which was frequently encountered if they had to walk into a powder tray. This study shows that different regions of the IMM body vary in capacity to carry powder and that future research efforts should target these specific regions. While the proposed autodissemination system was optimized for management strategies of IMM, the results presented here can be used to develop novel management strategies for other insect pests.

4.1 Introduction

Since the phase-out of methyl bromide in stored product environments researchers have put considerable efforts into alternative approaches for controlling serious postharvest pests, such as the Indianmeal moth (Mohandass et al., 2007; Phillips, 2006). One such experimental approach has been lure and kill (Nansen and Phillips, 2004), whereby the main component of the female-produced sex pheromone, (Z,E)-9,12-tetradecadienyl acetate (commonly referred to as ZETA) (Brady et al., 1971; Kuwahara et al., 1971), is used to attract male moths to an insecticidal bait. Such targeted approaches have the advantage of keeping potentially harmful active ingredients

within bait stations or otherwise out of the way of staff and food products (Lanier, 1990). In addition, it is a way to expose target insects to high dosages of active ingredients at point sources, so that the likelihood of the insects acquiring a lethal dosage is high. However, a limitation of most lure and kill approaches, especially when developed for control of moths, is that only males are controlled. It would, therefore, be a considerable advantage if, instead of killing males that get into contact with the active ingredient, they could stay alive and perform natural mating behaviour long enough to transfer sufficient active ingredient to conspecific females. Such a variant of the lure and kill technique is autodissemination, where active ingredients, *i.e.* entomopathogens, are spread by inoculated individuals to conspecifics (Soper, 1978). The process of infecting individuals may be achieved by placing infective life-stages of the entomopathogen within a bait station to which the target pest species is attracted using a pheromone lure (Pell et al., 1993). The station is designed such that the inoculated pest may leave the dispenser and horizontally transfer active ingredients during normal conspecific interactions such as mating and lekking.

In experimental approaches where insects are exposed to carrier powders, marker dyes can be incorporated into the powder mix to measure the amount of powder taken up by test insects using spectrophotometry (Barton et al., 2006). From analyzing the amount of marker dye present on the test insects, it is possible to determine how much powder is taken up from treated surfaces and from horizontal transfer between conspecifcs (Baxter et al., 2008; Nansen et al., 2007b). Whilst this technique is useful to determine the level of gross contamination of a test subject, it does not distinguish between different regions of the body such as dorsal and ventral surfaces. The process is also destructive meaning that the same individual subject cannot be followed over time.

The objectives of this research were to establish a technique based on image analysis to determine the regions where a carrier powder is taken up and horizontally transferred by IMM males to conspecific females. Characterisation of which parts of the body hold powder most effectively and where it becomes transferred during mating is essential for the design of an optimised autodissemination system for IMM and other important insect pests. While this study exclusively addresses the development and optimisation of an autodissemination system to be used in management of IMM, the overall approach is relevant to a number of insect pests both within and beyond stored product environments.

4.2 Materials and Methods

4.2.1 Test insect

See Appendix I for details of the culturing of insects.

4.2.2 Carrier powders

See Appendix II for details of the carrier powders Entostat.

4.2.3 Powder dosing

A 20 mg sample of dyed powder was measured into a plastic general-purpose container with hinged lid (57 mm length x 17 mm diameter) (code 216-3136, from VWR International; www.vwr.com). The powder was applied to the sides of the container using a plastic (polypropylene) drinking straw in a circular movement to create a continuous film of powder. A single virgin moth was held in a glass 5 ml sample tube and then tipped into the powder tube. Once in the treatment tube, the glass vial was quickly reversed so that the base formed a seal over the end of the tube. The tubes were held in place as still as possible, for 1 min, before the moth was allowed to climb back into the glass holding tube. To mitigate the risks of powder contamination, treatments were applied in a different room to the observational phase of the experiment and powder sample tubes were disposed of after a single replicate.

4.2.4 Image acquisition

Moths were anaesthetised using humidified carbon dioxide rather than immobilising them by exposing them to a low temperature, as previous observations had shown that the moths' antennae were more likely to fold against the body when chilled, preventing a full-view image from being obtained. On occasions where moths had either become immobilised with their antennae adjacent to the body or where they could not be placed into a suitable position for imaging (*i.e.* repeatedly rolling onto their side) the replicate was discarded. Individual moribund moths were placed onto non-reflective black card and photographed both dorsally and ventrally using a Motic DM-143 digital dissection microscope (Motic, Barcelona, Spain) fitted with a Stockeryale UV microscope circular illuminator (Stockeryale LiteMite™, model 10 high frequency; Stockeryale, Salem, NH, USA) as the sole illumination source of the stage. All image acquisition was carried out within a darkroom booth. The microscope was connected to a PC (via USB) held outside the booth so that light from the monitor did not contribute to the stage lighting. Image acquisition was controlled from the PC using the software Motic Images Advanced 3.2. All images were stored on the PC as bitmaps (.bmp), of 640 x 480 pixels in size.

4.2.5 Image calibration

To separate powder fluorescence from that of auto-fluorescence of the moth, an image calibration protocol was developed. This consisted of placing a 5 x 2 mm piece of translucent double-sided sticking tape gently onto the surface of the dyed Entostat powder to obtain a consistent thin covering of powder onto the tape. This was then stuck to a piece of black card, with the powdered side uppermost. An anaesthetized moth was then placed onto the card with care being taken not to contaminate the moth with powder. Images were collected of both dorsal and ventral sides of individual moths. This image was then referred to as the 'calibration image' (see fig. 4.1A). The calibration image bitmap was opened in the computer software 'ImageJ' v. 1.39q (National Institutes of Health, USA) and converted to a 32 bit greyscale image. The threshold function was adjusted so that only pixels from the powder and not moth auto-fluorescence were highlighted on the image. A note was made of the threshold settings and the procedure repeated for the second calibration image showing the moth from a ventral view (see fig. 4.1B). The second calibration image was set to the same threshold levels and the image checked to ensure that only powder fluorescence was being recorded. Using the powder-coated sticking tape as a calibration scale, the ImageJ software's calibration function was globally invoked. Moribund moths were then imaged under the same conditions (e.g. focus, zoom, lighting) under which the calibration image has been taken. The microscope and ImageJ settings were re-calibrated every 3-5 images to maximize repeatability. Overall, the number of pixels that constitute a 5 mm length of powder on the card base varied by 4% across all the images taken, therefore, it can be said that there is a 4% margin of error between all the images taken (see fig. 4.1C).

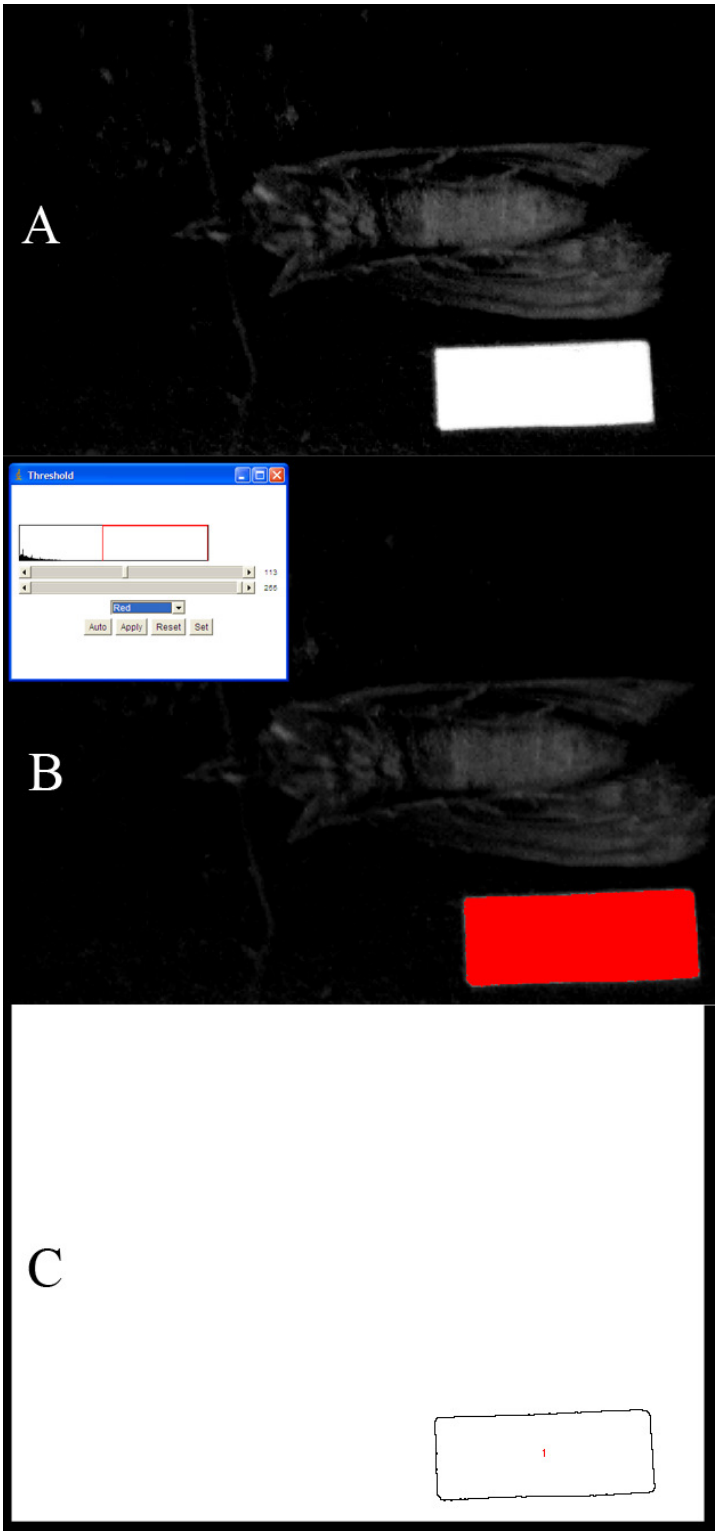


Figure 4.1(A,B,C). Image analysis calibration procedure (ventral view only shown in this figure). **(A)** An untreated moth was imaged from both the dorsal and ventral side with a 5 x 2 mm piece of adhesive tape coated in dyed Entostat powder. **(B)** The threshold settings (window inset) were set within the software package ImageJ (1.39q) so that only pixels from the powder were counted and that light reflected from the moth was not included in the calculations. **(C)** Using the 'analyze particles' procedure in ImageJ, only the wavelength from the powder was processed and a calibration bar was formed.

4.2.6 Measuring powder contamination on the moth

Using ImageJ, the "Analyze Particles" function was invoked and the software set to "Show Outlines" and results to appear in "Separate Windows". A map of the powder was generated (see fig. 4.2) with a results page showing the area (mm^2) of powder and corresponding reference number on the map. Using Adobe Photoshop® CS2 v 9.02, for PC, (Adobe Systems Incorporated, San Jose, CA., USA) the powder map was overlaid onto the original .bmp image (using layers with the map layer set at 50% transparency) so that it was possible to determine which regions of the moth were contaminated with powder. The total surface coverage of powder was calculated for three body regions – head, antennae, body (thorax, abdomen and legs). To determine the total area of each body region the external circumference of either the head, antennae or body was marked using the pen tool in Adobe Photoshop®. This area was then filled using the paint-bucket tool and the image exported as a bitmap. The image was opened in ImageJ, where it was converted to 32bit greyscale and the total area of the fill calculated using the particle analyzer command as above. The area of powder contamination was then expressed as a proportion of the total body area.

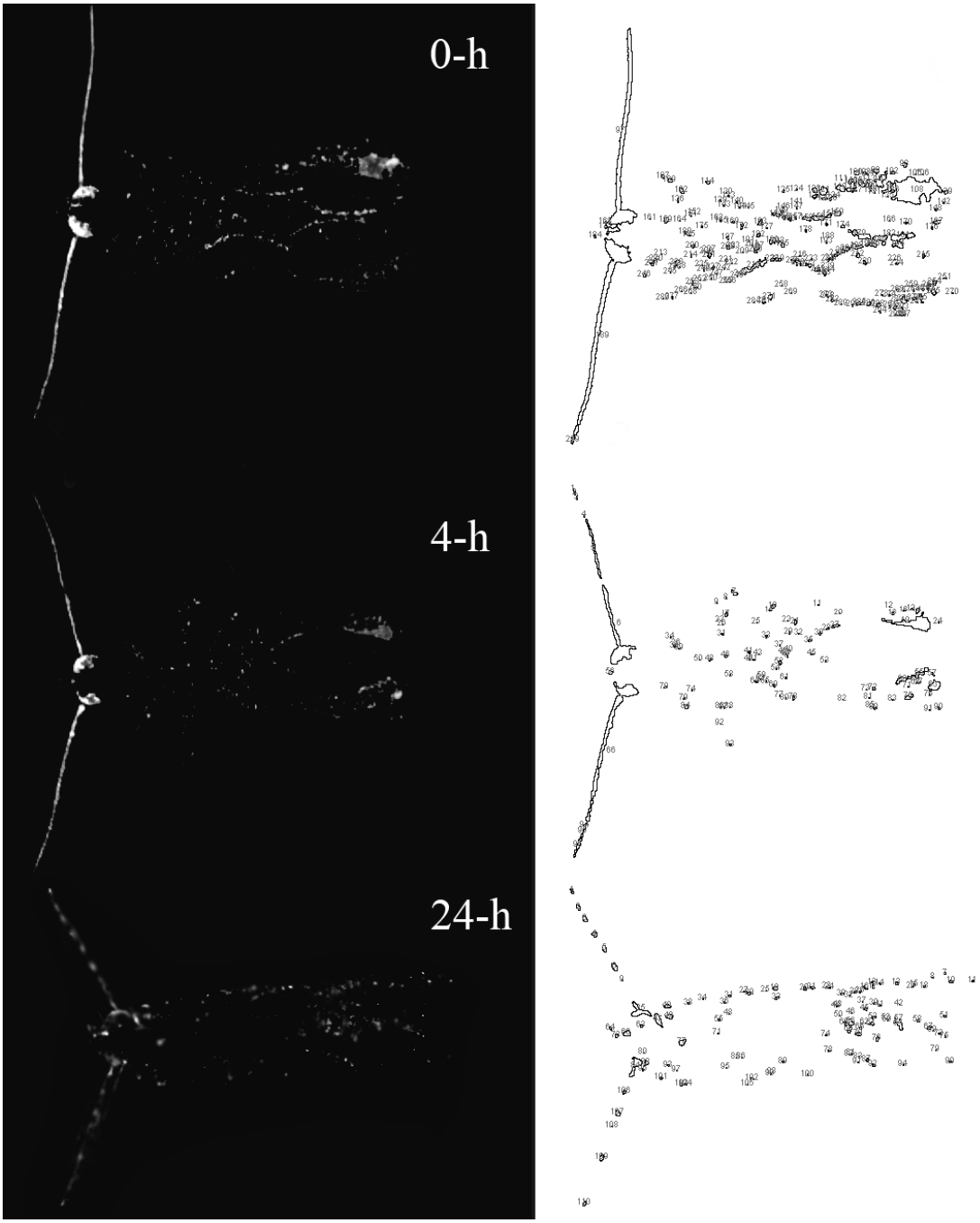


Figure 4.2. Image analysis using ImageJ. The left-hand pictures (taken under a UV and blue visible light source) is an image of a *Plodia interpunctella* moth contaminated with visible light emitting powder when excited by ultraviolet wavelengths. The picture was then processed in the software package ImageJ invoking the 'analyse particles' commands. The analyse particles function produces a map and statistics of the powder (right-hand images). This selection of images, although not the same moth, shows how powder take up and losses may be calculated over a 24-h period.

4.2.7 *Experiment 1. Colocalization of the Entostat™ Powder and Dye.*

The aim of this experiment was to determine whether powder and dye were collocated (*i.e.* both dye and wax present in the same particle), as subsequent powder uptake assays were based on the assumption of direct correlation between the two. Using a Leica TCS SP2 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) and a multiphoton laser (Broadband Mai Tai™ laser, Spectra Physics, Newport Corporation, CA, USA) a spectral dye separation was performed with Leica Confocal Software (v. 2.61 build 1537). For this the laser wavelength of the multiphoton was set to 720 nm and lambda series were acquired of the Entostat, the dye, and the Entostat/dye mixture, at x20 magnification. Each lambda stack covered an emission range of 400-540 nm and comprised 10 images taken at constant increments. Based on the lambda stacks taken of the wax preparation and of the dye preparation emission profiles were generated for each of the two fluorescent components. These spectra served as a reference which allowed us to analyze the lambda stacks of the Entostat/dye mixture. As a result, spectral dye separation provided two images, each containing only the share of one of the fluorescent components (fig. 4.3 A, B). The overlay of the two images (fig. 4.3 C) shows that there is a colocalization of the Entostat Powder and dye, with the dye being incorporated into the larger wax particles. The degree of colocalization on a pixel to pixel basis is demonstrated in the cytofluorogram shown in fig. 4.3 D.

For further quantification two additional methods were deployed, both based on the analysis of z-stacks of images taken of the Entostat/dye preparation at x40 magnification. Each z-stack comprised 10 images taken at 2 µm increments along the z-axis, each image with two colour channels that had been acquired with optimized settings for imaging of the wax, and the dye, respectively. In the first type of analysis three images were selected and the separate "powder image" (green channel) and its corresponding "dye image" (red channel) were saved in jpg-format and imported into ENVI 4.3 for Windows (Research Systems Inc., Boulder, CO). On each of the three "powder images", about 40,000 pixels were selected (total image was about 140,000 pixels) representing the entire range from very dark to very bright pixels and the random selection option in ENVI 4.3 was used to choose 5,000 of the 40,000 pixels, and the random selection was exported to a txt-file. The exact same 5,000 pixels were also selected from the corresponding "dye image". The txt-files exported from ENVI include x- and y-coordinates for each of the 5,000 pixels, which allows for direct comparison of fluorescence values in pixels from the two images, and a linear regression analysis was used with fluorescence values of the "dye image" as response variable and those from the "powder image" as explanatory variable. - An additional analysis of colocalization was made using the Manders' method. For this the ImageJ software plugin, JACoP (Bolte & Cordelières, 2006) was used to measure the extent of colocalization by generating the Manders' coefficient which measures the degree of

colocalization of objects in confocal dual-colour images (Manders et al., 1992). The confocal image *z*-stack, taken as described above (total of 10 images taken at 2 µm increments), were opened as individual *.lsm files as with the dye image or the wax image representing one channel each. The two images were analyzed by invoking the JACoP plugin which generated scatter plots and Manders' correlation coefficients. Briefly, Manders' coefficients range from 0 to 1, with 1 demonstrating a high degree of colocalization.

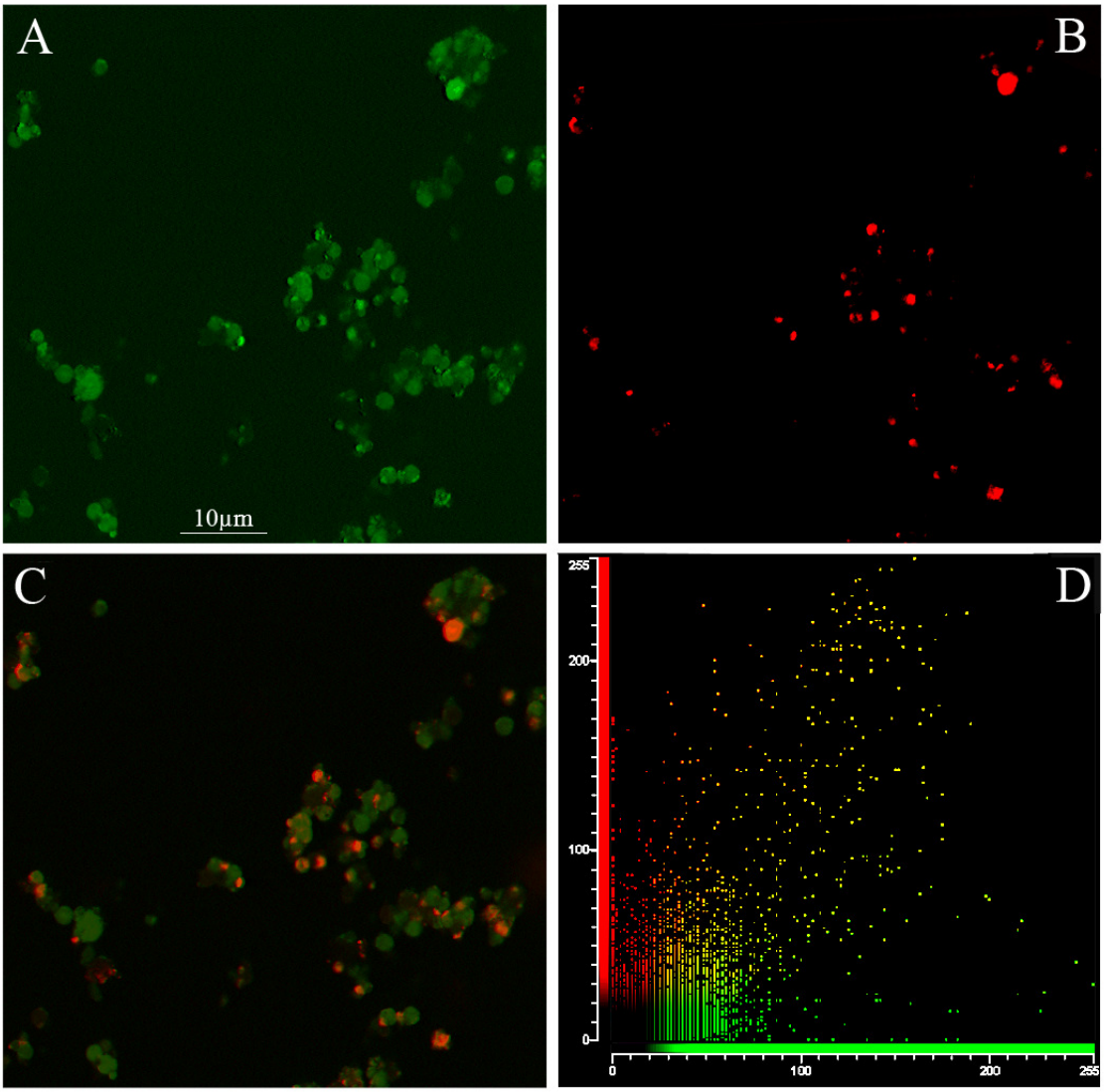


Figure 4.3. Confocal microscope analysis to measure the extent of Entostat and globrite dye colocalization. **(A)** an image of Entostat dyed with 10% Globrite dye is taken at optimal excitation and emission to display only the Entostat powder; **(B)** is the same image, except taken at optimal settings to show just the dye; **(C)** overlay image shows both dye and powder location; **(D)** is the cytofluorogram determining the extent of colocalization.

4.2.8 Experiment 2. Powder retention analysis on the same test subjects by both image analysis and spectrophotometry techniques to compare the alternative approaches.

Individual virgin male moths (<48 h old) were dosed with powder as above and immediately housed individually into polypropylene food storage containers (26 cm x 26 cm x 26 cm) for either 1, 4, 24, or 48 h. The entire cage was held at 24-27°C, 60-80% r.h. and under a 16:8 light/dark cycle. After treatment the moths were immobilised with humidified CO_2 and imaged. After image acquisition, moths were subjected to spectrophotometric analysis (Baxter et al., 2008; Nansen et al., 2007b; Barton et al., 2006; Armsworth et al., 2006) in order to compare the image analysis data to that of an alternative technique. Individual moths had their antennae and heads excised from the body using a scalpel (cleaned in 99% ethanol between incisions) and each body part was placed into an Eppendorf tube containing 1 ml of 99.5% ethanol (Sigma-Aldrich, UK). Blank cuvettes containing 1 ml of 99.5% ethanol were scanned at an excitation of 385 nm wavelength and emission wavelength of 450 nm using a PerkinElmer Luminescence Spectrometer LS5OB fluorometer and these readings set as a background level and automatically subtracted from future measurements. Serial dilutions of Glo-Brite® dye in the range 0.1-1.9 µg/ml were prepared in 99% GC-grade Ethanol and five replicates per concentration scanned to produce a calibration curve. As a control, five individual moths with their antennae and heads excised from their bodies were placed in a 1 ml Eppendorf tube containing 1 ml of ethanol and subjected to vibration-plate mixing for 30 s. A 0.5 ml sample of liquid was removed from the tube and scanned to determine if there was any background emission. As the excised body parts did not demonstrate any significant readings beyond that of background scatter, the above method was followed for each of the treated moths' body parts (*i.e.* a pair of antennae, head or body). Where the intensity of emission was greater than the fluorometer could read appropriate serial dilutions were carried out. There were 10 replicates for each sampling point at 1, 4, 24 and 48 h after treatment.

4.2.9 Experiment 3. Powder retention over time

Moths were treated using the same procedure as described in experiment 2, except that only image analysis was performed. The procedure now also involved placing the dosing tubes with the anesthetised moths for 30 s on a vibration plate to achieve a more consistent uptake of powder over the entire *P. interpunctella* body. Moths were again, anesthetised at time points 1, 4, 24, or 48 h after treatment and digital images taken (details as above). After imaging, moths were returned to a new container to prevent re-contamination. There were 20 replicates.

4.2.10 Experiment 4. Powder transfer between conspecifics

P. interpunctella males were treated with powder as in experiment 2 and placed into a 9 cm plastic petri dish containing an untreated virgin female moth. The dishes were observed for up to 2 h for any mating activity, if the moths did not couple within this time then the replicate was discarded. If moths did couple, then the dish was left for 24 h before the female moths were immobilised and imaged. In addition, it was speculated that treated *P. interpunctella* males might deposit dyed powder residue on surfaces within the 24 h time period and that females might acquire powder that way as well (in addition to acquisition from mating/direct contact with treated males). Consequently after each 24 h trial with a treated male and an untreated female, an untreated male and female moth were released into the same cage for 24 h before these moth individuals were immobilised and subjected to imaging. There were 18 replicates of this study.

4.2.11 Experiment 5. Powder take up from a dispenser

A plastic funnel trap measuring 170 mm (d) x 270 mm (h) (Black Stripe Trap, Cooper Mill Ltd., Ontario, Canada) was adapted into two distinct types of powder dispenser each containing 1 g of dyed powder; station A, the powder tray dispenser, where the moths had to walk over the powder to reach the pheromone lure and, station B, the powder funnel dispenser, where moths were only inoculated with powder if they entered the funnel of a bucket-trap (see fig. 4.4 for details). Trials were conducted inside a glass wind tunnel [60 cm (height) x 60 cm (wide) x 150 cm (depth)] similar to the one used by El-Sayed et al., (1999). The wind tunnel has a push/pull system of variable speed fans, a charcoal filtration system at each end and steel mesh mounted in both ends of the tunnel to generate laminar flow. The airflow was kept at 35 cm sec^{-1} for all experiments and the tunnel was illuminated by an overhead red light at 30-50 lux. Room temperature was maintained at 26–28^{o}C and humidity at 30–42% r.h. A fresh pheromone lure consisting of a rubber septum (Exosect Ltd., Colden Common, UK) containing 0.1 mg of (Z,E)-9,12-tetradecadienyl acetate (ZETA) (Bedoukian Research Inc., Danbury, CT, USA) was used for each replicate and left in the activated wind tunnel for 5 min before introduction of a moth to standardize the release rate. Between replicates the walls and base of the tunnel were wiped with fresh paper cloth and ethanol (99%) to prevent contamination and the paper base of the tunnel was replaced. Virgin male *P. interpunctella* <2d were placed in individual holding tubes and were released one at a time into a plastic weighing boat (which stimulated them to fly once they reached the upper edge) 100 cm downwind of the station in the wind tunnel. If the moth had not entered the station within 1 minute of release the replicate was discarded and the moth removed from the wind tunnel. Once a moth entered the station it was given up to 5 minutes to leave the station unaided, after which it was recaptured into a holding pot, anaesthetised and imaged, or

stored in alcohol for subsequent spectrophotometry analysis as in experiment 2. If the moth had not left the station within 5 minutes then this replicate was discarded.

A random testing sequence of either tray or funnel stations, image analysis or spectrophotometry was adopted. All experiments were carried out within 2 h of the onset of scotophase and there were 20 replicates per treatment.

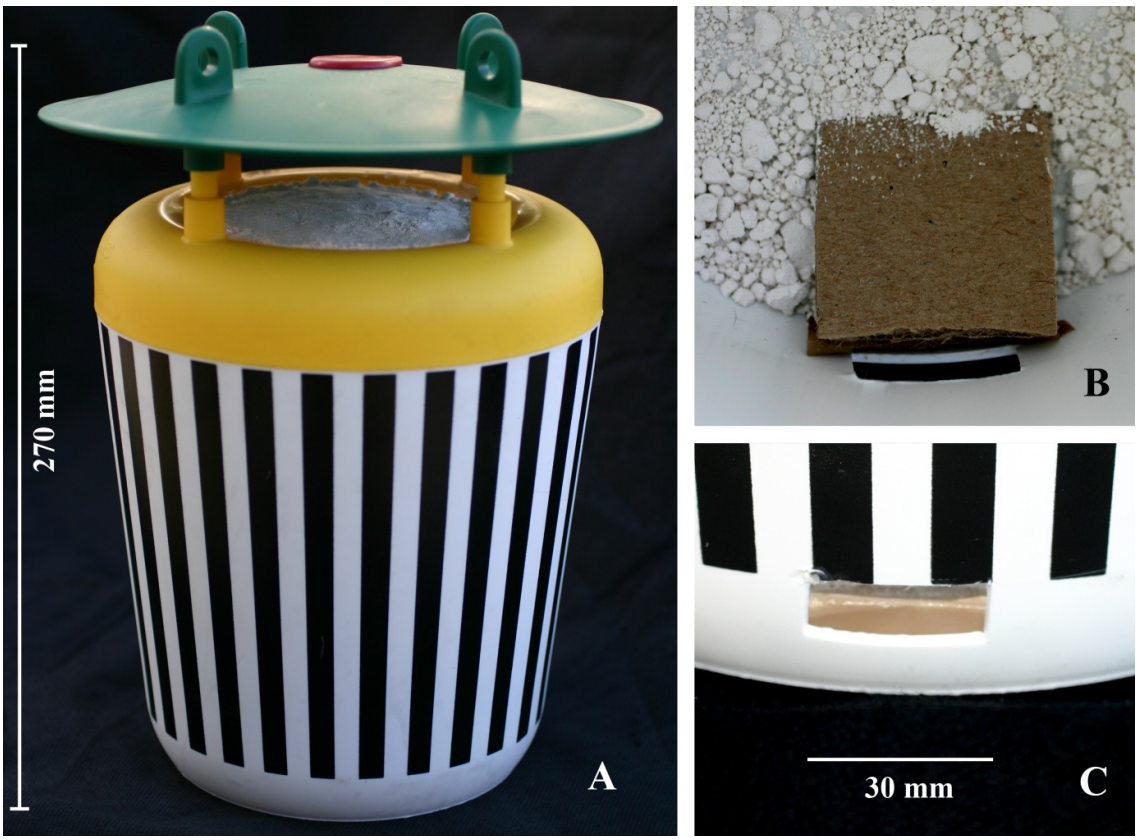


Figure 4.4 Prototype powder dispenser. Two types of prototype powder dispenser were evaluated – fig. A shows dispenser 1 – a powder tray inserted into the rim of the funnel station (and referred to in the text as the powder tray dispenser), whereas figs. B and C show dispenser style 2 – which is identical in appearance to dispenser 1 except that powder is held in the base and an escape ramp (B) and exit hole (C) enable *Plodia interpunctella* to exit the dispenser (referred to in the text as a powder funnel dispenser).

4.2.12 Data analysis

All statistical analyses were carried out using MINITAB v14 (MINITAB Inc, MINITAB Inc, State College, PA, USA).

In experiment 1, the relationship between corresponding pixels in images acquired with settings optimized to detect fluorescent light emitted from the wax or the dye, respectively, were compared using linear regression.

In experiment 2, Spearman's rank-order correlation was carried out between data from spectrophotometry (µg of powder per moth) and image analysis (mm^2 of powder coverage per moth). The analysis was repeated comparing the extent of correlation between the two techniques for each body region.

In experiment 3, the data was not normally distributed (Anderson-Darling test), had heterogeneous variance (calculated by Levene's test) and did not conform to sphericity assumptions (calculated by Mauchly's test). Therefore, a two-way non-parametric Friedman test for repeated measures was carried out to compare differences between the powder deposition on the dorsal and ventral portions of the moths over time (total amount of powder per moth (mm^2) versus dorsal or ventral surface with time since treatment (1, 4, 24 or 48 h) as the block. Further non-parametric *post hoc* analysis were carried out using Wilcoxon signed-rank for paired data tests on the amount of powder (mm^2) present on each body part at the above sampling points after treatment. As this is not a repeated measures approach the probability epsilon was adjusted using the Bonferroni method.

For experiment 4, Wilcoxon signed-rank test was used to compare the amount of powder on the dorsal and ventral surfaces of the body regions of the female moths that had successfully coupled with a treated male. Comparisons with the untreated control group females that were subsequently placed into the empty arenas after successful coupling of treated moths were made using Mann-Whitney *U* tests.

In experiment 5, the amount of powder deposited on male moths that had entered either the funnel or the 'walk in' tray dispenser was compared using Mann-Whitney *U* tests. Wilcoxon signed-rank comparisons were used to compare the amount of powder on the dorsal and ventral surfaces of the body parts of the male moths that had entered the funnel dispenser.

4.3 Results

4.3.1 Experiment 1. Colocalization of the Entostat™ Powder and Dye.

Images gained from spectral dye separation strongly suggest that wax and dye were collocated within the same particles (Fig. 3A-C). The cytofluorogram shown in Fig. 3 D, demonstrates that most pixels of the processed image comprise both a red and a green signal, often of similar intensity and then displayed in yellow in the $x = y$ region of the cytofluorogram. In addition to this, the further analysis carried out with ENVI 4.3 software, on three replicated analyses of powder, showed that there was a highly significant correlation between florescence values obtained with excitation optimized for the fluorescent dye and for Entostat (1. Adjusted R^2 – value = 0.46, F_{4999} = 4271.876, P <0.001; 2. Adjusted R^2 – value = 0.37, F_{4999} = 2982.332, P <0.001; 3. Adjusted R^2 – value = 0.37, F_{4999} = 2180.413, P <0.001). The mean Manders' coefficients also show a significant positive correlation between the dye and the wax (M_1= 0.773 (±0.04SE) and M_2 = 0.841(±0.04). Thus, it was found appropriate to assume that the fluorescent dye provided an accurate depiction of how much Entostat powder had been deposited on *P. INTERPUNCTELLA* individuals.

4.3.2 Experiment 2. Comparison of image analysis and spectrophotometry as a measurement of powder contamination

There was a significant correlation between dyed powder estimates based on spectrophotometry and image analysis (see table 4.1) (1-tailed, N = 40, r_s = 0.813, P = <0.01). In addition, separate analyses were conducted for different body parts, and there was a consistent significant correlation between the two estimates (body = 1-tailed, N = 40, r_s = 0.80, P = <0.01; head = 1-tailed, N = 40, r_s = 0.65, P = <0.01; antennae = 1-tailed, N = 40, r_s = 0.64, P = <0.01).

Table 4.1. Comparison of powder uptake by *Plodia interpunctella* (Hübner) body regions as measured by two different techniques – image analysis and spectrophotometry.

	Mean amount of powder (mm^2 or µg) determined as being on moth body regions over time (h) (±*SE*)					
Time (h)	Image analysis (mm^2)			Spectrophotometry (µg)		
	B	H	A	B	H	A
1	1.2 (0.2)	3.5 (0.5)	1.5 (0.1)	7.42 (1.0)	7.23 (0.6)	1.52 (0.1)
4	0.9 (0.2)	1.7 (0.2)	0.4 (0.0)	4.61 (0.7)	5.84 (0.4)	0.62 (0.0)
24	0.1 (0.0)	1.2 (0.3)	0.0 (0.0)	1.74 (0.4)	3.89 (0.3)	0.07 (0.0)
48	0 (0)	1.0 (0.3)	0 (0.0)	0.53 (0.1)	1.18 (0.1)	0.05 (0.0)

B = Body, H = Head, A= Antennae

4.3.3 Experiment 3. Powder Retention Over Time

Over a 48 h experimental period, moths had significantly more powder present on their ventral than dorsal surface ($\chi^2(4) = 1$, $P = <0.05$). *Post hoc* analysis tested at the 0.0125 level (Bonferonni correction), revealed that increased powder take up to the ventral surface was confined to the body and head regions at 1 h after treatment and the head region only at 4 and 24 h. There were no significant differences in powder deposition for any level at 48 h (see table 4.2).

Table 4.2. Comparison of powder loss rates from dorsal and ventral surfaces.

	Surface area of powder (mm^2) (±SE) present on P. interpuctella *body regions over time (h)*											
	1			4			24			48		
	B	H	A	B	H	A	B	H	A	B	H	A
Dorsal	0.75 (0.06)	0.30 (0.03)	0.92 (0.10)	0.45 (0.06)	0.16 (0.02)	0.05 (0.01)	0.04 (0.02)	0.05 (0.02)	0.07 (0.03)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Ventral	1.94 (0.50)	0.65 (0.06)	0.82 (0.80)	0.59 (0.05)	0.39 (0.14)	0.05 (0.01)	0.12 (0.03)	0.14 (0.03)	0.01 (0.01)	0.02 (0.01)	0.02 (0.01)	0.00 (0.00)
P-value	<0.001	<0.001	0.026	0.014	<0.001	0.889	0.016	0.006	0.315	NS	NS	NS
N for test and *W*	20, 8	20, 8	20, 18	19,39	20,0	13,48	14,13	17,18	14,36	-	-	-
Sig. With Bonferroni correction	*	*	NS	NS	*	NS	NS	*	NS	NS	NS	NS

B = Body, H = Head, A= Antennae, NS = Not significant, * = Significant

4.3.4 Experiment 4. Powder transfer between conspecifics

Of mated females, 61% had powder on them compared to 17% of the untreated females that had subsequently been placed (along with untreated males) in the same arenas (see table 4.3). The amount of powder on the treated and untreated moths (mm^2) was significantly different ($N = 18$, $U = 265.5$, $P = <0.05$) (see table 4.3). Within the treated group, powder was adhering to the ventral side and to both dorsal and ventral surfaces of the head. Statistical differences in the side of the insect to which powder was preferentially taken up was only demonstrated on the head ($N = 6$, $W = 21$, $P = <0.05$), where the ventral side appeared to hold more powder than the dorsal surface (see table 4.4). There were no significant differences recorded for which part of the body took up powder through horizontal transfer.

Table 4.3. Comparison of powder take up by females that have mated with treated males.

	Amount of powder (mm^2) *(±SE)*	
	Treated females	Untreated females
Percentage (%) moths with powder	61	17
Amount of powder (mm^2) *(±SE)*	0.09 (0.04)	0.02 (0.01)
P-value	0.0340	
N and *U*	18,265.5	

Table 4.4. Comparison of powder take up on body parts by females that have mated with treated males.

Surface area of powder (mm^2) (±SE) present on mated ♀ P. interpuctella *body regions (N=18)*						
	Control			Treatment		
	B	H	A	B	H	A
Dorsal	0	0	0	0	0.01 (0.01)	0
Ventral	0.02 (0.01)	0	0	0.04 (0.02)	0.04 (0.01)	0
P-value	NS	NS	NS	NS	0.036	NS
N for test and *W*	-	-	-	-	6,21	-

B = Body, H = Head, A= Antennae, NS = Not significant

4.3.5 Experiment 5. Powder Take Up from a Dispenser

The amount of dyed powder taken up by moths visiting the funnel dispenser was significantly higher than by those visiting the tray style of dispenser (N = 20, U = 210.0, P = <0.001), with a mean of 23.8 µg (±1.32 SE) and 0.18 µg (±0.07) respectively, per moth (see table 4.5). There were no significant differences in amounts of dyed powder taken up either dorsally or ventrally from moths entering either style of dispenser. Moths entering the funnel style dispenser took up, on average 1.91 µg/mm^2 (±0.43) body surface area of powder from the station. When calculated for each body region, the highest density coverage was on the head, 11.35 µg/mm^2 (±3.84), followed by the antennae, 3.59 µg/mm^2 (±0.84) and the body, 0.43 µg/mm^2 (±0.04). The head region took up significantly more powder per unit surface area than the other body regions when the moth had entered the dispensing station – head vs body: N = 20, W = 210, P = <0.001; head vs antennae: N = 20, W = 210, P = <0.001. Additionally, the amount of powder taken up by surface area was significantly more on the antennae than on the body (N = 20, W = 210, P = <0.001). With the tray style dispenser only the ventral side of the body region registered any powder take up with 0.01 µg/mm^2 (±0.009) coverage.

Table 4.5. Powder uptake from a prototype dispenser.

Surface area of powder (mm^2) (±SE) present on P. interpuctella *that have entered prototype powder dispenser (N=20) as determined by image analysis*							Mean amount of powder (µg) *(±SE)* per moth as determined by spectrophotometry	
	Funnel dispenser			Tray dispenser				
	B	H	A	B	H	A		
Dorsal	1.91 (0.27)	0.52 (0.04)	1.00 (0.09)	0 (-)	0 (-)	0 (-)	Funnel	Tray
Ventral	1.74 (0.18)	0.75 (0.05)	0.94 (0.10)	0.04 (0.01)	0 (-)	0 (-)	23.81 (1.32)	0.18 (0.07)
Mean time (s) *(±SE)* spent at dispenser		74.6 (15.8)			7.1 (1.2)			

4.4 Discussion

The main objectives in this study were to provide a quantitative and non-destructive method for determining dyed Entostat powder uptake to different areas of a *P. interpunctella* body, and to develop a prototype autodissemination station for powder delivery to parts of the *P. interpunctella* body where it will be effectively retained and horizontally transferred. The spatial relationship between the dye and the carrier powder is of critical importance, as introducing a dye as a marker is only representative if it is physically incorporated into the carrier powder being evaluated. This relationship has been assumed by previous authors (Armsworth et al. 2006; Barton et al. 2006; Nansen et al. 2007a, 2007b; Armsworth et al. 2008; Baxter et al. 2008) and this study is the first to actually quantify the positive correlation.

The image analysis technique presented here offers advantages over spectrophotometry in that it is both cheaper and quicker to run. It also offers wider possibilities as the sampling technique is non-destructive to the insects. This may be highly relevant when examining, for example, the uptake and distribution of powder within social insect communities. Rather than needing to extract and process samples back in the laboratory, imaging of brood cells or pollen stores, for instance, could be made *in situ* resulting in far less disturbance of the community and creating a more realistic experimental procedure. Non-destructive sampling may also provide investigators with a tool to determine the frequency of visits made by an individual to an autodissemination station. This would help answer wider questions to determine, for instance, the correct application rate of dispenser stations. Through re-sampling live individual *P. interpunctella* it may be possible to determine whether they are repeatedly returning to stations (*i.e.* the density of stations is too high) rather than horizontally transferring active ingredient to females. Finally, the image analysis system is easily adaptable to incorporate different dyes which reflect alternative light wavelengths, which may be distinguished by ImageJ. Through being able to track where the moth had been, preference experiments could be carried out to determine the efficacy of a particular dispenser station design, pheromone attractant loading and repellence of certain active ingredients, etc.

Using the image analysis technique, *P. interpunctella* were consistently shown to retain significantly more powder on their ventral than dorsal surfaces. *Post hoc* analysis showed that these differences were limited to the body and head regions but not present on the antennae. Powder appeared to accumulate directly on the cuticle of the eyes (see fig. 4.2) which, as they are more prominent when viewed dorsally, suggests being more visible from this perspective. These observations are supported by scanning electron micrograph images in Baxter et al. (2008), suggesting that powder is preferentially adhering to scale-less regions of the moth body such as eyes and tarsi.

Horizontal transfer of powder between conspecifics, which is critical to the success of an autodissemination program, was examined here using image analysis. In a previous study by Baxter et al. (2008) using the same dosing regimen as in the present study, the total amount of powder transferred from male to female was 0.67 µg (±17% SE). In these studies, female *P. interpunctella* took up significantly more powder to their ventral surface rather than to their dorsal side indicating that powder was initially horizontally transferred to the eyes, from where it then spread to the ventral side of the body. It was concluded that approximately the same amount of powder (by mm^2 surface area) is taken up by the head region as the body (see Table 4). However, given that the surface area of the head is smaller than the body, this equates to approximately 1.6 and 0.06% of the total area of the head and body, respectively. In terms of volume of powder per unit area of insect, the head region obtains more material through horizontal transfer than other body regions and, as *P. interpunctella* engage in 'head-thumping' during courtship (Phelan and Baker 1990), this would appear as the likely source of horizontal transfer of powder from male to female.

Plastic funnel traps were adapted to create two distinct types of powder dispensers which were evaluated by attracting male IMM within a wind tunnel to a sex pheromone lure placed in the station (see fig. 4.4). The two different designs (funnel and tray) were intended to evaluate general approaches to powder inoculation – one where moths fall into a pit of powder and leave through exit holes and another, where moths are required to walk through a tray of powder in much the same way as the current Exosex™ system operates for *Cydia pomonella* (L.). Spectrophotometry analysis revealed that moths which visited the tray style dispenser took up less than 1% of the powder present than the funnel dispenser moths. Previous studies (Baxter et al. 2008) showed that mating performance of male IMM was not affected by powder doses of up to 17.7 µg, however, doses higher than that may reduce both the moths' ability to mate and locate a pheromone lure; Nansen et al. (2007a) found similar results with the Grape Berry Moth, *Lobesia botrana* Den. and Schiff (Lep., Tortricidae) as did Armsworth et al. (2006) working with Mediterranean fruit flies, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae). The funnel station used here would need to be adapted by altering either the quantity or depth of powder in the base of station to reduce powder contamination per moth so that it reaches approximate parity with 17.7 µg per moth.

It was concluded that the funnel dispenser delivers powder to the key areas of the moth body where it is more effectively retained and more likely to be horizontally transferred than the tray station approach. Understanding where the dispenser powder deposits on the insect may be important depending on the application for the system as, for instance, in an 'autoconfusion'

mating disruption system (Nansen et al. 2007a), the aim would be to target pheromone impregnated Entostat to the antennae of a male in order to maximize chemosensory disturbance. Observations of moth behaviour around the tray-style dispenser suggested that they were repelled from walking into powder, as a brief touch of the tray appeared to be followed by orientation away from the station. Repellence from powder is likely, as particle films, particularly those formed from kaolin, are known to be repellent to a range of insect pests (Daniel et al. 2005) and widely used as barriers in pest control. It was, however, not possible to tell whether moths were making contact with powder before alighting, so the repellence may have been volatile related rather than physical avoidance. As an autodissemination station will contain active ingredients which *P. interpunctella* may find repellent, it would seem that a 'pit fall' style inoculation may be a more effective approach than a tray system as moths do not then have the opportunity to engage in avoidance behaviours.

Whilst the two techniques described here are useful indicators of powder adherence to IMM, there are potential sources of error in that not all wax particles may have dye present (see fig. 4.3) which is likely to lead to an underestimate of the amount of powder taken up. Additionally, incorporating dye into the powder matrix will alter the properties of the powder in terms of density, shape, particle size and electrostatic charge (see chapter 3) so the adhesive quantities of powder may vary significantly depending upon the addition of an active ingredient incorporated or admixed into the powder. Unlike the spectrophotometry technique, image analysis of powder uptake is also limited by the number of pixels of the digital images. With the equipment used here to image *P. interpunctella*, the area of one pixel equated to 0.7 μm^2 per pixel. This minimum detectable area is still smaller than the diameter of an average sized particle of Entostat powder (8 µm) but it does provide a lower limit for exactly how much material may be observed. Of course, this is only relevant for dyed powder which is in focus and other particles, out of focus, may go unrecorded or could even cause light to flare and register as larger surface areas than they actually are. However, the main disadvantage of the image analysis is that the three-dimensional properties such as depth of powder film, particles lodged out of view between scales, etc. will be unrecorded. Whilst it may not be a truly quantitative measurement of powder uptake, it can be considered a useful measure for determining where and in what ratio, powder is taken up by an insect.

Autodissemination is not a straightforward approach to pest management and the system is likely to fail should any of the individual factors within the required chain of events (*i.e.* pest attraction – inoculation – release – disseminate – action of insecticide) not be fully optimised. This study has demonstrated that the various *P. interpunctella* body regions have differing capabilities for carrying powder and that to target the most efficient 'carrier' region, the delivery system should

inoculate the insects 'heads'. It has been shown that this is unlikely to be achieved by using a delivery system that relies on *P. interpunctella* walking into powder and that, moreover, a pit-fall dissemination station was the most effective method of ensuring head contamination. At the time of writing there is only one commercially available insect pest control autodissemination product (Exosex®, www.exosect.com) and this is intended for use against the codling moth (*Cydia pomonella* (L.)). This system claims to inoculate the pest as they passively walk over the surface of the Entostat powder. However, results from this research suggest that this approach would be less than optimal for *P. interpunctella* as, not only will repellence from the powder be reduced, but also powder uptake itself will be more efficaciously distributed on the moth should a pit-fall inoculation be adopted.

5. The effect of *Beauveria bassiana* (Balsamo) on *Plodia interpunctella* (Hübner) fecundity, mortality and anemotactic flight behaviour

Abstract

Increased legislation and consumer demands for organic produce have meant that alternatives to chemical control are being sought for destructive pests of raw and processed foods such as *P. interpunctella*. In this chapter, the fungal entomopathogen, *B. bassiana* var. 'Naturalis' is evaluated for potential inclusion into a prototype autodissemination approach for biorational *P. interpunctella* control. Both male and female moths inoculated with approximately 5.3 x 10^6 cfu (colony forming units) of *B. bassiana* conidia, had 100% mortality at 168 h (compared to 53 and 43% in the male and female controls at the same time). There was no evidence that male moths were horizontally transferring a lethal dose of conidia to conspecifics, however, there were some indications that fecundity was affected by either male or female moths inoculated up to 48 and 96 h previously. Importantly, a rapid onset of sublethal effects of infection on anemotactic flight response was observed, where 24 h after inoculation just 40% (*SE*±6) of the treated males (compared to 100% in the control) orientated directly onto the pheromone lure. Infected moths also made significantly fewer turns per second during zigzagging lock-on flights and took longer to locate the lure. The co-formulation of conidia with Entostat™ was also shown to proportionally increase spore uptake by moths when compared to uptake with conidia alone, suggesting that cost-savings could be made through the use of this carrier.

5.1 Introduction

The use of entomopathogenic fungi in pest management is attracting interest from researchers due to the increasing regulatory constraints placed on traditional chemicals and fumigants, plus growing consumer demands for organic produce (Phillips, 2006; Schöller, et al., 2006). Unfortunately, technology transfer of this research into the market place, particularly in the food storage and processing area, has been limited (Phillips, 2006). There are many reasons for this, but a major concern is that entomopathogens are perceived to be slow to yield results when compared to chemical treatments, as the pest can take days die (St. Leger et al., 1996). However, the sublethal effects of entomopathogens are often overlooked when establishing the impact of a treatment on a pest population. These effects, although not as obvious as a death, may be substantially impacting

on host fitness and thus, will play an important role in suppressing the pest population (Torrado-León et al., 2006).

Seyoum et al., (2002), found that the desert locust (*Schistocerca gregaria* (Forsk.)) (Seyoum et al., 2002), was shown to be less able to conduct sustained flight activity when infected with the fungal pathogen *Metarhizium anisopliae* (Metschn.) Sorokin. Additionally, Eveleigh et al., (2007) found that female spruce budworm (*Choristoneura fumiferana* (Clem.): Lepidoptera) infected with the microsporidium *Nosema fumiferanae* (Thompson), were less able to instigate migration. It is not just dispersal that can be affected by sublethal infection, as Inglis et al., (1995) showed that fecundity of Orthoptera was significantly reduced by infection with the fungal entomopathogen *B. bassiana*. Sublethal effects of infection may not only be confined to the parent generation, as offspring of *B. bassiana* infected whitefly *Bemisia tabaci* (Gennadius) (Torrado-León et al., 2006) were shown to suffer from moulting defects.

In this chapter, the potential of *B. bassiana* var. 'Naturalis' as the active ingredient within an autodissemination station for the major Lepidopteran pest of raw and processed foods, the Indianmeal moth. A series of tests examining the potency of the isolate to adult moths, the concomitant effects on fecundity and the ability of infected individuals to conduct an anemotactic flight were evaluated. Additionally, as a carrier powder is likely to be an essential component of an autodissemination system (Baxter, et al., 2008) the uptake of conidia by moths when co-formulated with Entostat™ powder was assessed.

5.2 Materials and Methods

5.2.1 Test insects and rearing conditions

See Appendix I for details of insect culture and rearing conditions. For all tests virgin moths of 24-48 h old were used. In the fecundity experiment (5.2.8) where moths were dosed with conidia and left for up to 72 h before coupling with an untreated female, the control groups were similarly set-aside and, therefore, of the same age as the treatments. For the wind tunnel experiments (see 5.2.10) pre-selection of moths that were able to successfully locate a lure were undertaken on individuals 0-3 h old (all the moths used appeared to have successfully pupated and extended wings, etc.).

5.2.2 Supply of Entostat™ and Beauveria bassiana *conidia*

Entostat™ (see appendix II for details), was supplied by Exosect Ltd. (Winchester, UK). Spores were grown from Naturalis-L® (Intrachem Bio Italia SpA, Grassobbio**,** Italy), a commercially available 7.2% w/w suspension concentrate of *B. bassiana* var. 'Naturalis'. Spread plates were prepared applying 0.5 ml of the concentrate to the surface of Sabouraud's dextrose agar with 1% yeast extract (SDAY) and incubated for 1 week at 27^{o}C. A single isolate preparation of spores was taken from the spread plates and the cycle repeated, preparing dilutions of approximately 10 x 6^{8} cfu (colony forming units) per ml, to ensure purity of the isolate. Further plates were inoculated and the spores harvested after 1 week by tapping them off of the surface of the plate into a glass collection vial.

5.2.3 Inoculation of moths with conidia and formulation of spores with Entostat™

A 20 mg sample of conidia was measured into a plastic general-purpose container with hinged lid (57 mm length x 17 mm diameter) (code 216-3136, from VWR International; www.vwr.com). The conidia were applied to the sides of the container using a plastic (polypropylene) drinking straw in a circular movement so as to create a fine film of powder. A single virgin moth was held in a glass 5 ml sample tube and then tipped into the conidia tube. Once in the treatment tube, the glass vial was quickly reversed so that the base formed a seal over the end of the tube. The tubes were held in place as still as possible, for 1 min, before the moth was allowed to climb back into the glass holding tube. To mitigate the risks of spore contamination, treatments were applied in a different room to the observational phase of the experiment and powder sample tubes were disposed of after a single replicate. A positive control group of denatured conidia (autoclaved at 110^{o}C for 30 min) was used for each experiment. A pilot study revealed that the uptake of denatured spores to moths was sufficiently similar to that of the viable conidia making this a valid comparison treatment (I. Baxter, *pers. obs.*).

Six separate weight to weight (w/w) Entostat admixture formulations were tested; 100% conidia (Bb_{100}), 75% conidia : 25% Entostat (Bb_{75}), 50% conidia : 50% Entostat (Bb_{50}), 25% conidia : 75% Entostat (Bb_{25}) and a 100% Entostat control. A 1 g sample of the formulations were prepared by mixing the appropriate amount of powder into a glass vial (5 mL capacity) and agitating on a Whirlimixer™ mixing for 1 minute. See fig. 5.1.

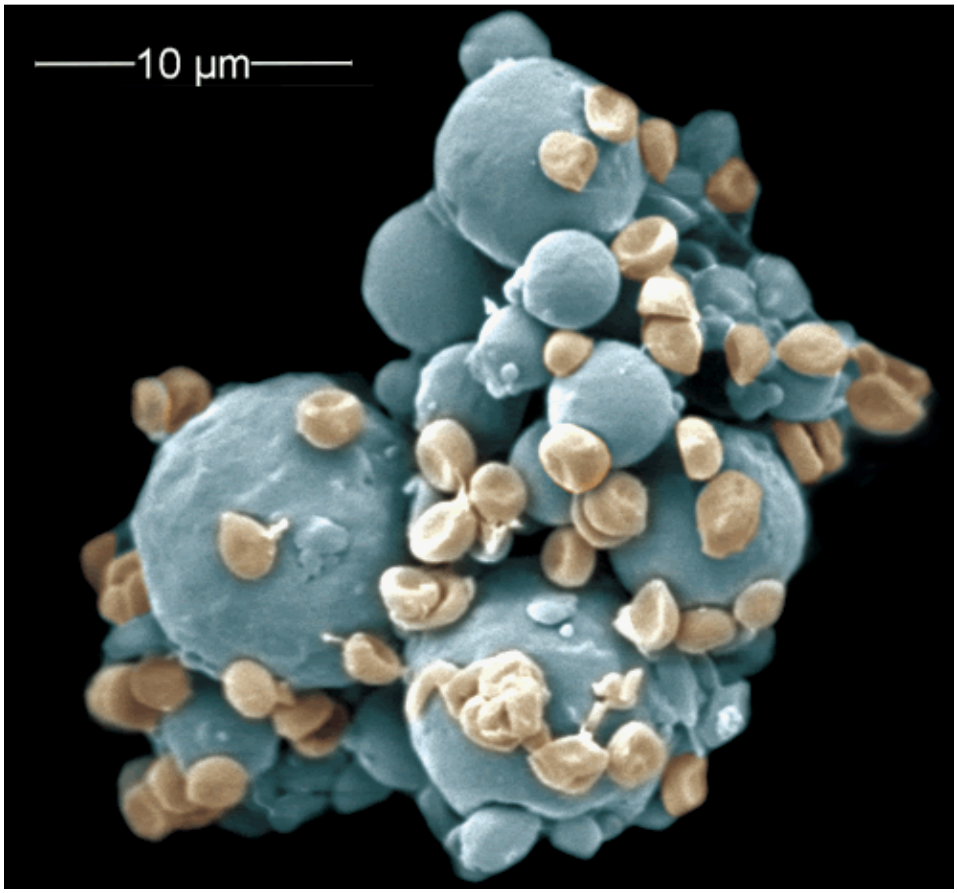


Figure 5.1. Electron-micrograph showing Entostat™ particles co-formulated with *Beauveria bassiana* (Balsamo) conidia (smaller colour shaded bodies)

5.2.4 Assessing conidia uptake by P. interpunctella

After exposure to conidia, moths were placed back into clean, 5 ml sample tubes, at which point they were immobilised by freezing at -20^{o}C for ca. 5 min. A 0.5 ml quantity of 0.05% TWEEN80™ solution in distilled water was measured into a 1.5 ml Eppendorf™ microfuge tube and the immobilized moth placed into the tube. The tube was agitated on a Whirlimixer™ plate for 1 min, after which a 0.1 ml sample of the Tween/spore solution was removed with a pipette and decanted underneath the cover slip of a haemocytometer (improved Neubauer Ruling, Hausser Scientific, PA., USA). Spores in each of the 25 reference squares were counted under a compound microscope at x250 magnification, resulting in a measurement of spores per 1 µl. In the Entostat formulations UV stage illumination was used as this helped to distinguish the reflective carnauba wax from conidia.

5.2.5 Assessing conidia viability

Conidia were diluted in sterilised distilled water and 0.05% TWEEN80™, until they formed a solution containing approximately 10 x 6^8 spores/ml. Spread plates of 0.5 ml of the conidia suspension were spread on Sabouraud's dextrose agar with 1% yeast extract (SDAY) and incubated for 18-24 h at 27°C. To assess viability, a glass cover slide was placed onto the surface of the media and 100 conidia examined for presence of growing germ tubes. A spore was determined as being viable when a germ tube was at least equal in length to the diameter of the conidia. In all experiments viability exceeded 90%.

5.2.6 Experiment 1. Formulated conidia uptake and retention by adult P. interpunctella

Groups of 10 virgin male moths 24-48 h old, were treated with either 100% spores (Bb_{100}), 100% Entostat (control), or ratios of spores to Entostat 75:25 (Bb_{75}), 50:50 (Bb_{50}), 25:75 (Bb_{25}). The moths were dosed with powder as per 5.2.3 by exposing them to 20 mg in a sample tube for 1 min. The moths were immediately immobilised by freezing and the numbers of conidia counted as per 5.2.4.

5.2.7 Experiment 2. Effect of B. bassiana *inoculation on mortality of adult* P. interpunctella

Thirty adult males and females were randomly selected from the separate-sex holding cages for treatment with either viable conidia or the positive control (heat denatured conidia). After exposure to conidia (as per 5.2.3), moths were held individually in plastic 568 ml pots, with a breathable paper lid at 26–28°C and humidity at 30–42%. Assessments of moth mortality were made at 1, 24, 48, 72, 96, 120 and 168 h after treatment. A further group of 10 moths were treated with conidia and the number of spores present on the moths calculated as per section 5.2.5. An untreated control group were also subjected to the same procedures to examine if background levels of spores or shed scales were inadvertently contributing to the results.

5.2.8 Experiment 3. Effect of B. bassiana *inoculation on fecundity of* P. interpunctella

The effect on fecundity of *B. bassiana* was examined with either male or female moths having been dosed with conidia either 24, 48, 72 or 96 h prior to coupling with an untreated conspecific. Moths were randomly assigned partners and the treated individuals were included in the experiment even if they had died during the period from inoculation. A positive control was included where both male and female moths were treated with denatured spores. Inoculated individuals were placed in a 9 cm plastic petri dish and, after 120 h the number of eggs laid by the female was counted by placing graph paper underneath the petri dish and viewing the eggs through a binocular microscope. A

subsection of 10 eggs was taken from each of five randomly selected replicates. The eggs were held for a further 72 h and the amount that hatched observed under the binocular microscope to calculate the proportion of viable eggs.

5.2.9 Experiment 4. Horizontal transfer of B. bassiana *between conspecifics of* P. interpunctella

Horizontal transfer was evaluated from treated male to untreated females and from treated male to untreated males. Firstly, a male moth was treated with conidia (viable spores in the treatment, non-viable for the control) at the 20 mg exposure rate (5.2.3) and placed in a plastic holding arena (polypropylene food storage container, 26 cm x 26 cm x 26 cm, Asda, Chandlers Ford, UK) with a single virgin female. After 24 h, the individuals were separated and housed in plastic 9 cm petri dishes with mortality assessments made every 3 days over a 12-day period. Secondly, prior to dosing with conidia, treated males were lightly anaesthetised using humidified CO_2 gas and marked on the apex of their wings using a nontoxic marker paint (Uni Posca, Bullet tip, medium line, Mitsubishi Pencil Co. Ltd., Japan) (the dot being <2mm diameter). A single treated male moth was placed in the plastic holding arena (as above) along with 6 untreated males. After 24 h, the treated moth (identified by the marking on his wings) was removed from the experiment and, as in the male-female assay, mortality assessments were carried out every 3 days over a 12-day period. Horizontal transfer was evaluated for 30 female moths, with experiments being carried out in groups of 10 for practical purposes ($N = 30$). There were 5 replicates of 6 male moths per container, for the male-to-male horizontal transfer ($N = 5$).

5.2.10 Experiment 5. Effect of B. bassiana *inoculation on anemotactic flight behaviour*

Trials were conducted inside a glass wind tunnel [60 cm (height) x 60 cm (wide) x 150 cm (depth)] similar to El-Sayed et al. (1999). The wind tunnel has a push/pull system of variable speed fans, a charcoal filtration system at each end and steel mesh mounted in both ends of the tunnel to aide laminar flow. The airflow was kept at 35 cm/sec and the tunnel was illuminated by an overhead red 120 cm strip light generating 30-50 lux inside the tunnel. The tunnel was lined with a white paper base to help contrast the moth for imaging via a overhead, wide-angled, low-light video camera (Sanyo VCC-4344, CCD camera, Sanyo UK, Watford, UK), which was used to capture the moths flights onto DVD for later analysis. Room temperature was maintained at 26–28^{o}C and humidity of 30–42% r.h.. A pheromone lure consisting of a rubber septum (Exosect Ltd., Colden Common, UK) containing 0.1 mg of (Z,E)-9,12-tetradecadienyl acetate (ZETA) (Bedoukian Research Inc., Danbury, CT, USA) was placed onto the tip of a glass pipette elevated 15 cm from the base of the

tunnel (see fig. 5.1). Lures were stored in the dark at -20^{o}C until 1-2 h before the experiment, when they were only handled using metal forceps which were washed in ethanol (99%) between replicates. Once mounted on the glass pipette, the lure was left in the active wind tunnel for 5 min to enable the pheromone release rate to settle. During this time, the walls and base of the tunnel were wiped with ethanol (99%) on a fresh paper cloth to prevent contamination. Steps were also taken to prevent releases of pheromone into the wind tunnel laboratory and, potentially, cause habituation of the male moths from the attractant. Firstly, the lures, which were replaced for each replicate, were held in individual glass tubes with a foil-lined screw top lid and were opened for the minimum time possible and always at the down-wind end of the wind tunnel so that volatiles would be drawn into the charcoal filter rather than the room. Moths awaiting testing were kept in clean plastic vials (as per 5.2.3) with the lids fastened whilst in the wind tunnel laboratory. For testing, moths were released into a plastic weighing boat (similar to Cox, et al., 2007) which stimulated them to fly when they reached the upper edge) 100 cm downwind of the lure (see fig. 5.2(iii)). All flight studies were carried out within 2 h of onset of scotophase.

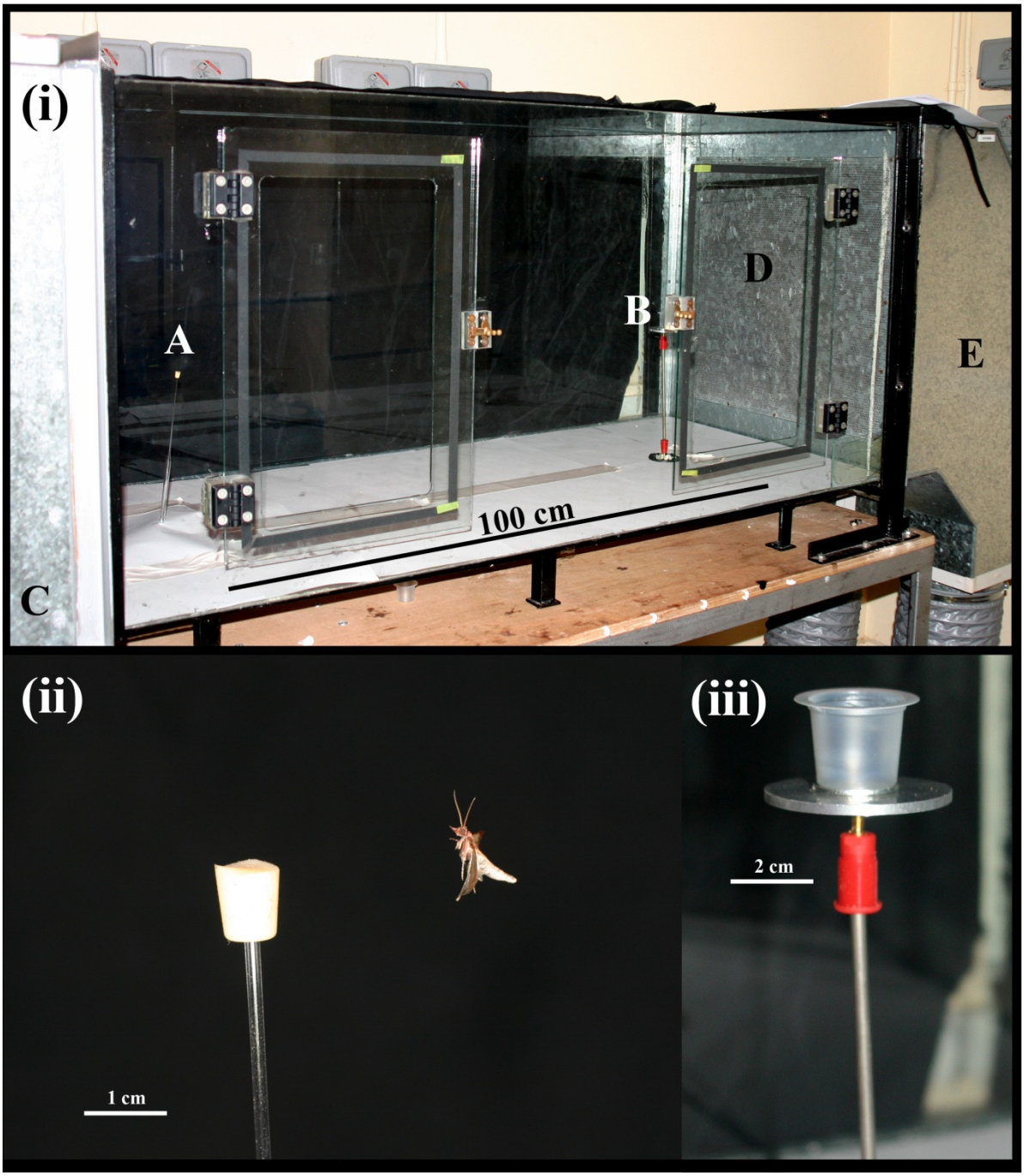


Figure 5.2. Wind tunnel. (i) A = septum containing pheromone; B = moth release platform; C = push charcoal filter; D = laminar flow grid; E = pull charcoal filter. (ii) *P. interpunctella* flying to lure. (iii) moth release platform

Pilot studies revealed that moths' ability to locate a lure varied considerably between individuals (I. Baxter *pers. obs*.) and Power-analyses (Doncaster & Davey, 2007) indicated that the amount of variation observed in these studies would require an unrealistic number of replications to be enable confidence in the experimental design Therefore, a pre-selection criterion of only using moths able to locate the lure within 30 sec was observed, as it was found that moths which reached this criterion were more likely to successfully locate the lure on subsequent days (I. Baxter *pers. obs*.). To limit the effects of pheromone habituation or exhaustion, the pre-selection flights were carried out 24 h prior to the wind tunnel experiments beginning. Moths that met the selection criterion were then dosed with conidia and assessed in the wind tunnel every 24 h until there was 100% mortality in the treatment group. Each moth was assigned an alpha-numeric identifier so that (1) it could be tracked throughout the course of the experiment and, (2) so that the sequence of moths tested could be randomised on a daily basis. Unlike the pre-selection criterion, moths were allowed up to 3 min to locate the lure (defined as making physical contact with the 1 cm cylindrical septum).

The flights were recorded onto DVD for later analysis and tracking using EthoVision™ v 3.1 (Noldus Information Technology, Wageningen, The Netherlands). Due to the constraints of filming the rapid movements of the moths under low lux conditions, the software was unable to reliably detect the moth. Therefore, the digital footage was edited in Adobe® Premiere Pro™ and a black sphere of equivalent size to the moth placed in a separate video layer, above the subject, every third frame (see fig. 5.3). This increased contrast to a suitable level for software analysis. The Ethovision software package was used to determine the time taken to fly to the lure by measuring the time at which the moth (black sphere) crossed a pre-defined start-point denoting the release platform and made contact with another defined area positioned where the pheromone lure was placed. The software also produced trail maps of each flight (see fig. 5.4). Data on distance travelled and number of counter-turns made during a locked-on orientation flight to the pheromone proved unreliable when analysed by the software (I. Baxter *pers. obs.*). Therefore, it was decided to manually record the frequency of counter-turns by playback of each event at 10% normal frame rate for each flight. A counter-turn was defined as an obvious change in direction resulting in movement across the x-axis of the wind tunnel. Data on distance travelled was not achievable with this set-up due to the design of the wind tunnel which did not allow for continuous viewing from the *y*-plain.

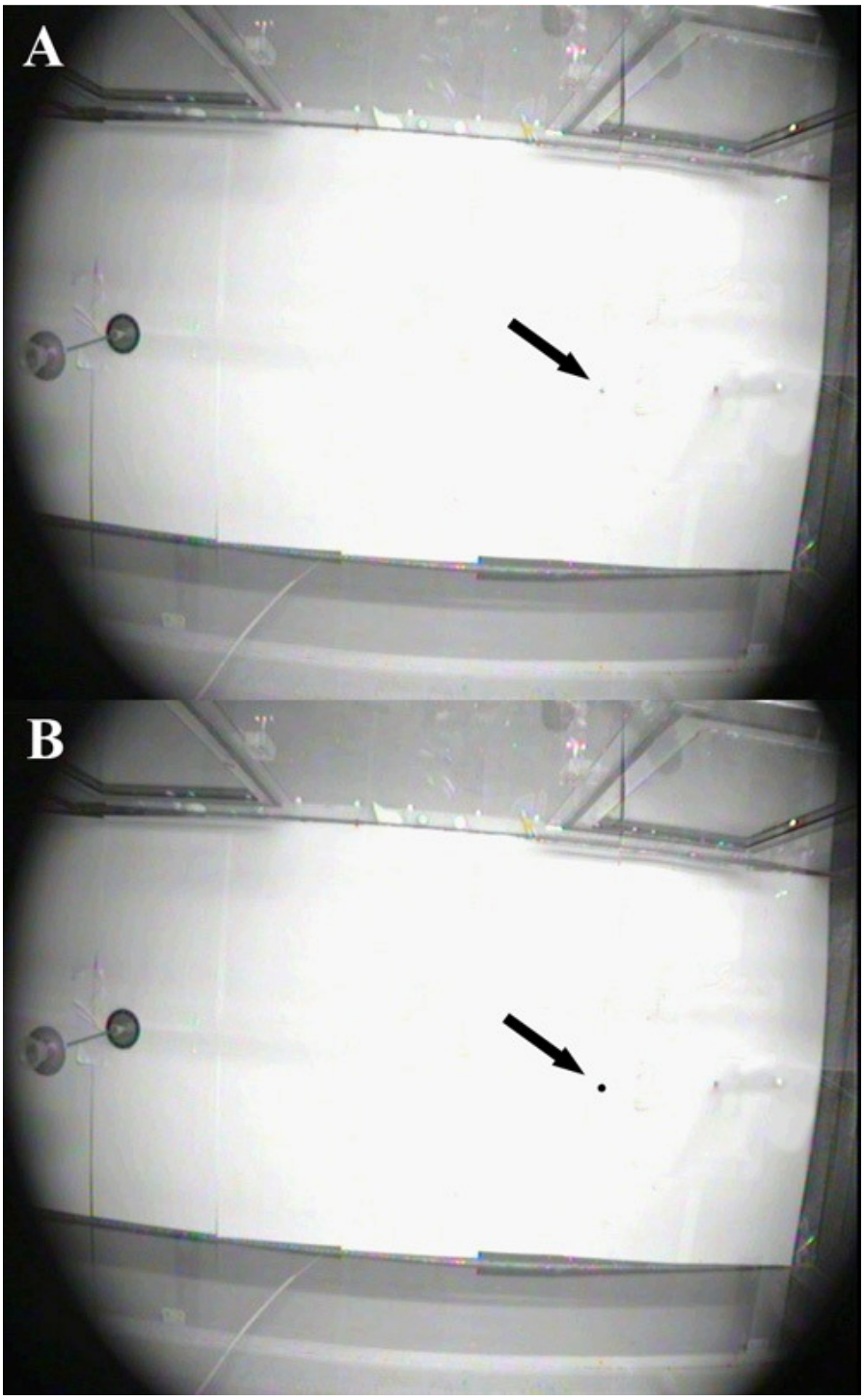


Figure 5.3. Still images from wind tunnel, (A) showing moth (marked with arrow) with weak contrast and, (B) with a black sphere of equivalent size edited onto the video in place of the moth in order to increase contrast for later analysis with EthoVision™

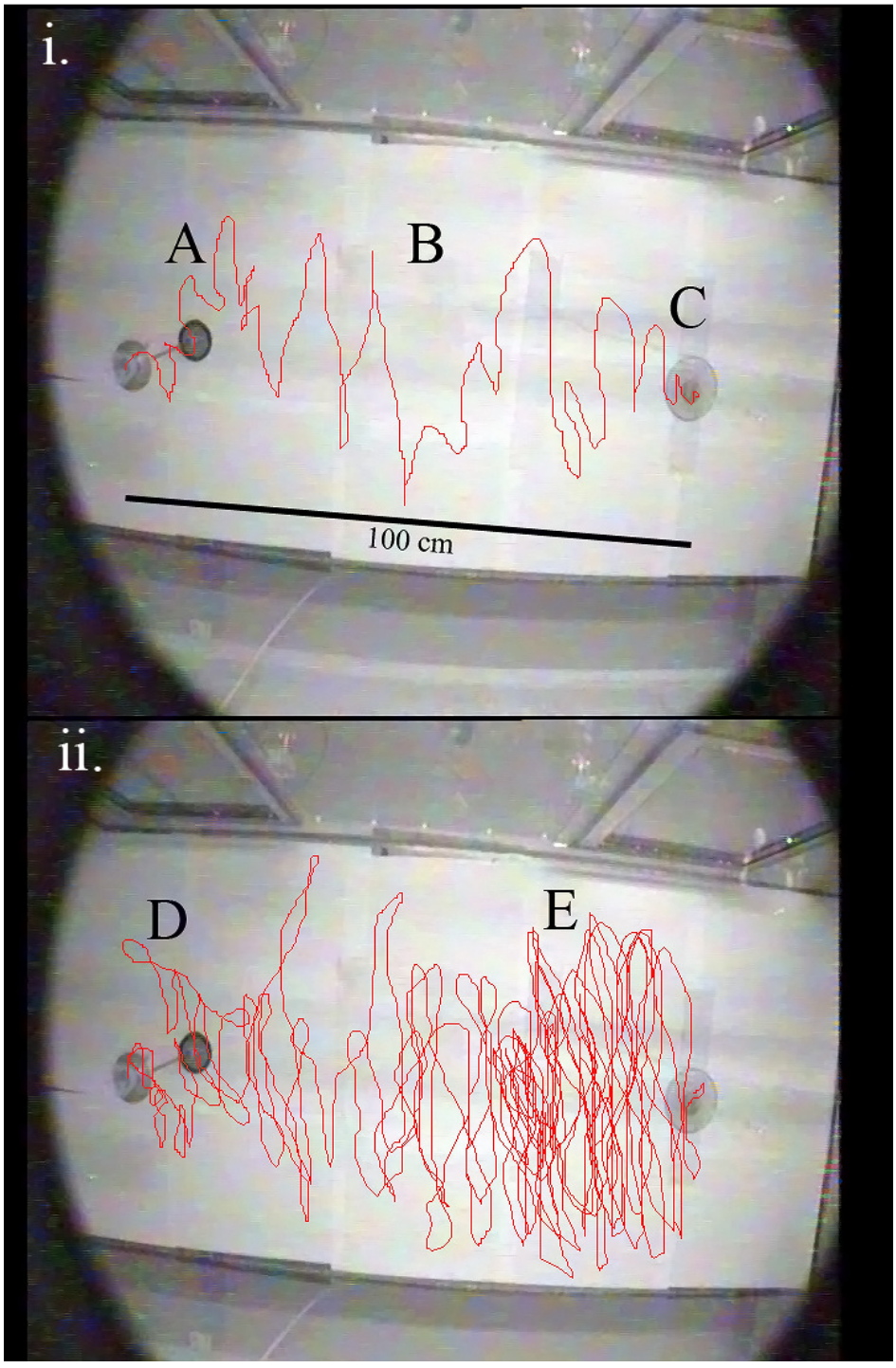


Figure 5.4. Flight trails (red line) of two 24 h old male *Plodia interpunctella* generated with EthoVision™ video-tracking software. Plate i. is a control moth which alights from the platform (A) and immediately commences zigzagging searching turns and counter-turns (B), before locating the pheromone lure (C) after 11 sec. Plate ii., the moth was treated 24 h previously with *Beauveria bassiana,* required much longer to locate the lure (152 sec), but made less frequent counter-turns. Treated moths appeared to spend more time characteristically zigzagging ca. 25 cm (E) downwind of the lure, less able to immediately locate the lure.

5.2.11 Data analysis

All statistical analysis were carried out using the software package MINITAB v14 for the PC (MINITAB Inc, v14, State College, PA, USA).

For experiment 2, the LT_{50} and LT_{95} (lethal time (h) for 50 and 95% of the *P. interpunctella* to die) was calculated from linear regression (log time vs log % mortality +1) using MINITAB.

In experiment 3, data were transformed to normalise distributions ($\log10(x + 1)$) and one-way ANOVA's (Analysis of Variance) conducted to compare the fecundity of each group at the three different inoculation times - 24, 48 and 72 h - prior to coupling. Tukey's *post hoc* test was used to establish which treatments were significantly different. Egg viability was compared using Mann-Whitney *U*-tests between the treatments and the control.

In experiment 4, mortality rates were compared using Mann-Whitney *U*-tests between the treatments and the control.

In experiment 5, the number of moths making contact with the lure was compared between the control and treatment group by independent samples *t*-test (distribution normalised by $\log_{10}+1$ transformation of the raw data). The time taken for moths to make contact with the pheromone lure was compared between treatment and control using independent samples *t*-test (distribution normalised by $\log_{10}+1$ transforming raw data). A comparison of the time difference taken for control moths to locate the pheromone lure between day sampling points 24, 48, 72 h was analysed using a repeated measures ANOVA (time (sec) normalised by $\log_{10}+1$ transformation). Only moths that had successfully reached the lure at all three time points were included in the analysis. Not enough moths reached the lure for a comparative analysis to be carried out for the viable conidia treatment group. A Mann-Whitney *U*-test comparison of the number of counter-turns made per second was compared between the treatment and the control at the 24 h time-point only, as there were not enough successful flights made in the treatment group to make comparisons on subsequent days. Further Mann-Whitney *U*-tests were performed to compare the number of turns per second made by the control group over subsequent days. Bonferroni correction levels on the probability epsilon were quoted as the experiment was a repeated measured design.

5.3 Results

5.3.1 Experiment 1. Formulated conidia uptake and retention by adult P. interpunctella

The results of the Entostat formulated conidia uptake experiments are shown in fig. 5.5. It was calculated that conidia uptake at the 20 mg exposure level was equivalent to 5.3 x 10^6 (*SE*±5%) cfu per moth. The background conidia counts from the untreated control group were 1.1 x 10^3 (*SE*±19%). Therefore, only 0.02% of the spores can be accounted for as either conidia already present on the moths or false recordings from scales or other debris.

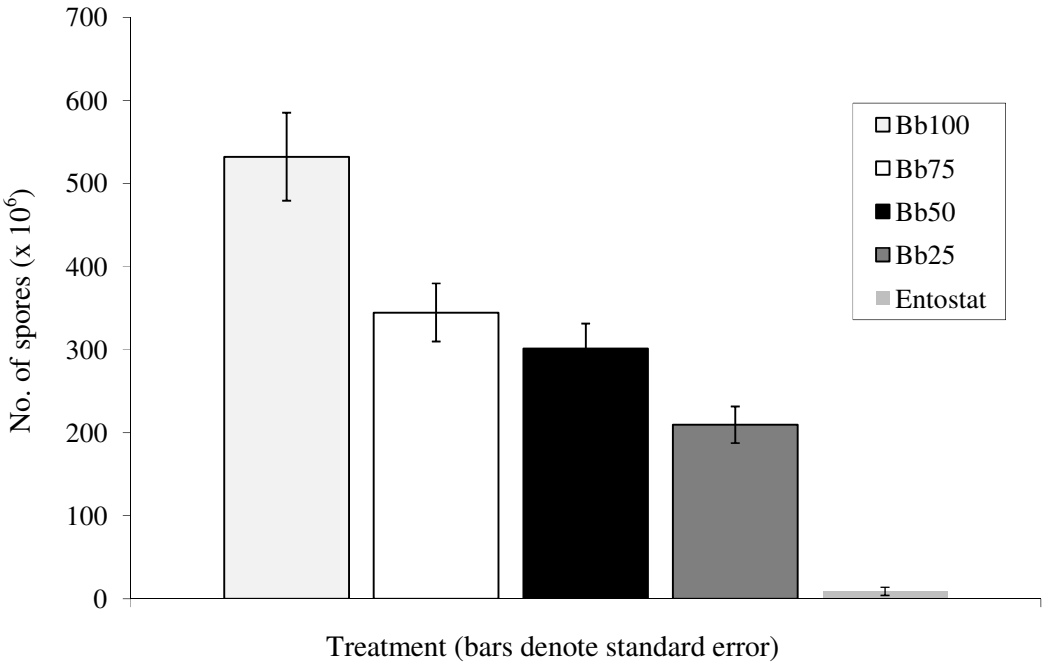


Figure 5.5. The mean number of conidia (±*SE*) recovered from Indianmeal moths exposed to 20 mg of spores formulated with Entostat™ in different ratios (25-75% - Bb_{25}, Bb_{50} and Bb_{75}), a positive control of 100% conidia (Bb_{100}) and a negative control of just carrier powder (Entostat) (N = 10).

5.3.2 Experiment 2. Effect of B. bassiana *inoculation on mortality of adult* P. interpunctella

The results of the mortality assay are shown in table 5.1. The LT_{50} and LT_{95} were calculated as being treatment male = 59 and 159 h for the male (Adjusted R^2 =0.87, F_6 = 40.4, P = 0.001) and 61 and 161 for the treated female (Adjusted R^2 = 0.86, F_6 = 37.4, P = 0.002).

Table 5.1. Mortality of male and female *Plodia interpunctella* treated with 5.3 x 10^6 (*SE*±5%) *Beauveria bassiana* var. 'Naturalis' conidia

N = 30		% mortality (treatment groups are Abbott's corrected*)						
		Time after treatment (h)						
Gender of treated group		1	24	48	72	96	120	168
Males	Control	0	3.3	6.7	6.7	10.0	10.0	53.3
	Treatment	0	6.7	16.7	16.7	60.0	86.7	100
	Abbott's corrected*	-	3.4	10.7	10.7	55.6	85.2	-
Females	Control	0	0	3.3	3.3	6.7	6.7	43.3
	Treatment	0	0	10.0	23.3	60.0	90.0	100
	Abbott's corrected*	-	0	6.9	20.7	57.1	89.3	-

*After Abbott, (1925).

5.3.3 Experiment 3. Effect of B. bassiana *inoculation on fecundity of* P. interpunctella

The results of the fecundity assay are presented in table 5.2. Fecundity was not significantly different for the groups coupled with conspecifics at 24 or 72 h after treatment with conidia. However, individuals coupled at 48 and 96 h after treatment showed a significantly reduced fecundity from that of the control (**48 h =** ANOVA: $F_{2,27}$ = 3.93, P = 0.032; **96 h =** ANOVA: $F_{2,27}$ = 12.87, P = <0.001) from that of the control. Egg viability was not significantly different for any treatment (*N = 5,W* = 27.5, *P = NS)*, but the analysis did not include the 96 h viable spore treatment moths as most individuals had succumbed to the entomopathogen.

Table 5.2. Fecundity of *Plodia interpunctella* after treatment with 5.3 x 10^6 (*SE*±5%) *Beauveria bassiana* var. 'Naturalis' conidia of either male or female moths 24-96 h prior to coupling with an untreated conspecific

N = 10		Mean number of eggs per female			
		Time after treatment (h)			
Gender of treated group		24	48	72	96
Males	$\bar{x}$ No. of eggs	119	133	92	17
	±*SE*	18	19	16	16
	% Egg viability	100	100	96	†
Females	$\bar{x}$ No. of eggs	146	136	81	7
	±*SE*	20	12	14	7
	% Egg viability	100	96	94	†
Control	$\bar{x}$ No. of eggs	155	188	126	106
	±*SE*	20	12	11	17
	% Egg viability	96	100	96	96

† Not enough dishes had eggs in to carry out meaningful viability assessments

5.3.4 Experiment 4. Horizontal transfer of B. bassiana *between conspecifics of* P. interpunctella

The results of the horizontal transfer assays are shown in table 5.3. None of the treatments showed significant differences in mortality from that of the control ($N = 30$, $U = 885.0$, $P = NS$).

Table 5.3. Mortality of *Plodia interpunctella* exposed to a male treated with conidia of *Beauveria bassiana*

		Mean % mortality				
		Time after treatment (days)				
Assay		0	3	6	9	12
Male horizontal transfer to males (±95%CI) $N = 5$	Control	0(-)	0(-)	0(-)	3.3(3.3)	20.0(20.0)
	Treatment	0	0	16.7(0)	20(6.5)	23.3(8.0)
	Abbott's corrected*	0	0	16.7	17.3	4.2
Male horizontal transfer to females (±95%CI) $N = 30$	Control	0(-)	0(-)	0(-)	3.3(7.4)	10.0(8.0)
	Treatment	0(-)	0(-)	0(-)	16.7(9.1)	16.7
	Abbott's corrected*	0	0	0	3.5	7.4

*After Abbott, (1925).

5.3.5 Experiment 5. Effect of B. bassiana *inoculation on anemotactic flight behaviour*

Significantly fewer of the *B. bassiana* treated moths completed an anemotactic flight within 3 mins to the pheromone source compared to the control ($t = 3.48$, DF = 15, $P = 0.003$). Results for the numbers of moths making contact with the pheromone lure are shown in figure 5.4. The time taken for moths to locate and make contact with the pheromone source is shown in table 5.4. Control moths' speed of pheromone orientation was shown to decline over time, as they took significantly more time to locate the pheromone at the end of the 72 h experimentation period (repeated measures ANOVA: $F_{1, 12} = 1142.462$, $P < 0.001$). The results for the anemotactic flight behaviour of the moths (frequency of counter-turns and turns/sec) are shown in table 5.5. Treated moths made significantly fewer turns per second than the control moths at 24 h after treatment ($N = 30, 12, U = 777.5$, $P = 0.0002$). Control moths made significantly fewer turns per sec as from 24 to 48 h after treatment ($N = 30, 24, U = 1101.0$, $P = <0.0001$, sig. at 0.016 level, Bonferroni correction).

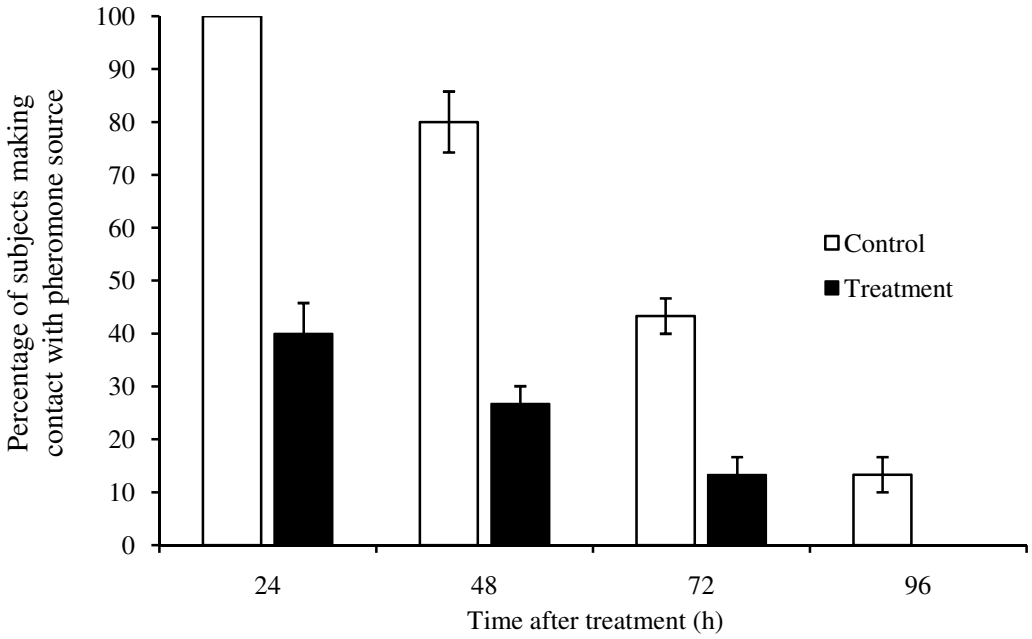


Figure 5.6. Percentage of moths orientating to and making contact with pheromone lure in wind tunnel within 3 mins of release. Moths were treated with ca. 5.3 x 10^6 cfu of *Beauveria bassiana* var. 'Naturalis' conidia. The control individuals were treated with heat-denatured conidia (error bars indicate standard error).

Table 5.4. Duration for *P. interpunctella* treated with *Beauveria bassiana* var. 'Naturalis' conidia (concentration of ca. 5.3 x 10^6) to locate and contact a pheromone lure within a wind tunnel.

	Mean Time (sec) to locate pheromone lure			
	Time after treatment (h)			
	24	48	72	96
Control (±*SE*)	30.9 (4.3)	41.2 (5.2)	65.8 (6.7)	59.5 (2.6)
Treatment (±*SE*)	50.3 (10.5)	49.25 (7.4)	92.3 (15.2)	-
P (*t* and *DF*)	<0.05 (*t* = -2.20, *DF* =19)	*NS* (*t* = -1.44, *DF* =16)	*NS* (*t* = -1.63, *DF* =8)	-

Table 5.5. Anemotactic flight behaviour of *P. interpunctella* treated with *Beauveria bassiana* var. 'Naturalis' conidia (concentration of ca. 5.3 x 10^6) flying to a pheromone lure within a wind tunnel.

		$\bar{x}$ frequency of counter-turns made during anemotactic flight to pheromone source		
Time after treatment (h)		24	48	72
Control	No. of turns (±*SE*)	63.2 (4.25)	65.8 (6.7)	59.5 (2.3)
	Turns/sec. (±*SE*)	1.9 (0.07)	1.5 (0.42)	0.9 (0.06)
	*N**	30	24	13
Treatment	No. of turns (±*SE*)	54.0 (10.2)	85.5 (12.8)	-
	Turns/sec. (±*SE*)	1.17 (0.16)	1.3 (0.30)	-
	*N**	12	8	4[†]

* Number of moths that located lure
[†] Too few subjects to include in analysis

5.4 Discussion

Researchers have investigated a number of diluents as additives to dry powder entomopathogic formulations in attempts to make production more economical or the system more efficacious against the pest (Lord 2005; Akbar, et al., 2004; Lord, 2001; Taborsky, 1992). Co-formulated powder additives may serve to protect conidia from biotic and abiotic factors which will impact upon product storage stability and efficacy of deployed materials in the field (Smith et al., 2006). In the first phase of this research, Entostat™ was evaluated as a carrier for conidia of *Beauveria bassiana* (Balsamo) to the Indianmeal moth (*Plodia interpunctella* (Hübner)). The Entostat/conidia formulations did not result in an increased uptake of conidia by the moths when compared to the 100% *B. bassiana* treatment. However, there were indications that Entostat was having a positive adjuvant effect as, although moths in the Bb_{25} treatment only took up 39% of conidia compared to the Bb_{100} treatment, the dosing tubes contained 75% w/w less spores, so the proportion of conidia uptake was better in the Entostat formulated product. Data suggested that lower concentrations of conidia to Entostat resulted in an increased proportional uptake of conidia as both Bb_{25} and Bb_{50} resulted in a proportionally higher uptake of conidia, whereas Bb_{75} recorded 10% less proportional uptake. Boucias et. al., (1988), determined that electrostatic charge was not important in the

attachment of conidia to host cuticle, in which case it is unlikely that charge separation from Entostat to spores is synergising pathogen uptake. It is, however, possible that tribo-charged Entostat (Baxter et al., 2008) is enhancing attraction of spores which, themselves are associated with the surfaces of the carrier powder (see fig. 5.1) and spores are attracted to the insect with the carrier powder along the principals of charge attraction reported in chapter 2.

From these experiments it is concluded that Bb_{25} was the most effective and economically viable of the formulation rates evaluated for a prototype, conidia-based, autodissemination station. However, further work would be required to examine whether lower ratio mixes of conidia to Entostat could further optimise the proportion of spore uptake. Additional studies examining spore viability under realistic environmental conditions would also be beneficial to shape the future direction of this programme. For example, Entostat, being a hydrophobic wax, may offer protection from moisture which, in-turn, could prevent premature germination of the conidia and improve product shelf-life. A successful autodissemination approach along these lines will certainly need a reliably manufactured product with adequate shelf- and field-life before it can become and established tool for the PCO.

Adult male and female Indianmeal moths treated with *B. bassiana* conidia var. 'naturalis', showed similar mortality curves, with LT_{50}'s at ca. 60 h and LT_{95}'s at ca. 160 h, but, in addition to the characteristics of the isolate, a number of other factors may affect the potency of the entomopathogen – (1) the amount of active ingredient the target pest has been exposed to, (2) dietary stress and, (3) relative humidity :-

(1). In these experiments the amount of conidia moths were exposed to was constant and a concentration gradient was not evaluated. However, as previous authors have shown and, as would largely be expected, increasing spore concentration often results in a faster LT_{50} (Devi and Maheswara, 2006). It is not known whether increasing the spore concentration in these studies would result in a faster mortality rate, but as inoculated moths appeared well 'dusted' with spores over all sides of their body (I. Baxter, *pers. obs.*) it seems reasonable to assume the dosing regimen used here was at the higher end of what it is feasible to dose a Indianmeal moth.

(2). Indianmeal moths have adapted to live on a wide-range of foods (Rees, 2004), but as McVean, et al., (2002) showed in their studies on *P. interpunctella* and granulosis virus, individuals subjected to dietary stress were, surprisingly, less susceptible to infection with the virus. Whilst this has little consequence for these early-stage experiments, it is an important consideration of the differences

which may be encountered between laboratory strains reared on a nutrient-rich diet and those which may be encountered in a genuine infestation situation. Again, further studies would be beneficial using multiple strains of moth harvested from infested warehouses.

(3). Experiments involving entomopathogens are frequently carried out under high humidity to favour germination of spores (Lord, 2007). This leads to questionable results and has little application for the system outside of a laboratory environment. The experiments carried out here were conducted under a relative humidity of 30-42%, as this was determined as being realistic to that of a UK food processing factory or storage facility (P. Abbott, Statfold Seed Oil Ltd., *pers. corr.*). The availability of moisture will have an effect on the LT_{50}, as it has been well documented that fungal entomopathogens require high humidity to germinate (Gillespie & Crawford, 1986). However, the moths themselves are also dependent on humidity for survival. Somewhat unexpectedly for a moth that has evolved as a pest of dry bulk-storage food facilities, Howe (1965) classified the Indianmeal moth as needing moderate humidity (r.h. ca. 40%) for optimum survival. It is important that all components of the autodissemination system, which certainly includes the fungal isolate, are capable of activity against the pest under a range of realistic environmental conditions for it to be accepted as a viable control tool.

In examining the sublethal effects of *B. bassiana* on fecundity, it was considered unlikely that either sex of *P. interpunctella* inoculated ≥96 h prior to coupling would be capable of producing viable offspring, as the majority of treated moths in this experiment were either dead or moribund at this point. There were indications that fecundity was marginally affected with two of the four sampling points showing significantly less fecundity than the control, however, there were no significant differences depending on whether it was the male or female moths that received the conidia treatment. There were also no significant differences in egg viability at 24-72 h and too few eggs to gain a meaningful sample at 96 h, suggesting that both infected male and female moths could be viable mating partners for up to 72 h after inoculation. From the standpoint of using this isolate in an autodissemination approach to moth control, inoculated fecund moths may be producing viable offspring for up to 72 h before the onset of *B. bassiana* infection renders them unable to mate. These results also show that a female moth whom has become infected with *B. bassiana* during courtship, will most likely lay a full batch of viable eggs before the infection impacts on fecundity. On this basis, there is unlikely to be a significant impact on the overall Indianmeal population based on the autodissemination of this particular isolate of *B. bassiana*.

In experiment 4, the mortality of moths exposed to spores by horizontal transfer either during courtship or by containment with a treated individual was examined. There was no evidence that a lethal dose was transferred to any conspecifics during either of these studies. However, the design of these studies does not take into account secondary recycling of spores via sporulating cadavers. Under the conditions evaluated here (26-28^{o}C, 20-42% r.h.) moths did not show evidence mycosis which would be needed to recycle infection to conspecifics. Indeed, only when humidity was increased to ≥70% were hyphae visible (I. Baxter, *pers. obs.*) (see. fig. 5.7). Under appropriate environmental conditions, infected moths could go to act as foci of infection once they have died and the fungus forms infective bodies (Thomas et al., 1995). However, it is unlikely that the high humidity required for mycosis in these studies is typically achieved in a UK food storage/processing plant.

Figure 5.7. Adult *Plodia interpunctella* (Hübner) showing mycosis from *Beauveria bassiana* infection.

Anemotactic flight behaviour was shown to be significantly affected by sublethal infection with *B. bassiana*. As Nansen et al., (2007a) found that *Lobesia botrana* Den. and Schiff, which were treated with Entostat powder did not fly readily in the wind tunnel (possibly attributed to particles

obstructing antennal receptors), a positive control treatment of heat-denatured conidia was used to mitigate changes in behaviour observed from the presence of particles on the moth. The viable conidia treated moths demonstrated a considerable reduction in successful anemotactic flights 24 h after treatment, with just 40% (*SE*±14.5%) locating the lure, compared to 100% of the control. The trend continued up to 72 h after treatment, after which none of the viable treated moths made contact with the pheromone lure compared to 43% of the control. Interestingly, those moths that did reach the lure took both significantly longer than the control and made fewer counter-turns per sec while in flight. This is best illustrated in the EthoVision™ paths shown in figure 5.4. In the control groups it was also possible to observe the effects of aging on flight performance. Significantly fewer moths successfully located the lure within the 3 min time limit over the 96 h experimental period. Additionally, there were indications that older moths were making fewer turns and counter-turns per second as they aged, with the control moths making an average of 1.9 (*SE*±0.07) turns per sec at 24 h, falling to 0.9 at 72 (±0.06) h. Shi and Njagi (2004) found that Locusts (*Locusta migratoria manilensis* (Mayen 1835)) infected with the protista *Nosema locustae*, had weaker amplitude electroantennogram responses than untreated comparisons. As image analysis (chapter 4, figure 4.2) clearly shows carrier powder adhering to the antennae of the moth, it is possible that the pure conidia preparation used here also adheres in a similar pattern and that it is the early onset of disease that is impacting on chemoreception.

In addition to the effects on chemoreception, the impact of sublethal *B. bassiana* infection on the bodies' energy resources must also be considered (Seyoum et al. (2002). Lacey et al. (1995), evaluated the dispersal of Japanese beetle (*Popillia japonica* (Coleoptera)) infected with *M. anisopliae* and found that less than half of the beetles recaptured from the treatment group. They did, however, recapture some treated individuals 1 km from the release point, which drew them to conclude that the delayed time to death and distance over which they dispersed would potentially make *M. anisopliae* an effective dissemination agent. Keil et al. (2001) showed that age has an effect on codling moth locomotion activity where individual males spent increased time flying until day 5, after which distance became reduced. From this, Keil et al (2001) concluded that codling moth are at peak dispersion at around 4 days after eclosion. From the results observed here with *P. interpunctella* it could be that although the moths fly for a longer duration as they get older, their ability to locate a pheromone source and commence co-ordinated lock-on flights reduces with age. Like the age-related effects, infection of *P. interpunctella* with the *B. bassiana* conidia appears to

induce effects akin to premature aging, whereby moths take longer to locate a lure, make fewer successful flights and make fewer counter-turns per second.

In summary, it would appear that the addition of Entostat as a diluent is a potential way of reducing the number of conidia in the preparation, which could result in a more effective manufacturing process. Secondly, although the *B. bassiana* var. 'Naturalis' isolate caused 100% mortality, the isolate was not sufficiently active against Indianmeal moth to be effective when horizontally transferred, meaning it is unlikely autodissemination will be effective in these circumstances. It seems unlikely that inoculating the foci moth with more spores would improve transfer rates as, (1) the moths in these experiments were determined as being near to saturation point and, (2) Baxter et al., (2008) showed that only 4% of the Entostat powder on contaminated males was transferred to females, which, although this is not directly comparable to conidia (due to differences in particle size and physical qualities), it serves as a guide as to what sort of levels of transmission may be possible. The sublethal effects of infection on anemotactic flight showed a significant impact on pheromone location 24 h after inoculation. Whilst this shows that the entomopathogen would be an effective primary insecticide it, again, would be problematic for the autodissemination concept where treated males require fitness to locate, mate and horizontally transfer active ingredient. This chapter justifies further research into the concept, concentrating on alternative active ingredients and the possibility of examining the secondary recycling of entomopathogen infection, both to conspecific adults and larvae.

6. The Critical Components of an Autodissemination System – an individual based modelling approach

Abstract

A prototype *P. interpunctella* autodissemination control system was critically evaluated using individual-based modelling (IBM). Data from previous chapters was incorporated into the model to calculate what proportion of a virtual, mixed-age population of 1000 male *P. interpunctella* would become contaminated with active ingredient (a.i.). It was calculated that using a fully optimised dispenser (where it is assumed that 100% of moths aged 0-24 h would visit) will result in approximately 62% of the virtual male moth population entering it over a 7 day period. Of those treated moths, a maximum of 39% (equivalent to 194 males) would be able to subsequently locate a female. This number declined rapidly as the population aged and as they either died or were less able to locate the pheromone source. Sensitivity analysis revealed that dispenser attractiveness was contributing more to the proportion of viable inoculated individuals than the deleterious effects of powder uptake observed in chapter 5. Finally, the proportion of female moths that became recipients of a.i. was calculated with the variable of 'number of times a male can transfer a.i.' set incrementally between 1 and 4. It was determined that increasing the number of transfer events had a relatively small effect on a.i. uptake by females, as the ability of male *P. interpunctella* to locate a calling female declined with age to the point where an inoculated individual is very unlikely to locate a female after 2-d. The rapid decline in *P. interpunctella* ability to locate a pheromone source and the relatively short life-cycle of this pest suggests that it is not an ideal candidate for autodissemination.

6.1 Introduction

Individual based models (IBMs) allow for the heterogeneity of single agents to be expressed and researchers can examine the impact of individual behaviours on the rest of the population (Macal and North, 2006). Simple spreadsheet methods now exist to enable IBMs to be repeated many times over with changes in variables, using computer-based random sampling techniques (*i.e.* the Monte Carlo method). The large amount of simulations available to the modeller enable a wide range of probable outcomes to be examined. As insect pest populations are collections of many individuals of different sex and age, it makes IBMs an ideal approach for examining the effect of different control regimens on a species such as *P. interpunctella*, particularly where the often

overlooked sublethal effects of treatments are potentially having an impact on the population (Boots, 2004).

The concept of autodissemination-based control may be broken down into the following components – (1) attraction of the target pest, (2) inoculation of the pest, (3) release of the pest, (4) horizontal transfer and, (5) action of active ingredient. For the system to be successful, each of these steps must be fulfilled by a significant proportion of the target pest population. If the system fails at any stage prior to horizontal transfer then the control strategy is likely to fail full stop, as the polygamous nature of male *P. interpunctella* means that a single is capable of mating up to ten times and could produce over 2000 progeny (Brower, 1975). A *P. interpunctella* autodissemination system is likely to be based around the attraction of just the male moths to a synthetic analogue of the female-produced sex pheromone, (Z,E)-9,12-tetradecadienyl acetate 50 (commonly referred to as ZETA) (Brady et al. 1971; Kuwahara et al. 1971), as, like most other Lepidoptera, there is not an efficacious attractant to lure females.

In this chapter, data from the laboratory phase of this research is consolidated into a series of IBMs simulating components of the autodissemination system. Using this approach, parameters of the individual system components, *i.e.* station attractiveness, are evaluated for their impact on the overall likely success of a control programme. It is hoped that future research efforts may be better targeted by using IBMs to identify the component variables that impact most on system success.

6.2 Methods

6.2.1 Computer simulations

Simulations were carried out using the Crystal Ball® v. 7.3.1. (Oracle® Corporation, CA, USA) add on to Microsoft® Office Excel® 2007 (12.0.6300.5000). The software was set-up to run 5000 simulations for every assumption and the Monte Carlo simulations for each cycle set to follow the Anderson-Darling distribution. The base Excel spreadsheet, contained 1000 'moths' (assumptions and definitions are described for each model below) and were modelled for a time period of 7-days. Fitted line plots were carried out in MINITAB v14 for the PC (MINITAB Inc, v14, State College, PA, USA).

The core of the model followed the simple example of Anderson and May (1981), where they simulated the microparasite infection in invertebrate hosts. However, in this spreadsheet IBM model each 'virtual moth' was subjected to a series of logical functions. On model initiation each moth was randomly assigned an age between 1 and 7 days old and the probability that it

was "live" or "dead" calculated, based on age (see 6.2.2. for details). As calling by female *P. interpunctella* generally appears to peak during the 2-h upon onset and during scotophase, followed by a similar pattern at photophase (Huang, et al., 2004), the moths were programmed to have a maximum of two pheromone 'events' during a 24 h period. An event was classified as being either attraction to a female moth, or an autoconfusion dispenser. The level of attraction to a pheromone source was largely determined by age (see 6.2.2. for details), as previous work (chapter 5) demonstrated that a male moth's ability to locate a pheromone lure declined with age. The moths were modelled for a seven day period as the age-related effects re-applied to moth. Further modules examining behaviour under various autodissemination scenarios were incorporated onto this model with the details for each shown below.

6.2.2 IBM 1 – dispenser attractiveness

A schematic of IBM1 is shown in figure 6.1. Where m(A) is the attractiveness of the dispenser, therefore m(A) becomes a function of moth age, optimal station design, optimal station placement and density (*i.e.* the station is equally attractive throughout the entirety of our virtual infested warehouse) and station age (pheromone lures may become less effective over time). The model IBM1 tests the effect of m(A) on proportion of available males in a virtual population that will enter the dispenser. An m(A) of 100% assumes that all 1 d old male moths will be attracted to a dispenser in the virtual warehouse. At day 1 of the simulation the 1000 male moth population are randomly assigned an age between 1 and 7 days old and the ability of those moths to locate the lure in the dispenser decreases with age according the wind tunnel findings of chapter 5 (supplemented with data from pilot studies, I. Baxter, *pers. obs.*). The decrease in ability to locate the lure is summarized as:

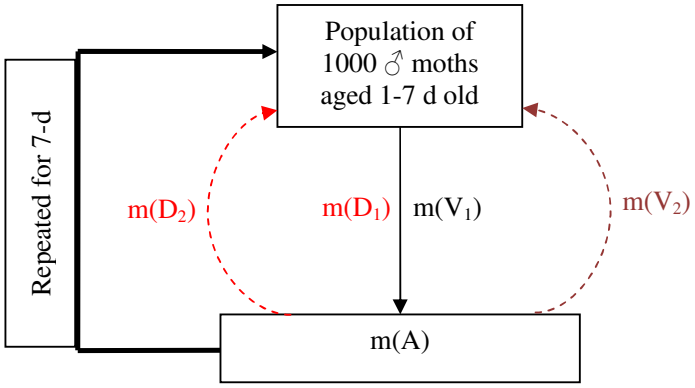
Day 1 = 100%, Day 2 = 80%, Day 3 = 40%, Day 4 =13%, Day 5 = 11%, Day 6 = 7%, Day 7 = 5% [1]

The simulation follows the individuals for 7-days and the proportion of the population that entered the station calculated. Moth mortality is also encompassed within a 'life-lottery' module, where the probability of death increases with age according to the control mortality of males calculated in chapter 5:

$$x = 6.275y - 12.28 \quad [2]$$

where x is male % moth mortality and y = time (Days). Simulations were carried out with m(A) set to increments of 0, 20, 40, 60, 80 and 100. Key assumptions are listed below:

- Moth ability to locate a lure decreases with age
- Moth chances of death increase with age
- Moths +7 days are either dead or will not be attracted to a station
- An m(A) of 100, means all male moths 0-24h old will enter station



m(A) = attractiveness of dispenser
$m(D_1)$ = Natural age related mortality of moths on day 1
$m(D_2)$ = Natural age related mortality of moths on subsequent days (2-7)
$m(V_1)$ = Moths visiting dispenser is a function of moth age and dispenser attractiveness on day 1
$m(V_2)$ = Number of moths visiting dispenser is a function of attractiveness and moth age subsequent days (2-7)

Figure 6.1. Schematic of IBM1. See section 6.2.2.

6.2.3 IBM 2 – Fitness of treated males

IBM1 calculated the proportion of males that visit the dispenser when m(A) ranges in efficacy from 0-100%. However, this does not necessarily mean that all males have an equal chance of mating success. In IBM2, the probability that individual treated males are able to successfully locate a female is evaluated and accounts for the age-related decline in ability to locate a pheromone source (*i.e.* a dispenser or a female) as per [2].

Key assumptions:

- Moth ability to locate a female decreases with age
- A female moth is similarly attractive as a pheromone lure
- An m(A) of 100, means all male moths 0-24h old will enter station
- Model population of 1000 male moths, no immigration

6.2.4 IBM 3 – Effect of carrier powders/active ingredients on moth fitness

When uptake is excessive, carrier powders have been shown to have a deleterious effect on *P. interpunctella* mating behaviour (Baxter et al., 2008). Additionally, it seems reasonable to

assume that the addition of certain a.i.'s will also have a deleterious effect of male moth fitness. In this model, the function c(A) is incorporated, where c(A) is the proportion to which treated moths ability to locate a female is impaired by exposure to the carrier powder and a.i. Data from chapter 2, showed that, although mating was not significantly reduced in the 20 µg treatment, that the propensity was reduced by 14.4% (*SE*±8.5) in the treatments, compared to that of the control. In the 100 and 200 µg powder treatments (which were significant from that of the control), reduction in mating propensity was 20% (±2.6) and 23.6% (±6.6), respectively. This simulation defines the reduction of mating propensity as an assumption based on the responses observed in Chapter 2, where moths that have visited the dispenser have been exposed to powder with equivalent uptake of either the 20, 100 or 200 µg. The assumption for c(A) is selected during the Monte Carlo simulation as being within the normal distribution range of the mean and standard error as defined above.

Key assumptions:

- m(A) is 100% attractive
- Powder uptake results in reduced mating frequency.

6.2.5 IBM 4 – Sensitivity analysis of dispenser attractiveness vs. effects of powder on ability of male moths to locate a female

Sensitivity analysis was carried within the Crystal Ball® software to identify which of the two variables, either m(A) (dispenser attractiveness) or c(A) (deleterious effects of powder contamination), were having the most influence on the forecasted overall proportion of moths able to locate a female moth (from IBM3). Within the model two assumptions were declared, m(A) and c(A), at identical rates, *i.e.* m(A) was 50% optimal and c(A) was 50% deleterious, meaning that treated male probability of locating a female was reduced, accordingly. The assumptions were selected according to a normal distribution within Crystal Ball®, during the 5000 Monte Carlo-based simulations (see fig. 6.2).

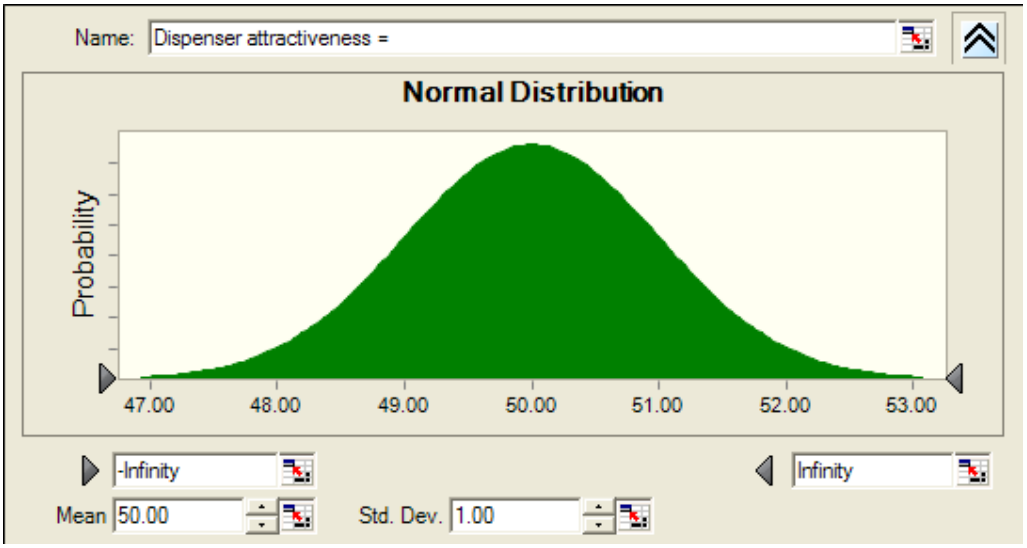


Figure 6.2. Within Crystal Ball® software, variables may be set according to distribution profiles such as that shown above. The variable 'dispenser attractiveness' is set to 50% efficient with the actual output calculated 5000 times by Monte Carlo simulation based on the distribution shown above.

6.2.6 IBM5 – Effects of horizontal transfer on powder dissemination

An additional module was incorporated into IBM2, whereby the number of times a male moth could horizontally transfer a "lethal" dose of powder to a model female moth was able to be set. The model assumed that a male moth may have a maximum of 2 pheromone attraction events (either to a female, the dispenser, or a combination) during a 24 h period, as mating is known to occur primarily during the onset of scotophase and photophase (*i.e.* dusk and dawn). As with previous models, the ability to locate a pheromone source decreased with age as the possibility of death increased.

Key assumptions:

- Moth ability to locate a female decreases with age
- A female moth is similarly as attractive as the pheromone lure in the dispenser
- The dispenser optimally contaminated all visiting moths
- Model population of 1000 male moths, no immigration
- There was no decline in transfer levels over time – a moth that visited a station just once on day 1 was equally capable of horizontal transfer as one that had become inoculated 12 h previous.

6.3 Results

6.3.1 IBM 1 – dispenser attractiveness

A fitted line plot showing the effects of altering dispenser attractiveness are shown in fig. 6.3. As the model is a mixed age population and male Indianmeal moth ability to locate a lure declines with age (chapter 5), the model predicts that a maximum of 62% (*SD*± 1.5) of available males would enter the dispenser over a 7-day period.

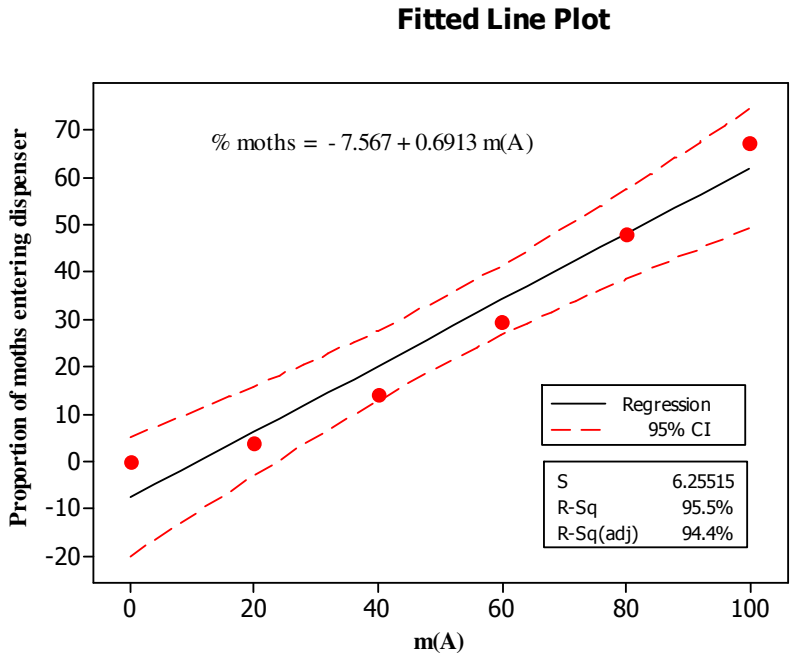


Figure 6.3. Monte Carlo Simulation showing the effects of dispenser attractiveness, m(A), on the proportion of male Indianmeal moths of a mixed –age population entering a dispenser.

6.3.2 IBM 2 – likelihood of a treated male locating a female

Table 6.1 shows the likelihood that a treated moth, from the model population (of mixed age on day 1) is able to locate a female. A fitted line plot defined the relationship as:

Log % of male moths able to locate a female = 1.932 – 0.2659*d* [3]

where *d* = time in days after introduction of the dispenser.

Table 6.1. Monte Carlo Simulation (N = 5000) showing the likelihood that a treated male will be able to locate a female (brackets denote standard deviation).

	Time after introduction of dispenser (days)						
	1	2	3	4	5	6	7
Percentage of treated moths able to locate female (Std. Dev.)	38.7 (1.3)	24.5 (1.1)	14.3 (0.8)	7.4 (0.5)	3.2 (0.3)	1.0 (0.1)	0.1 (0)
Predicted actual number of moths treated moths able to locate a female (initial population = 1000)	194	92	38	13	3	<1	<1

6.3.3 IBM 3 – Effect of carrier powders on moth fitness

Figure 6.4 shows the predicted effect of three levels of carrier powder contamination on the likelihood that a treated male will locate a calling female.

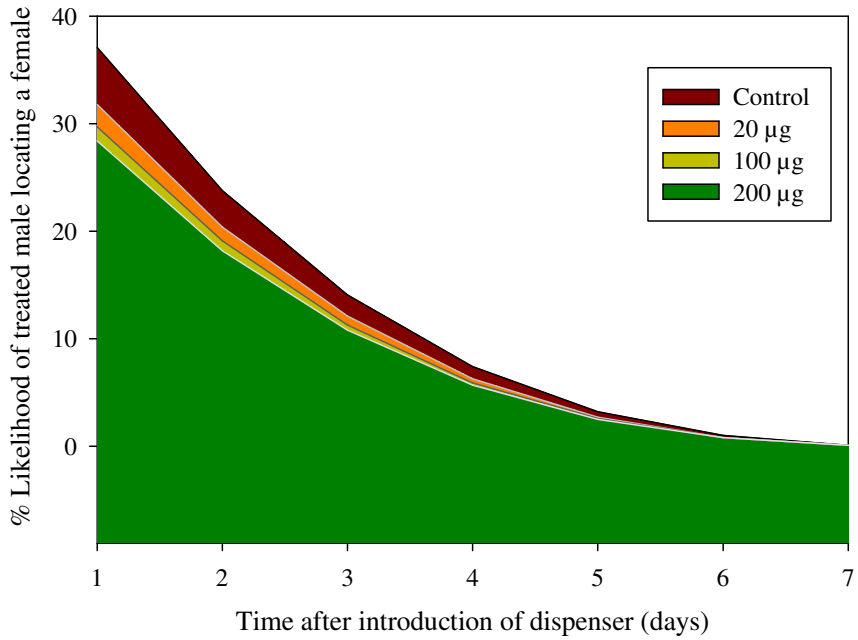


Figure 6.4. Individual based model predicting the proportion (%) of treated male moths able to locate calling female after exposure to different quantities of carrier powder

6.3.4 IBM 4 – Sensitivity analysis of dispenser attractiveness vs. effects of powder on ability of male moths to locate a female

The sensitivity analysis of m(A) vs c(A) is shown in figure 6.5.

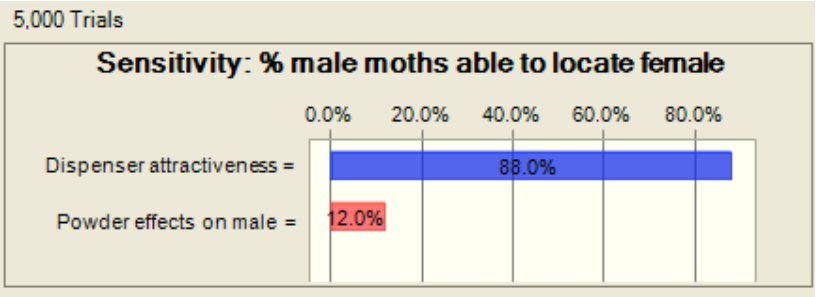


Figure 6.5. Sensitivity analysis showing the relative contribution to the model made by each of the variables – m(A) dispenser attractiveness and c(A) the Powder effects on male to locate a female.

6.3.5 IBM5 – Effects of horizontal transfer on powder dissemination

The effect of multiple horizontal transfer events from single moths is shown in table 6.2.

Table 6.2. Crystal Ball® software based Monte Carlo Simulation (N = 5000) calculating the effect of increasing the number of lethal doses of powder that could be transferred by a model moth over a 7-d period.

	Number of lethal doses a single treated moth is capable of horizontally transferring (simulation of 1000 moths, run 5000 times)			
	1	2	3	4
Number of treated male mating events over 7-d	342	367	376	379
Standard deviation	27	24	29	26

6.4 Discussion

Monte Carlo simulations developed in the software programme Crystal Ball® are frequently used in pest management to account for elements of uncertainty (Peterson and Hunt, 2003), which in the present example is largely attributed to the age-related ability of individual male *P. interpunctella* to locate a pheromone source (*i.e.* either a female moth, an autodissemination station or neither). However, approaches taken by other authors when modelling pest populations frequently include immigration and natural population growth from within the cohort as a contributory factor to the age-structure of the model (Hastings, 1997), which was not included in this example for two reasons. Firstly, the aim of producing the model was to examine the impact of changing certain parameters, such as station attractiveness or deleterious effects of powder contamination, to determine which factors impact most upon the pest – a model incorporating population growth was not necessary for these factors to be investigated. Secondly, in the UK, permanent infestations of *P. interpunctella*, and therefore, permanent pest populations, are not typically encountered by the warehouse manager (*P. Abbott, Statfold Seed Oil Company, UK, pers. corr.*) and infestation usually occurs when eggs and larvae are inadvertently imported along with product from warmer countries. This results in the pest emerging largely synchronously and being of similar age. Therefore, modelling the infestation as a closed-cohort was deemed an acceptable approach under these circumstances.

The model predicts that a maximum of 62% of male moths will visit the dispenser during a 7-day period if the m(A) was 100% attractive. This is below the ca. 90% attraction required for a male-only based control system to work, should Brower's (1975) observations on the polygamy of male *P. interpunctella*, be typical of an infestation. Therefore, it may be concluded that the horizontal transfer of active ingredient from males to females is essential for this control technique to have an impact on the population and that a 'male-annihilation' technique is unlikely to work. IBM2, calculates the proportion of treated moths that are likely to locate a calling female, based on the decline in moth ability to locate a pheromone source as determined in chapter 5. Here, it is shown that the availability of treated moths to horizontally transfer a.i. to females declines considerably, due to the effect of aging on ability to locate a pheromone source. In this model, the age-related increased chance of death and decreased ability to locate a pheromone source, resulted in just 13 of the 1000 moths being both inoculated and likely to locate a female after 3-d. Data from the model suggests, as expected, that it is the younger moths recently emerged, that will have the greatest capacity to autodisseminate active ingredient.

For autodissemination to be effective the target pest must become inoculated with active ingredient and not loose fitness to the point that the individual becomes unable to mate or not an attractive partner. IBM3 examined the impact of different levels of powder dosing on the overall proportion of treated males that could locate a female. Data from chapter 2 on the propensity of

treated male moths to mate is incorporated into the model. As figure 6.4 shows, powder uptake had little overall impact on ability of male moths to locate a female, at the 20 mg exposure rate (equivalent to ca. 17 µg Entostat per male moth), with treated males around 5% less likely to mate than untreated. However, higher exposure rates saw differences of up to 12% with that of the control. As with the other IBMs presented here, the declining ability of the moth with age is the dominant factor and there is very little difference between any of the treatments after 3 days. In IBM4 the deleterious effects of treating a moth, were contrasted with dispenser attractiveness, m(A), to determine which has a bigger influence on the overall number of female moths that are likely to become inoculated with a.i. Sensitivity analysis determined that the overall proportion of treated females was 88% attributed to m(A) and 12% to the effects of powder uptake, thus showing that future research efforts should concentrate on ensuring the dispenser is optimised for attraction.

In IBM5, the number of horizontal transfer events that a single treated male could achieve were set between 1 and 4. As with the previous IBMs, male ability to locate either a female or the dispenser declined over time, at a rate calculated from the findings presented in chapter 5. It was found that the total number of horizontal transfer events was only increased by 9% over a 7-d period by increasing the number of times a male could transfer a lethal dose from 1 to 4. It is concluded that this seemingly modest increase is largely due to age-related reductions in their ability to locate a pheromone source preventing the majority of moths >2 d from locating a pheromone source. Secondly, as the model programmed the moths to be capable of just two pheromone attraction events in one 24 h period (either mating or attraction to a dispenser), the competition provided by the dispenser lures (assumed to be equally as attractive as a female moth) meant that 50% of the moths capable of locating a pheromone source were drawn back to the dispenser rather than distributing the active ingredient to conspecifics. This observation raises an important issue in that an autodissemination based system is likely to cause a certain degree of mating disruption as dispensers will be masking the calling of females. This has implications for the evaluation of a prototype system in the field, as experiments would need to be designed such that they took into account the low-level of mating disruption in order to accurately evaluate the powder-based product. Additionally, a major drawback of autodissemination as a commercial control is highlighted here as an essential monitoring tool for the warehouse manager is a pheromone attractant on a glueboard. Where the dispenser is also competing for male moths it is likely to impact upon historical pest monitoring thresholds that trigger more substantive control measures.

The calculations made using the model here, suggest that standard autodissemination, where a.i. is passed from male to female, is unlikely to have a major impact on a pest population of *P. interpunctella*. It would appear that the age-related decline in male moths' ability to locate a

pheromone source and the relatively short lifespan means that treated males do not have many opportunities to horizontally transfer material. For this system to be successful it is likely some role for secondary cycling of infection from treated males to larvae would be required for the system to make an impact. Alternatively, one must consider what level of control is attainable by a passive, biorational system such as this? If the aim is to completely control pest populations with similar efficacy to fumigation then, clearly, the modelling approach suggests this will not work. If the system is intended to provide background control, perhaps increasing the interval between conventional treatments, then there might be an opportunity for an autodissemination approach if low-level control is economically viable. A model should serve as a guide and, although frequently misapplied and misinterpreted (Godfray & Rees, 2002), it would be unwise to wholly discredit the potential of autodissemination on the basis of the aforementioned model. However, on balance it would seem that there are other pests, with longer lifespans and where age-related decline in fitness is less pronounced, which could be better suited to this concept.

7. Discussion

Summary of key findings:

Chapter 2: The potential of two electrostatic powders as the basis for an autodissemination control method of *Plodia interpunctella* (Hübner)

- Uptake of both Entomag and Entostat powders by male moths caused a reduction in mating frequency, at high doses (>20 µg powder per moth)
- Uptake of both Entomag and Entostat powders caused a reduction in the number of male moths that could locate a pheromone source, at high doses (>20 µg powder per moth)
- Entomag was retained more effectively by the moths, with approximately 20 and 15% more powder (by weight) retained by males and females, respectively, over 48 h
- Greater volume of Entostat was horizontally transferred than Entomag (ca. 50% more per female)
- It was concluded that Entostat was a more suitable candidate for an autodissemination carrier powder, due to its more efficacious horizontal transfer rates

Chapter 3: The electrostatic attraction of two powders, Entostat™ and Entomag2, to the Indianmeal moth (*Plodia interpunctella*)

- The electrostatic charge attained by the Indianmeal moth was measured as being +2 x 10^{-11} Coulombs (C) when housed for 24 h on corrugated polypropylene (Correx®)
- After aerosolisation from Correx, Entostat was found to have more particles charging to the negative polarity than that of Entomag
- Modelling suggested that Entostat would be attracted to a charged moth over a greater distance than Entomag, making it more suitable as a potential carrier powder
- Modelling suggested that low density particles should be evaluated by future researchers investigating the role of electrostatic powder attraction to charged insects

Chapter 4: Using image analysis of powder deposition to evaluate two prototype autodissemination devices intended for the biorational control of *Plodia interpunctella*

- Entostat powder and Glo-Brite® dye were colocalised in the same particle, validating the concept of using spectral dye recovery as a measure of powder uptake
- A novel image analysis approach was developed to determine the body regions to which moths were taking up powder. Powder was taken up mostly to the eyes and, predominantly, the ventral side of the body. Powder was thought to horizontally transfer from the male's head to the female's eyes during courtship
- A pit-fall style dispenser was more effective at inoculating moths as the target region of the body (head) became contaminated, plus moths were unable to exhibit avoidance behaviour as they did from a tray-style dispenser, where they had to walk through the powder

Chapter 5: The effect of *Beauveria bassiana* (Balsamo) on *Plodia interpunctella* (Hübner) fecundity, mortality and anemotactic flight behaviour

- The co-formulation of conidia with Entostat was shown to proportionally increase spore uptake by moths and may provide a cost-effective carrier for entomopathogenic spores

- A mortality rate of 100% was observed after 7 d in both male and female moths that were exposed to 5.3 x 10^6 cfu *B. bassiana* var. 'Naturalis' conidia, held at 26-28^{o}C and 30-42% rh
- At the same uptake rate of 5.3 x 10^6 cfu, there was no evidence of a lethal dose by horizontal transfer to conspecifics
- Sublethal effects of infection showed that fecundity was reduced when either male or female moths had been inoculated 48 and 96 h previously
- The onset of sublethal effects of *B. bassiana* inoculation on anemotactic flight responses were rapid, with just 40% of the treated moths able to locate a pheromone source in a wind tunnel, 24 h after exposure
- Infected moths also exhibited different flight behaviour from that of the controls, by making fewer turns per second and taking longer to orientate to the pheromone source

Chapter 6: The Critical Components of an Autodissemination System – an Individual Based Modelling Approach

- The model suggested that 62% of a mixed age population of males would enter the dispenser if the system was 100% attractive to 1 day old moths. Where the ability of IMM to locate a pheromone source declines rapidly with age, few moths 3 d or older could locate the dispenser
- Modelling indicated that just 194 males (out of a virtual population of 1000) would locate a female after visiting the dispenser during the 7 d period
- Sensitivity analysis suggested that optimising the station for attractiveness to IMM was far more important for overall system success than concentrating on reducing the deleterious effects of powder contamination
- Increasing the number of transfer events (*i.e.* the number of times a male could transfer a lethal dose to a female) only improved system efficacy by ca. 10%, as the age-related decline of moths in their ability to locate a pheromone source proved a dominating factor in opportunities for horizontal transfer

General Discussion

The aim of the research undertaken here was to examine the concept of autodissemination as a biorational control strategy for *P. interpunctella* by breaking down the system into its essential component parts, (1) attraction of the pest, (2) uptake of a.i., (3) release of the pest, (4) horizontal transfer of a.i. and (5) effect of a.i. dissemination on the pest population. The approaches taken to undertake these evaluations have used both laboratory data and predictive modelling. The use of models in this work has served primarily as a tool to identify areas where future research efforts should be concentrated to maximise the efficacy of autodissemination. The major points highlighted by this research are discussed below within the context of the five major steps of creating a successful autodissemination system.

(1) Attraction of the pest

Autodissemination is only feasible where there is some way of attracting a significant proportion of the target pest population and the IBM presented in Chapter 6 (section 6.2.5) suggests that future research efforts should be largely focussed on this component. For *P. interpunctella*, it is only possible to attract the male sex in significant numbers as, like the vast majority of other Lepidoptera, there are very few effective female attractants (Knight & Light, 2001). If it were possible to attract females at a similar level to that of the males, then the system would be considerably more efficacious, as not only is the amount of material that is horizontally transferred extremely small (chapter 2, section 2.3.3), but the males' fitness to both mate (chapter 2, section 2.3.4) and locate a female (chapter 6, section 6.3.2) is affected by excess uptake of carrier powder and age-related decline. The lure within the autodissemination station itself will also suffer from degradation over time and this means that a station may vary in attractiveness during the course of deployment. If pheromone release rates are too high, as one may expect when the system is first deployed, then male moths may become habitutated (Howse, et al., 1998) and be prevented from locating the pheromone source. In contrast, over time, the release rate of pheromone from the lure is likely to decrease and the station will not constitute an attractive source for male *P. interpunctella* (Mankin, et al., 1980). Whilst *P. inertpunctella* appears to be adequately attracted to a single pheromone component, ZETA, other Lepidopteran species often require multiple pheromone components, which may span a range of molecular sizes and chemical groups (Cork, 2004). The release-rate characteristics from a matrix, such as the commonly used rubber septum, will almost certainly change over time where those components that are more volatile will release faster than others, making multi-component pheromone attraction even more problematic than the single one used here. An important consideration, however, is how attractive is the dispensing station in comparison to a calling female? As it is known that *P. interpunctella* females produce a four component blend of

pheromone (Zhu, et al., 1999), future research investigating attraction of *P. interpunctella* may find that the a.i. and age-related effects on lure location (Chapter 5 section 5.3.5) are less pronounced when working with the full blend of pheromone and additional research in this area is certainly warranted. Alternatively, autodissemination is, perhaps, better suited to pests where aggregation pheromones exist so that a greater proportion of female pests will become targeted as shown in the similar studies of Smith, et al., (1999).

(2) Uptake of a.i

In chapter 3 the saturation electrostatic charge attained by *P. interpunctella* was determined in order to calculate the distance over which an a.i. carrier particle could levitate to an insect. The purpose of carrying out this research was to identify what characteristics a carrier particle required to fully utilise electrostatic attraction to the pest. In concurrence with McGonigle and Jackson's (2002) findings with houseflies, it was determined that moths charged, in the majority, to the positive polarity when exposed to corrugated polypropylene. Therefore, candidate carrier powders would be required to charge, largely, to the opposite (negative) polarity for this delivery system to be exploited. It was found that Entostat did, indeed, have a heavily skewed charge distribution with over 80% of the particles charging negative when tribo-charged against corrugated polypropylene. It was concluded that where electrostatic attraction of particles to a target insect was the objective, then using Entostat in conjunction with corrugated polypropylene would provide an optimised solution. However, electrostatic attraction used in this manner could come at a cost as Maw (1964), determined that several pest species were repelled from charged surfaces. As the most important component of a *P. interpunctella* autodissemination system is the initial attraction event to the station, then further work should concentrate on whether or not the benefits of opposing charges on moth and insect come at the price of repellence from the station.

Uptake of carrier powder from tray style dispensers was small (chapter 2, section 1.3.6) and the moths displayed behavioural avoidance when presented with an opportunity to circumvent inoculation (chapter 4, section 4.3.5). The autoinoculation device was determined as being more effective when moths entered a 'pit-fall' style dispenser which, in addition to forcing moths to enter the powder, was shown to cause *P. interpunctella* to uptake powder uniformly over the body, especially to the regions where powder becomes horizontally transferred. Additional progress made in chapter 4, provides future investigators with a novel software-based approach, to measure the extent and body regions to where powder has adhered. In addition to measuring uptake, the validation of dye-carrier powder colocalization may be used to determine the consistency of the a.i./powder formulation. Whilst only used here to determine dye

colocalization, there is no reason why this approach may not be utilised for inclusions of conidia or other synthetic a.i.'s.

(3) Release of the pest

Using the pit-fall design with exit holes cut into the base (chapter 4, fig. 4), *P. interpunctella* were able to exit the station after inoculation. The station should be designed such that moths may easily exit the dispenser without exerting large amount of resources which may affect their fitness. Additionally, moths spending too much time attempting to exit the dispenser may miss the optimum mating times during the onset of scotophase and photophase. The prototype dispenser presented here may be improved by the addition of additional exit holes, although this must be balanced against the risk of operator exposure caused by accidental spillage of a.i. from its base.

(4) Horizontal transfer of a.i.

Approximately 4% of the Entostat powder present on the male moth was horizontally transferred to the female (Baxter, et al., 2008), this equated to 0.67 µg per female. For comparative reasoning the topical LD_{50} of technical grade Dimethoate a.i. to honeybees (*Apis mellifera* L.), an organophosphate to which bees are particularly sensitive, is 0.12 µg/bee (Anon, 1991). However, Entostat's co-formulation ceiling of 1% w/w of Entostat:a.i. (Exosect Ltd, *pers. com.*), means that a mixture exceeding 1% a.i. is unlikely to be feasible and, therefore, only ca. 0.007 µg of a.i. is likely to be transferred during mating. When placed in this context, the volume of a.i., presented as either conidia or synthetic insecticide, seems somewhat low to achieve mortality/fecundity effects on the recipient.

The a.i. evaluated in these studies was conidia of *B. bassiana*, var. 'Naturalis'. It was concluded that this isolate would not be an effective a.i. for a *P. interpunctella* autodissemination system, as there was no evidence that the quantities of isolate transferred could either cause mortality in conspecifics or reduce fecundity by sublethal infection in either male or female moths. Perhaps more importantly from the standpoint of autodissemination, was the rapid onset of deleterious effects on male anemotactic flight behaviour (chapter 5, section 5.2.10). The considerable sublethal effects of this isolate would severely impact on the likelihood of an inoculated male locating a female and horizontally transferring a.i. Whilst it is acknowledged that the experiments presented here evaluated treated male ability to locate a synthetic pheromone lure and not a calling female, the results strongly suggest fitness will be impaired during the early onset of infection, as little as 24 h after exposure. Further research may benefit from comparing the attractiveness of a calling female compared to a pheromone lure, as *P. interpunctella* mating is not just pheromone mediated, but also visual, behavioural and acoustic cues are important

(Phelan and Baker, 1990; Trematerra and Pavan, 1994). Two major factors will need to be considered when further evaluating horizontal transfer. Firstly, is either the horizontally transferred volume of conidia or ca. 6.7 nanograms of synthetic a.i. (*i.e.* 1% of the 0.67 µg per female) enough to cause the desired effect of reduction in fecundity of mortality? Secondly, are there any volatiles associated with the formulation (only applicable for synthetic a.i.) that may impact on the moths ability to locate a female? Only when it can be determined that either the a.i. or the formulation itself is not affecting moth fitness can it be concluded to be appropriate for inclusion into the autodissemination system.

(5) Effect of a.i. dissemination on the pest population

Modelling suggested that only ca. 20% of the females will interact with a treated male (chapter 5, section 6.3.2), which, as previously discussed, is unlikely to cause a major impact on a pest population regardless of how effective the a.i. is. The *B. bassiana* isolate used in this study did not appear to be a suitable candidate for the autodissemination system as there was no evidence of impact on mortality or fecundity from horizontally transferred conidia. Perhaps more importantly, were the adverse effects on pheromone lure location during early onset of disease, meaning that inoculated males fitness would be severely impaired. It is, however, acknowledged that these studies did not examine autodissemination in the manner that Soper (1978) and Vail et al., (1993) did, where they looked at dead infected individuals (secondary recycling) providing a foci of disease through contaminated cadavers infecting larvae. These studies were not carried out as it seems unlikely that the food industry would tolerate the presence of infected foreign bodies in foodstuffs. In a more appropriate setting, *e.g.* protecting animal feed or emergency food rations, where occasional moth cadavers may be tolerated, a biological-based system may provide effective non-chemical control providing the isolate is capable of infecting *P. interpunctella* larvae. There are few examples of vertical transmission (from parent to offspring) with fungal pathogens and, thus, it was not examined in this research. However, in chapter 1 (figure 1.4), maternal moth scales can be clearly seen adhering to the outside of the egg, which, may possibly provide a physical vertical transmission of pathogen. This would appear to have a slender chance of success, however, in light of the chapter 5 image analysis results which show that only a very small density of powder covers the ventral body. In contrast, there have been some reported cases of virus being vertically transmitted by adult Lepidoptera and, of particular interest, in *P. interpunctella* (Vail, et al., 1993). Potentially virus and protozoan (Shapas, et al., 1977) biological agents would have a greater chance of transmission from generation to generation, possibly as a result of their different pathology to that of the fungus.

The autodissemination system intended for use here, relies on the biological a.i. working very quickly on its female host, as the majority of oviposition takes place 24-72h after mating (Cook, 1999; Svensson, et al., 2002). As the mode of action of a biological a.i. is generally over a longer time than this, future research on this system may find that the appropriate candidate could come in the form of a synthetic insect growth regulator. Carlson, et al., (2001) reports a large reduction in fecundity when a relatively new class of insecticides, the diacylhydrazines, come into contact with a range of lepidopteran larvae. What makes these compounds of acute interest for potential future research on autodissemination is that they are essentially non-toxic to adult moths by either oral or contact administration (Carlson, et al., 2001). One compound in particular, methoxyfenozide, has now been registered for use in the UK as a spray treatment, for the control of codling moth in fruit (PSD, 2008). Future research on the dissemination of these types of a.i. combined with the carrier powders used here, is certainly warranted.

Conclusions

In theory, autodissemination is a near perfect concept for biorational pest control as it is safe to beneficial arthropods, a.i. is contained within a station making it safer for both field operatives and the consumer, it is highly suitable for resistance management strategies and is potentially labour-economical by providing long-lasting effects once placed in the field (Lanier, 1990). However, considering the body of research available on the subject (see chapter 1, section 1.5) the lack of a successfully established market-based product utilising autodissemination suggests that the theory and reality of this system are, at present, some way apart.

One of the major draw-backs of autodissemination is the complexity of the system in that the lure must be effective, pests must become inoculated without losing fitness, a.i. needs to be horizontally transferred in appropriate quantities and (particularly in the absence of bi-sexual attractants) the a.i. must exert an effect on the female between mating and oviposition, *i.e.* very quickly. It is concluded here that *B. bassiana* would not be an appropriate agent for *P. interpunctella* autodissemination-based control. However, new generations of synthetic a.i.'s may display some of the qualities required for system success. Additionally, where use of a biological agent is desirable, alternative pathogens such as virus and protozoa may be more appropriate, due to their greater capacity for vertical transfer (Fuxa, 1987). Autodissemination has the same basic rules for success that sterile insect release (SIT) does – the ratio of treated to untreated moths must be proportionally very high. Working with a closely related species to *P. interpunctella*, Hight et al., (2005) found that a ratio of 5:1 (sterile:wild) males were required to provide adequate control of the Pyralid, *Cactoblastis cactorum* (Berg). The modelling presented in chapter 5 suggested that this ratio would be unlikely to be achieved with the prototype system presented in this research.

An autodissemination system, regardless of the a.i. is likely to require a carrier powder to both reduce costs and protect the a.i. from biotic and abiotic factors. As already discussed, Entostat has been shown to be taken up by moths and horizontally transferred. Whether or not the electrostatic properties of the powder improve particle adhesion is not determinable from this research. Previous authors have shown success on pest insects with powders ranging from talcum powder (Yu and Brown, 1997) to diatomaceous earth (Mewis and Ulrichs, 2001), to glass (Lewis and Hughes, 1957). A common factor with all of these powders is their small size (<30 µm) (Golob, 1997) which, in conjunction with the findings of chapter 3, suggests that smaller sized and lower the density particles will have an increased likelihood of being attracted to the pest by virtue of electrostatic forces alone. Once the target is immersed in powder it is the Van der Waals-London interaction that is the dominant force of adhesion at this nano-level (Corn, 1966). Therefore, if the autoinoculation device is a pit-fall station, electrostatic attraction could be quite unimportant as the target pest is already in contact with the a.i. at which point adhesion of material is not necessarily electrostatic in origin. It may be more beneficial for future research in this area to focus on cheap, small diameter (<30 µm), sustainable sources of a low density, low volatile and low moisture absorbing carrier powder, rather than seeking out materials which generate a high surface to mass charge.

1. Appendix I – Culturing of *Plodia interpunctella*

The original culture of moths were supplied by the Danish Pest Infestation Laboratory, at Lyngby, Denmark. The moths were cultured at 26°C ± 3 °C, 50-70% r.h. The photoperiod of the insect rearing rooms was on a 16:8 h light/dark cycle, but as the room had un-screened windows the culture was also subjected to daylight. Larvae were fed on a diet of 'Chick Starter Crumbs' (a fortified mixture of wheat, soya and sunflower products), 'Good Lay' (a fortified mixture of wheat, maize gluten, sunflower, soya and vegetable oil) (both from Dukes and Botley, Southampton, Hampshire, UK), Whole Wheat (Street End Feeds, Bishops Waltham, Hampshire, UK) and glycerol (Sigma-Aldrich, UK) in a ratio of 2:2:1:1, respectively. Larvae were held in 0.5 L glass jars (VWR International; www.vwr.com) containing approximately 200 g of diet. Strips of corrugated card (ca. 20 cm x 3 cm) were placed into the jars to provide retreats for the moths to pupate. The jars were sealed with a breathable paper lid. Pupae were carefully excised from the corrugated card, separated by sex under a microscope and placed into a wire-framed cage (30 x 30 x 35cm) that was covered in a cotton mesh (Tubegauz® size T2, Seton Healthcare Group, Oldham, UK). To prevent habituation to pheromones male and female cages were not kept in the same room.

2. Appendix II – The Carrier Powders

Two powders were investigated, which were both manufactured by Exosect Ltd. The first powder, Entostat™, is a refined wax produced by the fronds of the Brazilian wax palm, *Copernica cerifera* Martius (Palmae). The powder particles are spherical with a diameter of 7.59 µm ± 3.0 (measured by Particle Technology Limited, Derbyshire, England) and a density of 0.97 g/cm^3. The second powder, Entomag™, is a proprietary metallic flake formed from iron milled with stearic acid. It has a density of 7.3 g/cm^3 and the flakes measure 9 x 3 x 2.5 µm (± 10%) (Anon, 2005). A second form of Entomag was used in chapter 3, referred to as Entomag2. Entomag2 was shown to have an average particle diameter of 2.42µm. Entomag2 is identical in constituents, density, etc. except that the particle is a sphere rather than a flake.

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