Student Report No. SR42

Investigating the effect of natural enemies and environmental conditions on soil populations of saddle gall midge (*Haplodiplosis marginata*)

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CONTENTS

1. Abstract

Saddle gall midge *Haplodiplosis marginata* (Diptera: Cecidomyiidae) is a pest of cereals across Europe. The occasional nature of this pest has resulted in limited and sporadic research activity. There remain important gaps in knowledge due either to a genuine lack of research or to previous research being difficult to access. These knowledge gaps make the development of effective control options difficult. As part of this project I have reviewed and consolidated the existing literature from research which spans several decades and encompasses many different countries so as to identify specific gaps in knowledge that need to be addressed. One of the major constraints in effective pest management of this species is a lack of appropriate tools for monitoring. Infestations may go unnoticed until galls are evident in the crop by which time the damage is done. Furthermore, if chemical controls are to be used, they must be timed to coincide with an appropriate life stage if they are to be effective.

Here, I have demonstrated how the emergence of adult *H. marginata* can be predicted on a yearly basis using a simple degree-day model and rainfall events. This will allow farmers to forecast the emergence of adults based on weather conditions and initiate inspections of the crop to check for egg-laying. This research also provides key insights into the development of this insect in the soil stage which is difficult to observe in the field. In this work I have also described the development of a pheromone trapping system for *H. marginata* based on the female sex pheromone. This trap provides a specific, highly effective means of monitoring *H. marginata* populations and greatly improves upon existing methods of trapping. Lures were optimised through field experiments which tested different lure types, loadings and formulations. Recommendations are provided for use of the trap itself based on experiments which determined how the position of the trap in the field influenced catch rate. Additionally, I have developed an assay to identify the presence of *H. marginata* DNA in the gut contents of arthropod predators. This relied on the development of species specific primers for use in PCR in order to amplify *H. marginata* DNA. Using this assay in the field, I have identified seven species of Carabid beetle that naturally predate on *H. marginata* larvae.

In all sections of this work I have addressed the implications of the findings in the context of *H. marginata* biology and ecology. Furthermore, I have described how this research can be used as the basis for and integrated pest management programme for this pest and proposed avenues for future research.

2. Introduction

Saddle gall midge (*Haplodiplosis marginata* von Roser; *Haplodiplosis equestris* Wagner) is a polyphagous pest of cereal crops across Europe. Between 1967 and 1972, severe outbreaks were reported in isolated areas across the UK (Golightly & Woodville, 1974; Woodville, 1968, 1970, 1973). The pest was not considered to be a problem again until 2010, when localised outbreaks were reported in central England (Allison, 2010; Case, 2011). Reports of the midge being present at lower levels have continued since this time (HGCA, 2012). The 40-year interval between economically damaging outbreaks of *H. marginata* in the UK and other European countries has resulted in a lack of continuity in research into this pest. For example, in the UK, prior to 2012 there had been no research published on *H. marginata* since 1974. A similar pattern can be observed in other European countries in which *H. marginata* has historically been economically damaging, with the last decade seeing an increase in research activity. The sporadic nature of this pest has frustrated research efforts as studies rarely coincide with serious outbreaks and long term information is sparse. Additionally, existing research on *H. marginata* is fragmented across several countries and several languages which, in combination with the age of the publications, can make accessing and translating them difficult, particularly where there is no digital copy available. The resulting knowledge gap has hampered attempts to respond to this re-emerging pest.

2.1 Life cycle of *H. marginata*

The life cycle of *H. marginata* was recently described in detail by Censier *et al.* (2015). *Haplodiplosis marginata* is a univoltine species with the flight period beginning as early as mid-April and lasting until the beginning of July depending on environmental conditions (Censier *et al.*, 2012) (Figure 1). Adults are short-lived and have limited dispersal ability. Lifespan estimates vary between 1 and 7 days (Njveldt & Hulshoff, 1968, Popov *et al.*, 1998) and flight distances average 18 m (Schütte, 1964), although male flight has been recorded as more than 120 m in some instances (Nijveldt & Hulshoff, 1968). Males generally emerge first and fly low to the ground in search of females (Skuhravý *et al.*, 1983). Females may undertake several short flights of 5 - 15 m when seeking a suitable oviposition site and appear to fly slightly higher than males (Skuhravý *et al.*, 1983; Skuhravý *et al.*, 1993). Adult *H. marginata* have been caught at heights of up to 6 m, meaning flight distances may be increased in high winds (Skuhravý *et al.*, 1993). Eggs are laid in a chain-like or raft-like formation along the leaf veins of cereals and grasses on either leaf surface (Dewar, 2012, Censier *et al.*, 2015). The location of oviposition is likely to depend on the position and angle of the leaf, apparently varying between crops (Nijveldt & Hulshoff, 1968). Barnes (1956) reports that females will preferentially lay on the topmost leaf, however this was only found to be true for spring barley in a later study by Nijveldt & Hulshoff, (1968), with the lowest leaf being more favourable for oviposition in wheat. Hatching occurs 1 - 2 weeks after oviposition depending on environmental conditions following which the larvae migrate down the leaf and begin to feed on the stem from beneath the leaf sheath

(Golightly & Woodville, 1974). Larval feeding on the stem results in galls 2 - 5 mm in length which appear as the elongated 'saddle shaped' depressions characteristic of this species. The larvae reach maturity between June and mid-July and drop from the stem to enter diapause in chambers in the soil where they overwinter (Golightly & Woodville, 1974; Skuhravý *et al.*, 1993). Pupation generally occurs the following spring, however larvae can remain in diapause in the soil for several years (Nijveldt & Hulshoff, 1968, Popov *et al.*, 1998; Dewar, 2012). Rarely, larvae can be found in cocoons in the soil stage (Censier *et al.*, 2014a).

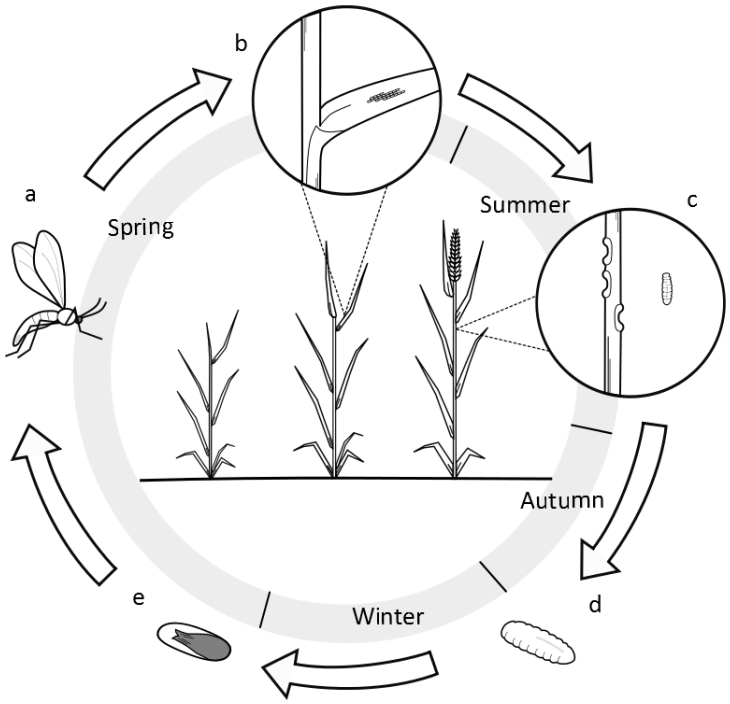

Figure 4. Life cycle of *Haplodiplosis marginata*; **A.** adult emergence, **B.** oviposition, **C.** gall formation and larval maturation, **D.** larval diapause, **E.** pupation.

2.2 Crop Damage

Crops most at risk are spring crops, particularly wheat and barley (Skuhravý *et al.*, 1983, Skuhravý *et al.*, 1993) but damage has also occurred in late sown (after mid-November) winter wheat and barley (Pope & Ellis, 2012; HGCA, 2012). Golightly and Woodville (1974) observed that damage is most severe when egg-hatch coincides with stem extension, whilst losses are incurred on crops that are in or beyond the booting stage at the time of larval infestation are minimal. Cereal crops are therefore most vulnerable to attack between growth stages 31-39 (Sylvester-Bradley *et al.*, 2008). Early sown spring crops appear to be less susceptible as the plant tissue is more mature at the time of egg hatch, potentially making it more difficult for the larvae to feed (Skuhravý *et al.*, 1993).

Where high population densities occur, there may be as many as 60 galls per stem (Skuhravá & Skuhravý, 2014). Galls are generally formed on the top three internodes where the plant tissue is least mature. A substance secreted by the larvae inhibits the development of epidermal cells in the immediate vicinity of the insect, while the surrounding tissues continue to develop, forming the gall (Nijveldt & Hulshoff, 1968). Development of vascular tissue is disrupted around the site of the gall, which can restrict the flow of nutrients to the ear. This can lead to shrivelled or underdeveloped grains (Golightly, 1979) and reductions in stem length (De Clercq & D'Herde, 1972; Popov *et al.*, 1998), ear length (De Clercq & D'Herde, 1972), and thousand grain weight (Woodville, 1968). Galling has been shown to result in reductions in grain number and thousand grain weight in wheat by 63% and 64% respectively (Popov *et al.*, 1998).

Destruction of the plant cuticle in the area of the gall leaves the plant vulnerable to secondary attack by bacteria or fungi, particularly in wet weather (Nijveldt & Hulshoff, 1968; Skuhravý *et al*., 1993; Eklund, 2005). Gall formation can also weaken the stem which increases the risk of lodging, where the stem breaks or bends so that the ear falls below the level of the combine and cannot be harvested (Woodville, 1970; Golightly & Woodville, 1974; Gratwick, 1992). This is of particular concern where attack coincides with a period of high winds and can be responsible for substantial yield losses.

2.3 Economic consequences

Estimates suggest that when the percentage of infested wheat stems reaches 70%, losses of 2.2 t/ha could occur (Skuhravá & Skuhravý, 2014). A recent study in Belgium showed a correlation between number of galls and yield loss in winter wheat, in the most severe case yields fell by 191 kg/ha (0.191 t/ha) for every increase of 100 galls per 100 stems (Censier *et al.*, 2016b). Past outbreaks of saddle gall midge in the UK have resulted in losses of 0.6 t/ha (Woodville, 1968). There are no published figures for yield losses incurred in the recent UK outbreaks, however, the recent HGCA survey anecdotally reports that 52% of respondents who observed saddle gall midge infestation observed subsequent yield loss. In the most severe case, there was an estimated 70% decrease in yield as reported by an agronomist in Buckinghamshire (Ellis *et al.*, 2014).

Estimates of thresholds of soil densities of larvae above which economic losses occur range from 12.4 million per hectare (Golightly & Woodville, 1974) to as little as 300,000 per hectare (Popov *et al.*, 1998). In terms of infestation, it has been estimated that more than three galls per stem causes significant damage and loss of yield (Skuhravý *et al.*, 1993). In Denmark, this threshold rises to five galls per stem (Woodville, 1973), in the UK it is between 4.5 and nine galls (Ellis *et al.*, 2014) and in Germany it is between five and ten (Schütte, 1983). The variation in these estimates demonstrates the current uncertainty surrounding the economic impact of this pest. Thresholds based on gall number are of limited use in pest management however, as control measures are likely to be ineffective at this stage. It is acknowledged that the actual damage caused depends on many factors

such as crop type, growth stage and weather conditions (De Clercq & D'Herde, 1972; Censier *et al.* 2015).

2.4 Current control methods

2.4.1 Cultural control

Agricultural systems in which cereal crops are grown continuously are particularly susceptible to outbreaks of *H. marginata* as high densities of larvae accumulate in the soil. Break crops are generally accepted as an effective means of reducing infestation by depleting larval soil populations (Censier *et al.*, 2016b). Skuhravý *et al.* (1993) showed that infestations of wheat varieties were greatly reduced when sown after non-susceptible crops such as alfalfa or potato rather than susceptible cereals. Even so, with the potential for *H. marginata* larvae to enter extended diapause, breaks of one year may not always be enough to reduce soil populations to below economically damaging levels. Field trials over six years in the Netherlands showed that a two-year break did not entirely eradicate *H. marginata* populations, and oats were often not particularly effective as a break crop despite being a relatively poor host plant (Nijveldt & Hulshoff, 1968). The introduction of the EU crop diversification requirement as part of the 2013 CAP reform aims to encourage farmers to grow a greater variety of crops by specifying a minimum number of crops and a maximum land cover amount for the two main crops (Regulation (EU) 1307/2013, 2013). This may result in fewer *H. marginata* outbreaks if continuous wheat systems are disrupted by widespread use of rotations and break crops.

2.4.2 Chemical control

Chemical controls applied directly to the soil are of limited efficacy, probably owing to insufficient penetration of the soil to the depths where overwintering larvae are found (Popov *et al.*, 1998). Foliar applications of organophosphates such as malathion and dimethoate applied to the crop have shown some efficacy against eggs and newly-hatched larvae of *H. marginata* on wheat in Romania (Popov *et al.*, 1998); and in the UK chlorpyrifos effectively reduced numbers of larvae and galls in wheat when applied at the visible flag leaf stage (GS 37) prior to its withdrawal (Roberts *et al.,* 2014). Control has also been achieved with pyrethroids such as alpha-cypermethrin (Popov *et al.*, 1998), and with deltamethrin, lambda-cyhalothrin and tau-fluvalinate on winter wheat (Censier *et al.*, 2012, 2015; Ellis *et al.*, 2014).

Early recommendations for chemical control advised using persistent insecticides and to time applications for three to five days after the first adults were recorded or when the eggs were found on 20% of leaves (Skuhravý *et al.*, 1993). There is a limited timeframe for application as once the larvae are beneath the leaf sheath they are protected from contact-acting insecticides (Gratwick, 1992). Repeated applications may be warranted as adult flight can persist for up to ten weeks (Censier *et al.*, 2012). Censier *et al.* (2012) found that treating the crop with pyrethroid insecticides

twice with a two week interval resulted in 75 - 87% efficacy based on reductions in the percentage of attacked stems and mean gall number per stem. In a further study the authors recommended treating the crop to coincide with peak adult flight (Censier *et al.*, 2016b). The authors, however, acknowledged that phenological monitoring was essential in order to synchronise applications with vulnerable life stages (Censier *et al.*, 2016b). Ellis *et al.* (2014) reported that chemical controls applied at the start of adult emergence resulted in the lowest yield loss, although treatments applied 7 – 10 days post emergence or when the first eggs were seen also reduced midge infestation. Ideally a forecasting model would be used to predict the onset of adult emergence and used to time in-field monitoring efforts on which chemical treatments may be based.

2.4.3 Natural enemies

Carabidae or Staphylinidae may contribute some degree of population control having been observed feeding on *H. marginata* larvae at the soil surface (Golightly & Woodville, 1974; Skuhravý *et al.*, 1993). Species from these families have similarly been shown to feed on orange wheat blossom midge larvae (*S. mosellana*); a species that shares many characteristics with saddle gall midge (Holland *et al.*, 1996). Larval stages may be parasitised by *Chrysocharis amyite* and *Platygaster taras* (Baier, 1963; Skuhravý, 1982), although research suggests that saddle gall midge mortality due to the latter is only 1 - 2% and the former only attacks larvae found on wild grasses as females are unable to penetrate the leaf sheaths of cereals with their short ovipositors (Nijveldt & Hulshoff, 1968; Woodville, 1968; De Clercq & D'Herde 1972). Parasitism of *H. marginata* eggs by a novel parasitic hymenopteran was found in Belgium in 1965. The species was described as *Platygaster equestris* in reference to the host's earlier name (*Haplodiplosis equestris*) and was found to parasitise up to 10% of *H. marginata* eggs (Spittler, 1969). An unidentified Chalcidid in Austria was found to parasitise up to 23% of *H. marginata* eggs according to Faber (1959 cited in Nijveldt & Hulshoff, 1968). Another *Platygaster* species was observed in 1966 attacking *H. marginata* larvae in the Netherlands, parasitising between 1 and 40 % of larvae. Within a year, populations of the parasitoid overtook that of *H. marginata* although it is not clear whether declines in the latter were because of parasitism alone (Nijveldt & Hulshoff, 1968). *Holarcticesa clinius* is also recorded as a parasitoid of *H. marginata* in the Universal Chalcidoidea Database (Noyes, 2017). Although populations of such parasitoids may help to keep *H. marginata* numbers in check, there is little evidence to suggest that any of these species would be appropriate for use as biological controls.

2.5 Influence of environmental conditions on *H. marginata*

Like many Cecidomyiidae, outbreaks of *H. marginata* are highly sporadic. Populations fluctuate from year to year and in the absence of a single correlating biotic or abiotic factor, predictions of future population size are difficult (Woodville, 1973; Basedow, 1986). Numbers of larvae in the soil can increase gradually over several years or rapidly within a generation (Basedow, 1986). High larval population densities in the soil can result in outbreaks (Skuhravý *et al.*, 1993), however, the level of

damage further depends on elements such as reproductive success, crop susceptibility and weather conditions

2.5.1 Effects of temperature and moisture on *H. marginata* development

Skuhravý *et al.* (1983) have reported high larval mortality in the soil stage after recording emergence levels of just 5-12% in field experiments in Slovakia. It is not clear, however, what proportion of the population remained in diapause. Population declines have also been observed following unfavourable weather conditions such as cold temperatures and extremes of soil moisture content, however this is not always consistent (Woodville, 1973; Popov *et al.*, 1998; Skuhravý *et al.*, 1983, 1993; Pope & Ellis, 2012). There is evidence of larval resilience in the soil stage. Cold tolerance was observed in a laboratory experiment by Nijveldt and Hulshoff (1968), where 49% of larvae survived being in frozen clay soil after 48 days, however survival was zero after two weeks at -10 ˚C in further experiments by De Clercq and D'Herde (1972). *Haplodiplosis marginata* larvae may also survive periods of flooding: over 50% of 100 larvae were able to survive immersion in water for 28 days. This supports field observations of larvae surviving in flooded soils (Nijveldt & Hulshoff, 1968) but disagrees with a recent UK study by Pope and Ellis (2012) who observed high levels of larval mortality following heavy rainfall. Additionally, very wet weather in summer may cause eggs to be washed off the leaves before hatching (Gratwick, 1992). On the other hand, very hot and dry summers may result in egg or larval desiccation (Eklund, 2005). The prevalence of *H. marginata* in heavy soils that contain a high proportion of clay (Golightly & Woodville, 1974) is thought to be due to the higher moisture content of heavy soils protecting the larvae from desiccation (Andersson, 1969). Nonetheless, some drought tolerance has been recorded in experiments by Nijveldt and Hulshoff (1968): after 14 days of drought under controlled conditions, 52% of 600 larvae developed into adults while 15% remained in diapause. Larval survival dropped to 11% after 60 days of drought (Nijveldt & Hulshoff, 1968). It was thought that, as with the Cecidomyiid wheat blossom midges *Sitodiplosis mosellana* (orange wheat blossom midge) and *Contarinia tritici* (yellow wheat blossom midge) the larvae overwinter in cocoons, however cocoons have only ever been found in three field populations; one each in the UK (Barnes, 1956), the Netherlands (Nijveldt & Hulshoff, 1968) and Belgium (Censier *et al.*, 2014a). Cocoon formation is therefore considered to be rare in this species and is likely to be a response to drought, preventing desiccation (Nijveldt & Hulshoff, 1968; Censier *et al.*, 2014a).

Temperature and moisture are likely to be closely linked to the termination of diapause (Gratwick, 1992). *Sitodiplosis mosellana*, *Contarinia tritici* and *Contarinia sorghicola* (sorghum midge), also in the supertribe Cecidomyiidi, all require an interaction between temperature and moisture for diapause termination and adult emergence (Basedow, 1977; Baxendale & Teetes, 1983; Oakley & Ellis, 2009, Jacquemin *et al.*, 2014). Increased soil moisture may make it easier for larvae to move up through the soil profile to pupate whilst rising temperatures are likely to trigger the end of diapause for this species. This is supported in the literature, with numerous reports of warm,

humid conditions prevailing shortly before an outbreak (Gratwick, 1992). It has been observed that under laboratory conditions, diapause in *H. marginata* is not terminated below 10°C (Baier, 1963) with larvae unable to survive prolonged temperatures of 5°C or 30°C (Nijveldt & Hulshoff, 1968). If conditions are too dry, about 75% of the larvae will remain in diapause for another year (Dewar, 2012) but even in suitable conditions, some 20% of larvae may extend diapause (Popov *et al.*, 1998). Extended larval diapause has been observed in other cecidomyiids (Harris & Foster, 1999). The orange wheat blossom midge, *S. mosellana* can remain in the soil within cocoons for ten years or more (Oakley & Ellis, 2009). The duration of diapause in *H. marginata* has been shown to extend to at least six years, and is likely to vary according to both biotic and abiotic factors (Nijveldt & Hulshoff, 1968).

2.6 Summary

Integrated pest management programmes could offer a viable solution to reducing the risk and mitigating the consequences of future *H. marginata* outbreaks. In order for this to happen however, the current knowledge gaps concerning the biology and ecology of this insect outlined by this literature review need to be addressed. In particular, a breadth of research needs to be conducted over several years at a number of locations to understand how this species interacts with the wider environment and the implications for farmers. This information can be used as the foundation for management strategies and ecologically based IPM programmes. The ultimate aim for any IPM programme should be to consider the ecosystem as a whole, encompassing multiple pests within cropping system. This is not possible, however, without first conducting species-level research to understand each individual component of that ecosystem. This thesis therefore, aims to broaden existing knowledge of *H. marginata* and deliver a critical first step towards the development of IPM-compatible control options.

2.7 Thesis aims and objectives

Aims: To expand upon existing knowledge of the biology and ecology of *Haplodiplosis marginata* and improve current monitoring methods for this pest to aid the development of an effective integrated pest management programme

Objectives:

1. Study the effects of meteorological and soil conditions on the development of *H. marginata* in the soil stage through to adult emergence over three years. Use this information to develop a model based on thermal accumulations and other environmental factors in order to predict yearly adult emergence.
2. Use electroantennography to assess the responses of male *Haplodiplosis marginata* to the major and minor components of the female sex pheromone. Test optimum

pheromone formulations, loadings and dispenser types in field experiments to develop an effective lure for trapping male *H. marginata.*

3. Identify the main factors affecting pheromone trap catch with the chosen lure in the field and determine a recommended best practice for users.
4. Identify the predatory natural enemies of *Haplodiplosis marginata* through the development a PCR-based assay to identify the presence of *H. marginata* in the guts of field-caught arthropod predators.

3 Materials and methods

3.1 Objective 1 – phenological forecasting

3.1.1 Preliminary degree day emergence model

Field data 2014 and 2015

A study was completed to assess the feasibility of developing a model to reliably predict the adult emergence of *H. marginata* in the UK. Approximate dates of *H. marginata* emergence were established for sites across the UK in the years 2014 (four sites) and 2015 (seven sites). Emergence traps (2014) and pheromone traps (2015) were placed in fields in mid-April and monitored on a weekly basis. Emergence traps consisted of an upturned seedling tray which was coated on the underside with insect barrier glue (Agralan Ltd, Ashton Keynes, UK) and secured on the soil surface by wooden stakes. Pheromone traps consisted of a standard red delta trap with a removable sticky insert (Agralan Ltd, Ashton Keynes, UK) hung on a fibreglass cane. Pheromone lures comprised a polyethylene vial containing 0.5mg (*R*)-2-nonyl butyrate placed in the centre of the trap (Natural Resources Institute, University of Greenwich). The date midway between when midge were first found on the trap and when the trap was last checked was used as the emergence date. Hourly soil temperatures and daily rainfall data were obtained from the Met Office MIDAS network of weather stations (Met Office, 2012). Each station was within 20 km of each field site. The distance of the meteorological stations to the emergence sites is likely to be a source of error, however the data are representative of that which would be available to farmers in order to use the model.

Preliminary model development

Two emergence models were produced based on degree day models previously developed for *S. mosellana* in Canada (Elliott *et al.*, 2009) and Belgium (Jacquemin *et al.*, 2014). The first model used hourly soil temperatures to calculate the accumulated degree days above a base temperature from 1st March until the date of emergence for each site. Degree hours were calculated by subtracting the base temperature from the mean hourly temperature and summing all positive values. The total was then divided by 24 to convert it to degree days (Cesaraccio *et al.*, 2001). The

mean number of degree days was then used to predict emergence dates for all sites. Base temperatures ranging from 0 – 10 °C were tested to determine the best model. The 1st March was chosen as a date at which any diapause requirements for this insect are likely to have been met, as is the case with *S. mosellana*, and there are no references to post-larval development occurring prior to this date in the field. The second model incorporated rainfall data as current evidence suggests that moisture is important in the onset of *H. marginata* emergence (Nijveldt & Hulshoff, 1968; Gratwick, 1992; Popov *et al.*, 1998). The first date on which rainfall occurred after the mean daily soil temperature rose above a predetermined threshold was used as the date of biofix. Here we are using the term 'biofix' to represent the estimated date at which pupation begins. The date of biofix was then used to calculate accumulated degree days above a base temperature until emergence (as previously). Mean daily soil temperature thresholds of 5 - 12 °C were tested, along with degree day base temperatures of 0 – 10 °C to determine the best model.

For both models, the predicted dates of emergence were compared against the observed dates for the sites sampled in 2014 and 2015. The standard deviation of the differences were calculated to determine the accuracy of each model as described by Elliott *et al.* (2009). Previously recorded emergence dates were used for model validation. The models were used to predict emergence dates for *H. marginata* in North Bedfordshire for sites sampled in 1971 and 1972 (Woodville, 1973), although daily soil temperatures were used for the degree day calculations due to the unavailability of hourly data. The models were further validated against emergence data for at site at Aylesbury from sampling done in 2012 and 2013 (Pope & Ellis, 2012; Ellis *et al.*, 2014).

3.1.2 Modelling peaks in *H. marginata* emergence

Field data 2015 and 2016

Following the success of the emergence model, further data were collected over two years to investigate if peaks in emergence could be predicted. *Haplodiplosis marginata* activity was monitored over the entire flight season at three sites in the UK: Buckinghamshire (Bucks) and Oxfordshire (Oxon) in 2015, and additionally Wiltshire (Wilts) in 2016. Pheromone traps were placed in two fields at each site which were all in wheat with the exception one field at Bucks in 2015 which was in field beans and at Oxon in 2016 which was in oilseed rape. The trapping period began approximately a week prior to the start of the flight season (mid-April to May) and sticky cards were changed every 3 - 4 days for 8 weeks, after which they were changed weekly until emergence ceased. The same pheromone lures were used throughout the field season. Numbers of *H. marginata* caught at each trapping interval were counted. Hourly soil temperatures and rainfall data were obtained as described.

Model development

Peaks in *H. marginata* activity were identified from catch numbers and the start and end dates were approximated as occurring midway between counts. The first model assumed a straightforward relationship between degree day accumulations from a single date of biofix to the start of each peak (Fig 1a). Here, different DD accumulations do not represent exact physiological requirements but are used to approximate the time to emergence for groups of insects experiencing different temperatures lower down the soil profile. The second model (Fig 1b) assumed equal DD accumulations between each rainfall event and the subsequent peak, as described by Jacquemin *et al.* (2014) from observations of *S. mosellana* emergence.

The same biofix was used as the start of DD accumulations for both models, defined as the date of first rainfall on or after 1st March. Here, biofix represents the time when conditions were suitable for pupation to occur post-diapause. The chosen biofix assumes the diapause requirements for *H. marginata* would have been met prior to 1st March as described in section 2.1.2. It also assumes moisture is necessary for pupation to occur, as with models of *S. mosellana* development (Oakley *et al.*, 1998; Elliot *et al.*, 2009). Degree day (DD) accumulations were calculated above 0°C as described in section 2.1.2, having been determined to be the most appropriate base temperature from the preliminary model (section 2.1.2). Rainfall events were classified as daily rainfall over 1 mm following 3 days without precipitation. The threshold of 1 mm was used to account for inaccuracies in monitoring equipment. For both models, the coefficient of variation of DD accumulations was calculated for all sites and years.

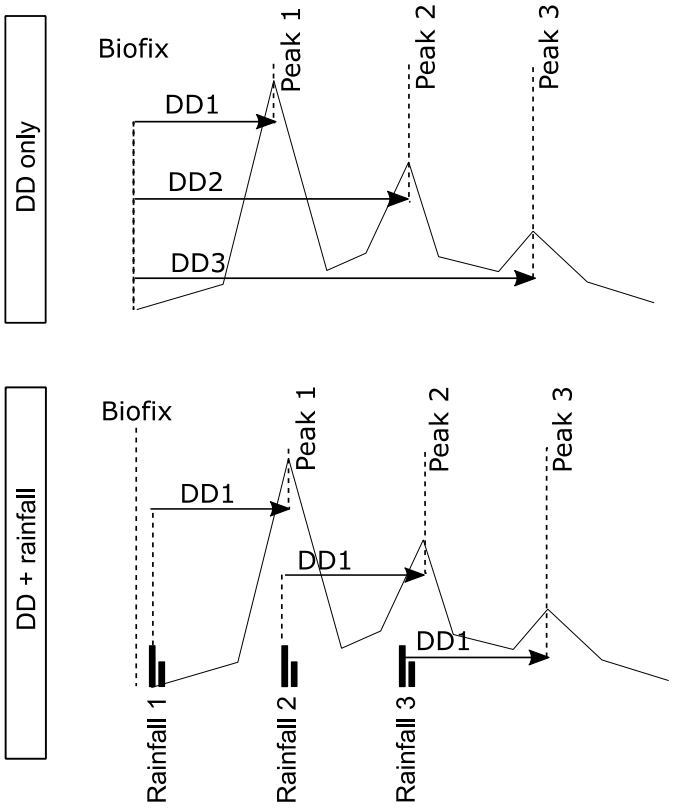

Figure 1. Representation of two different models to predict peaks in *Haplodiplosis marginata* emergence. DD refers to degree day accumulations with numbers indicating unique DD values.

3.1.3 Cumulative percentage emergence model

Pooled field data for each site and year were used to calculate the cumulative percentage emergence at each monitoring interval. Degree days were then calculated from the pre-determined date of biofix for each site and year. Four models were tested to determine which one best described the relationship between degree days and cumulative percentage emergence, all having previously been used successfully to describe insect development. A two-parameter Weibull function was used:

(1) $y = 100(1 - \exp(-(x/\alpha)^{\beta})$

Where y is the cumulative percentage emergence, x is cumulative degree days and α and β are model parameters. A modified bimodal model developed by Kim *et al.* (2000) was used:

(2) $y = \alpha_1 \left\{ \left[\frac{1}{1+\exp\left[-\frac{x-\beta_1}{\gamma_1}\right]} \right] + \left(\frac{\alpha_2}{\alpha_1}\right) / \left[1 + \left(\frac{x}{\beta+\Delta\beta}\right)^{\gamma_2}\right] \right\}$

Where y is the cumulative percentage emergence, x is cumulative degree days and α to γ are model parameters as follows: α_1 and α_2 are the height of the first and second peaks respectively; β is the time in DD of the first peak; $\Delta\beta$ is the difference in DD between the first and second peak; γ_1 and γ_2 define the steepness of the first and second slopes respectively. Initial parameter estimates were made using the methods described in Kim *et al.* (2000). Both functions were fitted using nonlinear least squares regression. Two generalised linear models were also performed with binomial errors and logit or probit links (Forrest & Thomson, 2011). Model selection was done by comparing the adjusted r-squared and root mean square error (RMSE) values of models fitted to observed data (Damos & Savopoulou-Soultani, 2010; Parker *et al.*, 2011). The chosen model was validated against previous sites and years for which the date of *H. marginata* emergence is known and compared with the previous emergence model from section 2.1. All statistical analyses were done in R-3.3.1 (R Core Team, 2016).

3.1.4 Soil textural analysis

Ten individual soil samples of approximately 200g were taken from each field studied in 2016. Soil was removed using a trowel at a depth of 10 cm by walking a 'W' transect through the field and sampling at regular intervals. Samples were pooled for each field to create a composite soil sample which was air dried at 25 °C for 7 days and then sieved through a 2.0 mm sieve to remove stones. Three 10 g samples were taken from the sieved soil for analysis. Soil texture was determined by mechanical analysis using the methodology described in Benton Jones (2001). Organic matter was removed prior to analysis by boiling the samples in hydrogen peroxide. Fractions based on particle size were then separated out according to the different settling velocities in a column of water. Soil

organic matter content was determined by the loss on ignition method described by Ben-Dor and Banin (1989). Soil pH was measured using a Jenway 1305 pH meter with an epoxy bodied gel filled reference electrode.

3.1.5 Crop damage assessment

Tillers of wheat at growth stage at GS92 were sampled at random by walking along the tramlines of each field and sampling a stem at arm's length into the crop every 10 steps. In 2014, 25 galled and 25 non-galled stems were sampled from both fields at the Oxon site. In 2015, this was increased to 30 stems but samples were only taken from Field 1a due to the other being in field beans. In 2016, 30 stems were taken from each field, and additionally from field 3b at the Wilts site. Measurements were taken of ear length, stem height from the first node to the ear, grain number and grain weight per ear. In galled stems, the number of galls was also recorded. Evidence of damage by *Sitodiplosis mosellana* was recorded in 2016 when it became apparent that this pest was also infesting the crop at the Oxon and Wilts sites. Linear models on untransformed data were used to determine any differences in the measured parameters between galled and non-galled stems. Linear models were also use to identify any correlation between the number of galls and the crop measurements on damaged stems. All statistical analysis was done in R v.3.3.1 (R Core Team, 2016).

3.2 Objective 2 – Pheromone lure optimisation

Sections 3.2.3 – 3.2.7 performed by Professor David Hall, NRI, University of Greenwich

3.2.1 Insects

Larvae of *H. marginata* were collected from soil samples taken from affected fields between November 2013 and May 2014 and stored at 4 °C for a minimum of 3 months. Each larva was transferred to an individual plastic container (1.5 cm diameter, 2.5 cm high) of moist sterilised compost covered with a fine mesh and maintained at 20 °C, 60% r.h., and L16:D8 photoperiod, until adults emerged.

3.2.2 Pheromone collection

Volatiles were collected from individual virgin adult males and females separately, within 48 h of emergence. A single live midge was used per collection and was placed in a cylindrical glass vessel (5.3 cm diameter, 13 cm long; Hamilton Laboratory Glass, Margate, UK) with a glass frit and activated charcoal filter at one end (20 × 2 cm, 10-18 mesh; Fisher Chemicals, Loughborough, UK) and a collection filter at the other. The collection filter consisted of a Pasteur pipette (4 mm i.d.) containing Porapak Q (200 mg, 80-100 µm; Waters Associates, Milford, MA, USA) positioned between two glass wool plugs (Supelco, Gillingham, Dorset, UK). Air was drawn through the charcoal filter into the vessel containing the midge and out through the collection filter using a vacuum pump (M361C; Charles Austen Pump, Byfleet, UK) at a rate of 0.5 l per min. Collections

were made continuously for a period of 48 h. Five collections were made from males and four from females. Volatiles were desorbed from the collection filters with dichloromethane (1.5 ml), concentrated under a stream of nitrogen, and refrigerated prior to analysis.

3.2.3 Coupled gas chromatography-mass spectrometry (GC-MS)

Aliquots of volatile collections were analysed using a Varian 3500 GC coupled to a Saturn 2200 MS (Agilent Technologies, Stockport, UK) operated in electron impact mode. A polar or non-polar GC column was used (30 m × 0.25 mm i.d., 0.25 µm film thickness) coated with DBWax (Supelco) or VF5 (Agilent), respectively, and the oven temperature was held at 40 °C for 2 min and then programmed at 10 °C per min to 240 °C. Compounds were identified by their mass spectra, their GC retention indices relative to the retention times of *n*-alkanes and comparison of retention indices and mass spectra with those of authentic synthetic standards.

3.2.4 Coupled gas chromatography-electroantennography (GC-EAG)

Antennal responses of male and female *H. marginata* to collections of volatiles from females were measured by EAG coupled with an Agilent 6890N GC with fused silica capillary columns (30 m × 0.32 mm i.d., 0.25 µm film thickness) coated with polar DB Wax (Agilent) and non-polar SPB1 (Supelco). Injections were splitless (220 °C) for the polar column and with programmed temperature vaporising injector (held at 50 °C for 0.2 min and then programmed at 600 °C per min to 220 °C) for the non-polar column. The carrier gas was helium (2.4 ml per min) and the oven temperature was held at 50 °C for 2 min and then programmed at 10 °C per min to 250 °C. The ends of the GC columns went into a push-fit Y-connecter that lead through a second Y-connector fitted with two equal lengths of deactivated fused silica capillary going to the flame ionisation detector (FID) and a glass T-piece, splitting the GC effluent 50:50. The effluent was collected in the T-piece for 17 s before being blown over the antennal preparation for 3 s in a stream of air (200 ml per min) (Cork *et al.*, 1990).

The antennae were prepared by excising the head from a live specimen, then removing one of the antennae and the tip of the remaining antenna using a sharp microscalpel. Antennal responses were recorded using an INR-2 micromanipulator assembly (Syntech, Hilversum, The Netherlands). Two newly-pulled glass capillary electrodes were filled with an electrolyte solution of 0.1 M KCl with 1% polyvinylpyrrolidine (BDH Chemicals, Poole, UK) added to prevent evaporation. These were attached to silver wire electrodes mounted in micromanipulators. The insect preparation was mounted between the two glass electrodes with the head in the reference electrode and the distal end of the antenna in the recording electrode. The antennal responses were amplified 10× and converted to digital format through the second detector channel of the GC. Data from FID and EAG were captured and processed with EZChrom Elite v.3.3.1 software (Agilent).

3.2.5 Enantioselective gas chromatography

Enantoselective GC was carried out on a CP-Chirasil-Dex CB column (25 m × 0.32 mm i.d., 0.25 µm film thickness; Varian/Agilent) with He carrier gas (2.4 ml per min), split injection (220 °C, 20:1), and FID (220 °C). The oven temperature was held at 60 °C for 2 min and then programmed at 5 °C per min to 200 °C.

3.2.6 Chemicals

Unless otherwise stated, all chemicals were obtained from SigmaAldrich (Gillingham, UK) and were at least 98% pure. Racemic 2-nonyl butyrate was prepared by esterification of 2-nonanol with butyric acid in the presence of N,N'-dicyclohexylcarbodiimide (DCCD) and 4-dimethylamino-pyridine (DMAP) in dichloromethane (Neises & Steglich, 1978). The product was obtained in 93% yield after purification by flash chromatography on silica gel eluted with 2% diethyl ether in hexane and kugelrohr distillation (at 70 °C and 0.03 mm Hg). ^{1}H and ^{13}C nuclear magnetic resonance (NMR), infrared (IR) and mass spectral (MS) data were in agreement with those reported by Censier *et al.* (2014b).

Racemic 2-nonyl butyrate was resolved into the two enantiomers by stirring with a catalytic amount of lipase acrylic resin from *Candida antarctica* yeast in phosphate buffer (1 M K_2HPO_4) for 6 h with monitoring by enantioselective GC, which selectively hydrolysed the (*R*)-enantiomer (Hall *et al.*, 2012) The product was chromatographed on silica gel eluted successively with 2, 5, 10, 20, and 50% diethyl ether in hexane to give (*S*)-2-nonyl butyrate (98.7% enantiomeric excess by enantioselective GC) and (*R*)-2-nonanol. The latter was esterified as above to give (*R*)-2-nonyl butyrate (98.9% e.e.). Racemic 2-heptyl butyrate was prepared similarly from 2-heptanol. This was resolved into the enantiomers with lipase from *C. antarctica* to give the (*S*)- (97.8% e.e.) and (*R*)- (98.2% e.e.) enantiomers.

3.2.7 Pheromone dispensers

Two different dispenser types were tested: polyethylene vials (26 × 8 mm, 1.5 mm thick; Just Plastics, London, UK) and white rubber septa (20 × 10 mm; International Pheromone Systems, The Wirral, UK). Dispensers were loaded with the pheromone dissolved in hexane (100 µl) and the solvent was allowed to evaporate. Release rates were measured for dispensers loaded with 2-nonyl butyrate (1 mg) and maintained in a laboratory wind tunnel (27 °C, 2.2 m s^{-1} wind speed). Duplicate samples were removed at weekly intervals and the remaining pheromone was extracted individually in hexane (5 ml) containing dodecyl acetate (1 mg) as internal standard. Extracts were analysed by GC with FID on a capillary column (30 m × 0.32 mm i.d., 0.125 µm film thickness) coated with DB5 (Agilent) with splitless injection (220 °C) and the oven temperature held at 50 °C for 2 min and then programmed at 10 °C per min to 250 °C. The amount of pheromone remaining in lures returned from field trapping tests was measured similarly.

3.2.8 Field trapping experiments

Field trapping experiments were all carried out at sites with known soil populations of *H. marginata*. Five experiments were performed. Experiments 1, 2, 3, and 4 were carried out in Oxfordshire, UK (51°55"N, 1°10"W). Experiment 5 was carried out in Buckinghamshire, UK (51°37"N, 0°48"W). All fields were in winter wheat and the experiments were conducted during part of the flight season of *H. marginata*, coinciding with wheat growth stages 39-59 (Zadoks *et al.*, 1974).

For each experiment, pheromone dispensers were placed in standard red delta traps (Agralan, Wiltshire, UK) containing a removable sticky insert (15 × 15 cm). Polyethylene vials were used as dispensers for all experiments with the exception of experiment 1. Traps were hung from fibreglass canes and positioned at the height of the wheat ear. For experiments 1-4, traps were laid out in a randomised complete block design with 10 m between traps and 50 m between blocks. Adult *H. marginata* were identified based on antennal and genital morphology (Harris, 1966) and counted using a bifocal microscope.

Experiment 1 – pheromone dispensers

Catches of male *H. marginata* in traps baited with racemic 2-nonyl butyrate (1 mg) formulated in the two types of pheromone dispenser, rubber septa, and polyethylene vials, were compared with catches in an unbaited trap. Traps were laid out in four replicated blocks and were in place between 15 May and 19 June 2014 and the sticky inserts of the traps were changed after 6 days, at which time the treatments were re-randomised within the blocks.

Experiment 2 – pheromone chirality

Catches in traps baited with lures containing (*R*)-2-nonyl butyrate (0.5 mg), (*S*)-2-nonyl butyrate (0.5 mg), the racemic mixture (1 mg), and an unbaited trap as control were compared. Traps were laid out in four replicated blocks and were in place between 5 and 19 June 2014.

Experiment 3 – effect of minor components

The effects of addition to (*R*)-2-nonyl butyrate (0.5 mg) of two minor components were tested: (*R*)-2-nonanol and (*R*)-2-heptyl butyrate, each at 2% of the major component, separately and in combination. These treatments were compared with lures containing (*R*)-2-nonyl butyrate (0.5 mg), lures containing the racemic mixture (1 mg), and with an unbaited trap as control. Traps were laid out in 10 replicated blocks and were in place between 18 and 29 May 2015. The sticky inserts of the traps were changed on days 4 and 9 of the experiment, with the treatments re-randomised within the blocks after each change.

Experiment 4 – pheromone loading

Trap catches with lures containing loadings of 2.5, 0.5, 0.05, and 0.005 mg of the major pheromone

component, (*R*)-2-nonyl butyrate, were compared. Traps were laid out in 10 replicated blocks and were in place between 2 and 11 June 2015. The sticky inserts of the traps were changed on days 4 and 8 of the experiment, with the treatments re-randomised within the blocks after each change.

Experiment 5 – comparison with other traps

Numbers of midges caught in delta traps baited with lures containing (*R*)-2-nonyl butyrate (0.5 mg) were compared with existing trapping methods, i.e., unbaited sticky traps and water traps. Standard yellow insect sticky traps (25 × 10 cm) were mounted on fibreglass canes at crop height. Water traps (Nickerson Brothers, Lincoln, UK) comprised a yellow bowl (25 cm diameter, 10 cm deep), partly filled with water to which several drops of Fairy dishwashing liquid were added, and mounted on a cane at crop height. All three traps were compared in two 3 × 3 Latin squares. All traps were checked at weekly intervals between 11 and 29 May 2015.

3.2.9 Statistical analysis

Numbers of *H. marginata* caught per day for each trap were log(x+1) transformed to improve the homoscedasticity of the data and were analysed with a two-way ANOVA with treatment and block as factors. The least significant difference (LSD) test was used to test for significant differences between means ($\alpha = 0.05$). All analyses were done in R v.3.2.2 (R Core Team, 2015). Results in experiment 5 were not analysed statistically due to the extreme heteroscedasticity of the data.

3.3 Objective 3 – Pheromone trap development

3.3.1 Field sites

Three sites known to have populations of *H. marginata* located in Oxfordshire (51°55"N, 1°10"W); Buckinghamshire (51°37"N, 0°48"W) and Wiltshire (51°2"N, 1°57"W) were selected. Pheromone dispensers were placed in red delta traps (Agralan, Wiltshire, UK) fitted with a removable sticky insert (15 cm x 15 cm). Polyethylene vials (see above for description) containing (*R*)-2-nonyl butyrate (0.5mg; 98% enantiomeric excess) synthesised as described previously (Rowley, 2016) were used as lures for all experiments. Unless otherwise stated, traps were suspended from canes at the height of the ear of the wheat crop. The three nearest weather stations to each field site from the Met Office MIDAS dataset (Met Office, 2012) were used to record mean wind speed and prevailing wind direction for the duration of the trapping periods. Adult *H. marginata* were identified and counted using a bifocal microscope. All statistical analyses were done in R 3.3.1 (R Core Team, 2016). Linear mixed effects models were fitted with the lme function from the nlme package (Pinheiro *et al.*, 2016) and post-hoc multiple comparisons (Tukey's Contrasts) were performed using the glht function from the multcomp package.

3.3.2 Field experiments

Lure longevity

Traps were positioned in two fields of winter wheat: one each at the site in Oxfordshire and Buckinghamshire, between 3rd May – 1st July 2016. Traps were positioned along two parallel transects 20 m apart with each trap suspended at the height of the ear. Each transect consisted of four traps were placed at intervals of 40 m. Traps placed at the same distance along each of the two transects represented a pair, each trap baited with either a pheromone lure that remained in the trap throughout the season or a lure that was replaced weekly. New lures were replaced on days 6, 13, 20, 29, 34, 43, 50 and 59 of the experiment. Each time the lures were replaced the sticky inserts of all traps were renewed and the positions of each pair of traps switched in order to reduce positional effects. Trap height was adjusted each week to match the growth of the crop. At the end of the experiment the remaining pheromone in each of the aged lures was extracted individually in hexane (5 ml) containing dodecyl acetate (1 mg), which was used as the internal standard. Extracts were analysed by GC with FID on a capillary column (30 m x 0.32 mm i.d. x 0.125 µ film thickness) coated with DB5 (Agilent) with splitless injection (220°C) and the oven temperature held at 50°C for 2 min and then programmed at 10°C/min to 250°C. Numbers of *H. marginata* caught per day for each trap were log transformed to improve the homoscedasticity of the data. Linear mixed model, with pair as a random effect was used to analyse the effect of field, days elapsed and lure type (old or new) on catch. The total catch of traps with old lures was calculated as a percentage of the total catch of traps with new lures for each time period. A linear regression of this data against days elapsed was used to analyse the effect of time on lure performance.

Trap height

Traps were positioned at the site in Oxfordshire between 13th – 19th May 2016 in two adjacent fields. One field was in winter wheat and the other in spring wheat. Traps were laid out in two 4 x 4 Latin squares, one in each field with at least 200 m between the two squares. Traps were positioned 0 cm, 40 cm, 80 cm and 120 cm from the ground. Treatment 0 cm was below the height of the crop in both fields. Treatment 40 cm was at the height of the ear in the field of winter wheat but wasabove crop height in the field of spring wheat. Treatments 80 cm and 120 cm were above crop height in both fields. Sticky cards were removed and numbers of male *H. marginata* counted on day three and at the end of the experiment. Treatments within each Latin square were re-randomised on day three. Both sets of counts were used in the analysis. Numbers of male *H. marginata* caught in each trap were log(x+1) transformed to improve the homoscedasticity of the data. Data were analysed using a two-way analysis of variance (ANOVA). Tukey's honestly significant difference (HSD) test was used to compare means of different height treatments overall and between fields.

Distance from field margins

Traps were positioned at all three locations in fields of winter wheat between 19th May – 1st June 2016. A transect of three traps were positioned at 20 m intervals on a line running perpendicular to the field margin. The first trap in the transect was placed in the margin itself. Transects were placed on field margins of each aspect (north, south, east and west facing) in each of the three fields giving a total of 12 transects. Transects were classified as upwind, downwind or crosswind according to the prevailing wind direction for the experimental period and sticky inserts were changed on a weekly basis. A linear mixed model, with transect as a random effect, was used to analyse the effect of trap position in relation to wind direction and distance from the field margin on catch. Diagnostic plots of residuals were used to check that the assumptions of the model were met. Multiple comparisons of means were used to test for significant differences in catch between traps at different distances from the field margin.

Range of interference

Traps were positioned in a field of winter wheat at each of the three sites between 1st – 22nd June 2016. In each field four hexagonal arrays of traps with an additional central trap were set up, so that all traps were equidistance apart with at least 80 m between arrays (Elkinton & Cardé, 1988; Wedding *et al.*, 1995). Each array of traps had a different inter-trap distance (treatment) of 5 m, 10 m, 20 m and 40 m, with each treatment occurring once per field. The sticky inserts of all traps were changed three times at one week intervals and on each occasion the treatments within each field were re-randomised. There were a total of nine replicates of each inter-trap distance. The central trap was kept in the same location regardless of the inter-trap distance. Catch data were log-transformed and the relationship between inter-trap distance and mean catch of the outer and central traps was investigated using a linear mixed effects model with array as a random effect. Both downwind traps of one of the 20 m arrays had significant outliers during one trapping period. These traps were determined to be unduly influencing the fit of the models, and it was therefore decided that these should be removed prior to analysis. Diagnostic plots of residuals were used to check that the assumptions of the models were met.

3.4 Objective 4 – Identification of natural enemies through gut analysis

Sections 3.4.1 – 3.4.4 work done in collaboration with Janetta Skarp, Imperial College London, as part of an AHDB summer studentship project

3.4.1 Insects

Haplodiplosis marginata larvae were extracted from soil collected from fields in Oxfordshire and Buckinghamshire, UK, between April and June 2015 and kept in plastic containers of moist, sterilised compost at 4°C until use. Pitfall traps were used to collect adult *Nebria brevicollis* (Fabricius) beetles from fields at Harper Adams University, UK, in June 2015. Beetles were kept in clear plastic

containers at 20°C, 16:8 L:D, 60% RH and fed *ad lib* on *Tenebrio molitor* (Linnaeus) larvae prior to the feeding assay. Insect specimens used in cross-reactivity tests were collected by hand (Harper Adams University), pitfall traps and pan traps (Oxfordshire). These specimens were stored at -80°C prior to DNA extraction.

3.4.2 DNA Extraction

DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's supplementary protocol for insect DNA extraction. Whole specimens were first washed in fresh Tris-EDTA (TE) buffer and then ground with a sterile micro-pestle. For sequencing and assay cross-reactivity testing, individual *H. marginata* larvae and undissected invertebrates were used. For gut analyses of the beetles, entire guts were dissected out and used for DNA extraction. Extracted DNA was pelleted by centrifugation and resuspended in 100 µL TE buffer before being stored at -20°C until use. One negative control (no insect material) was included for every 20 extractions.

3.4.3 PCR amplification and sequencing of *H. marginata* COI region

A 521bp fragment of *H. marginata* DNA from the mitochondrial cytochrome oxidase subunit I (COI) gene was amplified using the universal insect primers C1-J-1718 and C1-N-2191 (Simon *et al.*, 1994; King *et al.*, 2010). Each PCR reaction (25 µL) comprised of; 1X PCR master mix (Invitrogen, Carlsbad, CA, USA), 0.625 U *Taq* polymerase (Invitrogen), 4 mM $MgCl_2$ (Invitrogen), 2.5 µg bovine serum albumin (Sigma-Aldrich, Dorset, UK), 0.05 mM dNTPs (Invitrogen), 0.1 µM of each primer and 2.5 µL of target DNA. PCR conditions consisted of an initial denaturation at 94°C for 2 min 30 s, then 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 45 s, followed by a final extension period at 72°C for 10 min. PCR products were separated on a 1.5% agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Fremont, USA) and photographed under UV light (Sint *et al.*, 2011). Unpurified PCR products were sequenced by Eurofins Genomics (Ebersberg, Germany) on a 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were deposited in the European Nucleotide Archive (accession number LT852755).

3.4.4 Primer design and PCR assay development

Species specific primers for *H. marginata* were designed from the sequencing products using the program Primer-BLAST (Geer *et al.*, 2010). Individual primer pairs were synthesised by Eurogentec Ltd. (Liège, Belgium) and validated for use using a T100 Thermal Cycler (Bio-rad, Watford, UK). Primer validation consisted of specificity testing against *H. marginata* and 40 non-target organisms from orders Diptera, Coleoptera, Hymenoptera, Hemiptera, and Araneae. The primer pairs which showed no cross-reactivity were selected and the optimum PCR conditions determined by altering the annealing temperature across individual reactions (55 °C to 77 °C). The primer pair with the

highest optimum annealing temperature was selected for use in the assay. Assay sensitivity was determined using a serial dilution of *H. marginata* DNA at concentrations from 10 ng μL^{-1} to 0.0001 ng μL^{-1}, with 10 replicates of each dilution.

3.4.5 Rate of digestion of *H. marginata* DNA in predator guts

The digestion half-life of *H. marginata* DNA in the guts of a predator was determined using the carabid *N. brevicollis*. The half-life is defined as the time at which *H. marginata* DNA can only be detected in 50% of the predators assayed post-feeding (Greenstone & Hunt, 1993). *Nebria brevicollis* specimens were first separated into individual clear plastic containers (10 cm diameter x 6 cm height) with moist cotton wool and starved for 5 days. Beetles were offered a single live larva of *H. marginata* at time 0h and were observed feeding. Any beetles that did not consume the larva within 15 minutes were excluded from the experiment. Beetles were kept at 20°C, 16:8 L:D, 60% RH for the duration of the trial. Beetles were killed in groups of 10 by freezing at 0h, 2h, 4h, 8h, 12h, 24h and 36h post-feeding, apart from the 24h group which had 9 beetles. Five beetles were not fed and killed at 0h. All specimens were stored at -80 °C and prior to DNA extraction (see section 2.2). PCR reactions proceeded as described in section 2.3. The percentage of the total insects screening positive for *H. marginata* DNA at each time point was analysed using a probit model to determine the time post-feeding at which the detection half-life occurred. Statistical analysis was performed in R v.3.3.1 (R Core Team, 2016).

3.4.6 Field survey

Pitfall traps were used to collect live Carabidae from the field in Oxfordshire. Five pitfall traps were placed in a cross-shaped array connected with barriers of galvanised lawn edging (10 cm height x 30 cm length) to improve the catch rate (Hansen & New, 2005). Each trap comprised a plastic beaker (8 cm diameter x 10.6 cm height) with small rocks placed in the bottom as refugia (Sunderland *et al.*, 2005) and a corrugated plastic cover (12 cm x 12 cm) positioned 5 cm above the trap on wire supports. Six arrays were used per sampling date making 30 traps in total, with at least 30 m between arrays. Traps were set in the late afternoon or early evening and collected before noon the next day. When the traps were emptied, live specimens were immediately placed on ice in an insulated container, prior to storage at -80 °C. Trapping took place on 2 occasions, 10 days apart, in early May 2016, with an additional collection made in late July using just 20 traps (4 arrays).

4 Results

4.1 Objective 1 – Phenological forecasting

4.1.1 Preliminary degree day emergence model

Across all sites and years, the date of emergence varied from 30th April at the earliest, until 19th May at the latest. For the first model, the mean number of degree days accumulated above 0 °C from 1st March until emergence was 588DD (± 9.7DD). A base temperature of 0 °C was chosen as it gave the best results in terms of predicted emergence date compared with the observed emergence date. For the second model, a temperature of 6 °C gave the best modelled results for the onset of pupation, followed by degree day accumulations above 0 °C for the completion of adult development. The mean number of degree days calculated from the date of biofix until the date of emergence for each site was 548DD (± 8.4DD). The first model was able to predict emergence at the sampled sites to within 5 days (± 4 days) and the second model to within 4 days (± 2 days). The standard deviation of the differences between the observed dates and model predictions was also smaller for the second model suggesting a higher degree of accuracy (Table 1). From the historical data, predictions for the date of emergence from both models were within 5 days (± 3.5 days) for all sites (Table 2).

Table 1. Dates of observed and predicted emergence for years 2014 & 2015 from sampled sites, and difference in days for each model

		Model 1		Model 2	
Site	**Observed emergence date**	**Predicted emergence date**	**Days difference (Obs – Pred)**	**Predicted emergence date**	**Days difference (Obs – Pred)**
2014					
Royston (Herts)	30th April (± 0 days)	29th April	+1	27th April	+3
Bicester (Oxon)	3rd May (± 3.5 days)	2nd May	+1	2nd May	+1
H. Wycombe (Bucks)	3rd May (± 3.5 days)	3rd May	0	4th May	-1
Aylesbury (Bucks)	3rd May (± 3.5 days)	3rd May	0	6th May	-3
2015					
Royston (Herts)	2nd May (± 2 days)	4th May	-2	2nd May	0
Bicester (Oxon)	2nd May (± 2 days)	6rd May	+4	3rd May	-1
H. Wycombe (Bucks)	2nd May (± 2 days)	4th May	-2	5th May	-3

Aylesbury (Bucks)	9th May (± 4 days)	4th May	+5	8th May	+1
Glemsford (Suffolk)	3rd May (± 3 days)	5th May	+2	2nd May	+1
Thirsk (North Yorks)	9th May (± 2 days)	12th May	+3	13th May	-4
Devizes (Wiltshire)	3rd May (± 3 days)	4th May	1	1st May	+2
Max. difference			**+5 (± 4 days)**		**-4 (± 2days)**
SD (obs-pred)			**2.43**		**2.17**

Table 2. Dates of observed and predicted emergence for years 2012 & 2013 and 1971 & 1972, and difference in days for each model

		Model 1		Model 2	
Site	**Observed emergence date**	**Predicted emergence date**	**Days difference (Obs – Pred)**	**Predicted emergence date**	**Days difference (Obs – Pred)**
2013					
Aylesbury (Bucks)	17th May (± 3.5 days)	19th May	-2	19th May	-2
2012					
Aylesbury (Bucks)	10th May (± 3.5 days)	6th May	+4	5th May	+5
1972					
N. Bedfordshire	19th May	16th May	+3	21st May	-2
1971					
N. Bedfordshire	18th May	19th May	-1	16th May	+2

4.1.2 Modelling peaks in emergence

Emergence began no later than 2nd May (± 3 days) at all study sites and continued until as late as mid-July. Total site catches ranged from 1,755 to 20,384 individuals over the entire flight season. The catch data revealed apparent 'waves' of emergence of *H. marginata* over time, with a maximum catch rate of 200 individuals per trap per day. The first peak generally occurred soon after the initial emergence, with smaller subsequent peaks occurring at two- to three-week intervals (Fig. 2). At the two sites for which data were obtained in both years, mean soil temperatures in April and May 2015 varied by only 2.33 °C whereas in 2016 the difference rose to 5.56 °C, reflecting a cooler April

and warmer May than the previous year. Mean daily rainfall at the two sites was higher in 2016, averaging 2.28 mm compared with 1.31 mm in 2015. The maximum daily rainfall of 33.8 mm occurred in 2016 at the Oxon site.

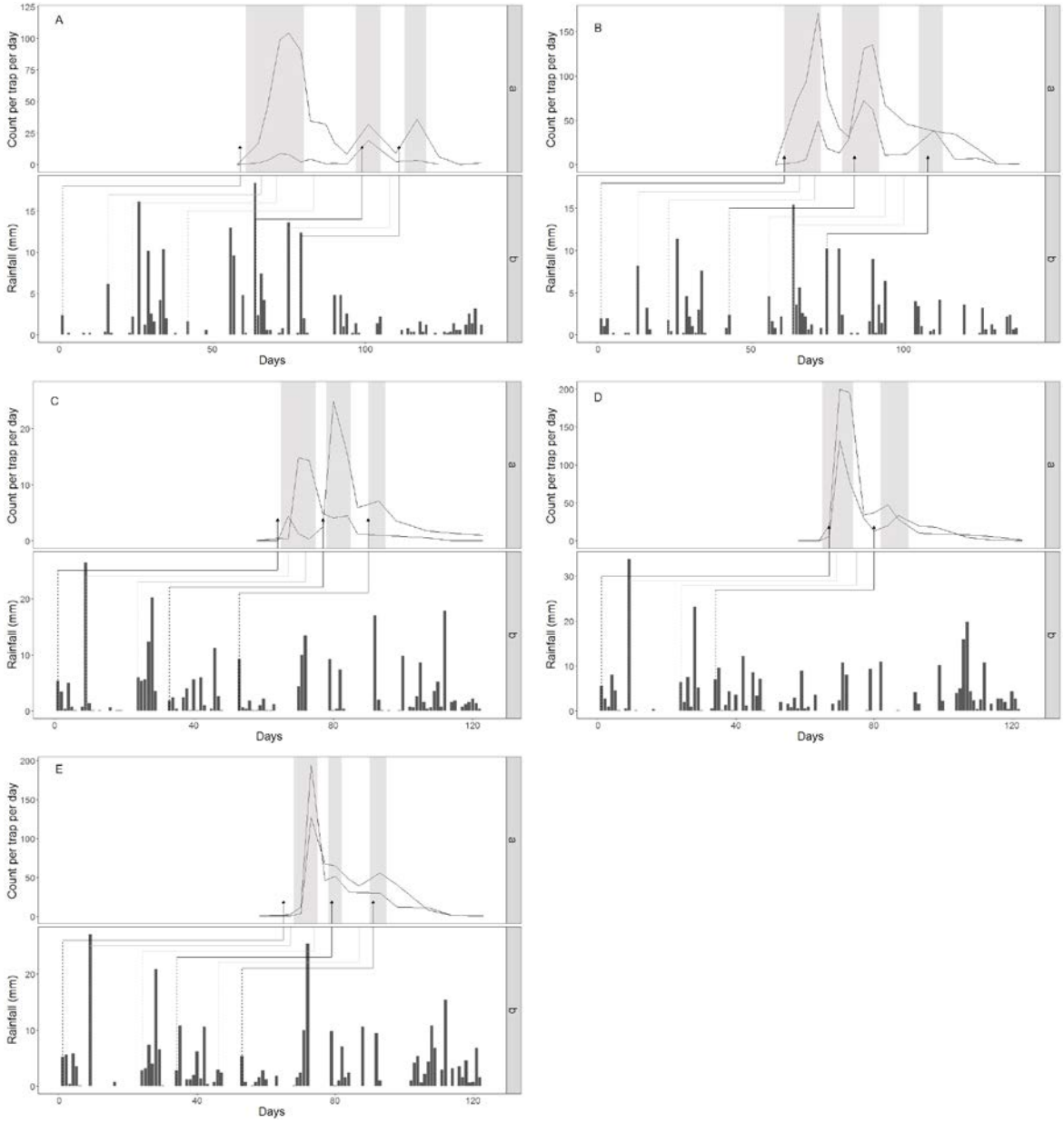

Figure 2. *Haplodiplosis marginata* catch per trap per day (panel a) and 24hr rainfall in mm (panel b) for each day of the trapping period. Black arrows represent inductive rainfall events, grey lines indicate non-inductive rainfall events. Horizontal lines represent degree day accumulations of 512 DD. A) Bucks 2015, B) Oxon 2015, C) Bucks 2016, D) Oxon 2016, E) Wilts 2016

The DD-only model showed an average accumulation of 528.25DD (± 7.69DD) between the biofix and the onset of emergence. Accumulations between the biofix and subsequent peaks however were more variable, averaging 796.82DD (± 39.14DD) and 1083.68 (± 54.81DD) for peaks 2 and 3 respectively (Table 3). Across all sites and years 46.7% of the identified rainfall events could be

linked to peaks in emergence (Fig. 2). The DD + rainfall model showed an average accumulation of 512.42DD (± 9.11DD) between a triggering rainfall event and a subsequent peak in emergence activity (Table 1, Fig. 2).

Table 3. Peaks in emergence: model development. Degree day accumulations for the periods between biofix and emergence peaks (DD-only model) and the periods between inductive rainfall events and emergence peaks (DD + rainfall model), calculated for each site and year.

4.1.3 Cumulative percentage emergence model

	DD only 1: Biofix - Peak			**DD + rainfall : Rain – Peak**		
	Peak 1 (DD1)	**Peak 2 (DD2)**	**Peak 3 (DD3)**	**Peak 1 (DD1)**	**Peak 2 (DD1)**	**Peak 3 (DD1)**
2015						
Bicester (Oxon)	537.24	764.91	1158.41	537.24	466.13	461.45
H. Wycombe (Bucks)	563.62	1054.46	1339.97	563.62	477.97	560.78
2016						
Bicester (Oxon)	484.68	746.86	-	484.68	551.44	-
H. Wycombe (Bucks)	534.13	694.30	926.43	534.13	486.59	516.16
Devizes (Wilts)	521.56	723.57	909.91	521.56	504.43	507.65
Mean	528.25	796.82	1083.68	528.25	497.31	511.51
SD	28.77	146.43	205.10	28.77	33.32	40.7
CV	**0.054**	**0.184**	**0.189**	**0.054**	**0.067**	**0.079**
				Mean (all peaks) (±SEM)		**512.42 (± 9.11)**

The bimodal model was the best fitting model based on the adjusted r-squared value, accounting for 92% and of the variation in the data (Fig. 3). The bimodal model and the GLM with probit link both showed similar RSME values (Table 4) and were selected for validation. The probit model predicted that 10% emergence of *H. marginata* would occur at 550DD post-biofix and the bimodal model at 576DD post-biofix. When validated against previous sites and years, the probit model predicted the date of emergence to within a maximum of 4 days (± 4 days) and the bimodal model to within 3 days (± 4 days), with the error reflecting uncertainty in the true emergence date as a result of the sampling interval. The bimodal model however had a lower standard deviation of differences between the observed and predicted dates indicating higher accuracy overall (Table 5).

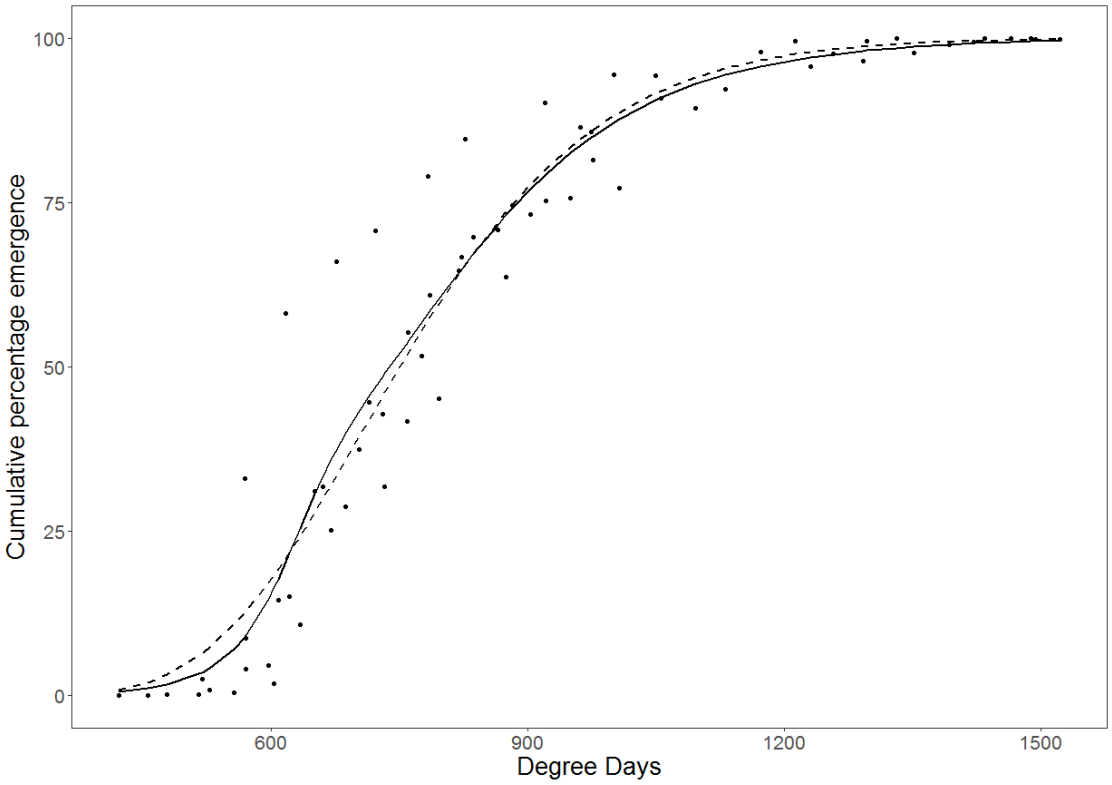

Figure 3. Percentage cumulative emergence of *Haplodiplosis marginata* as a function of accumulated degree days for all sites and years studied. Predicted emergence based on the probit model (dashed line) and bimodal model (solid line) shown.

Table 4. Parameter estimates and standard error (SE) for all four cumulative percentage emergence models. RMSE and adjusted R^2 values shown. Predicted DD accumulations required for 10%, 50% and 90% based on the two selected models.

Model	Parameter	Est. value	SE	RMSE	Adj. R^2	Model predictions	
Binomial GLM	α	7.126	0.01616	10.03	0.89		
	β	-47.16	0.10725				
Weibull	α	822.061	10.6095	10.67	0.91		
	β	4.696	0.3915				
Probit model	α	4.124	0.00845	9.98	0.89	10%	550.04
	β	-27.308	0.05616			50%	750.47
						90%	1023.93
Bimodal	α_1	25.409	48.365	9.79	0.92	10%	575.55
	β_1	623.233	29.473			50%	738.49
	γ_1	30.294	50.783			90%	1039.89
	α_2	75.063	50.114				
	$\Delta\beta$	187.541	136.260				
	γ_2	-7.322	3.286				

Table 5. Observed and predicted 10% emergence dates for probit model and bimodal model for all sites and years. Differences in days between observed and predicted dates shown. Error in brackets represents uncertainty in emergence dates which are given as a midpoint between sampling dates.

		Probit Model		Bimodal Model	
Site	**Observed emergence date**	**Predicted emergence date**	**Days difference (Obs – Pred)**	**Predicted emergence date**	**Days difference (Obs – Pred)**
2014					
Royston (Herts)	30th April (± 0 days)	27th April	3	29h April	1
Bicester (Oxon)	3rd May (± 3.5 days)	30th April	3	2nd May	1
H. Wycombe (Bucks)	3rd May (± 3.5 days)	30th April	3	3rd May	0
Aylesbury (Bucks)	3rd May (± 3.5 days)	2nd May	1	3rd May	0
2015					
Royston (Herts)	2nd May (± 2 days)	1st May	1	3rd May	-1
Bicester (Oxon)	2nd May (± 2 days)	3rd May	-1	5th May	-3
H. Wycombe (Bucks)	2nd May (± 2 days)	1st May	1	3rd May	-1
Aylesbury (Bucks)	9th May (± 4 days)	5th May	4	6th May	3
Glemsford (Suffolk)	3rd May (± 3 days)	2nd May	1	4th May	-1
Thirsk (N. Yorks)	9th May (± 2 days)	9th May	0	10th May	-2
Devizes (Wiltshire)	3rd May (± 3 days)	30th April	3	3rd May	0
2016					
Bicester (Oxon)	7th May	8th May	-1	10th May	-3
H. Wycombe (Bucks)	7th May	5th May	2	7th May	0
Glemsford (Suffolk)	5th May (± 3 days)	6th May	-1	8th May	-3
Devizes (Wiltshire)	10th May	7th May	3	8th May	2
Max. difference			**4 (± 4days)**		**+3 (± 4days)**
SD (Obs - Pred)			**2.19**		**1.81**

4.1.4 Soil analysis

Soil textural analysis showed some variation between field sites, with all fields being clays or dominant in clay particles (Table 6). All soils had an organic matter content of over 6%. The pH did not vary greatly across sites and was either neutral or slightly alkaline in all fields.

Table 6. Soil textural classification, percentage organic matter and pH for all fields. Figures represent the mean (±SEM) of three 10g composite soil samples.

Field (Site)	% sand	% silt	% clay	Classification	% Organic matter	pH
Field 1a (Oxon)	22.35 (±0.33)	35.65 (±0.46)	42 (±0.71)	Clay	8.77 (±0.10)	7.37
Field 1b (Oxon)	32.51 (±2.12)	28.12 (±1.07)	39.36 (±1.05)	Clay	8.71 (±0.02)	7.27
Field 2a (Bucks)	36.9 (±0.54)	38.63 (±0.09)	24.47 (±0.49)	Clay loam	6.87 (±0.04)	6.97
Field 2b (Bucks)	34.31 (±1.05)	32.73 (±0.52)	32.95 (±0.59)	Clay loam	7.85 (±0.15)	7.20
Field 3a (Wilts)	16.26 (±0.51)	48.08 (±1.18)	35.66 (±0.66)	Silty clay	6.63 (±0.02)	7.47
Field 3b (Wilts)	15.68 (±0.43)	48.51 (±0.30)	35.81 (±0.33)	Silty clay	5.96 (±0.06)	7.40

4.1.4 Crop damage assessment

A mean of 12.89 galls was found on damaged stems was, with little variation between sites and years. The maximum number of galls found on one stem was 34 however 83% of galled stems had ten galls or fewer and 55% had five or fewer. In year 3, 40% of all sampled stems had evidence of *S. mosellana* damage. Galled stems were shorter in length ($F_{1,333}$=46.44, P<0.001), had shorter ears ($F_{1,333}$=5.52, P<0.05), had fewer grains per ear ($F_{1,333}$=25.47, P<0.001) and lower grain weight per ear ($F_{1,333}$=6.75, P<0.01) when compared to non-galled stems (Figure 5). Removing data from year 3 in which *S. mosellana* damage was evident removed the effect of galling on grain weight ($F_{1,156}$=0.33, P=0.57) but had no impact on the other variables. There was no relationship between the number of galls and any of the measured parameters on galled stems.

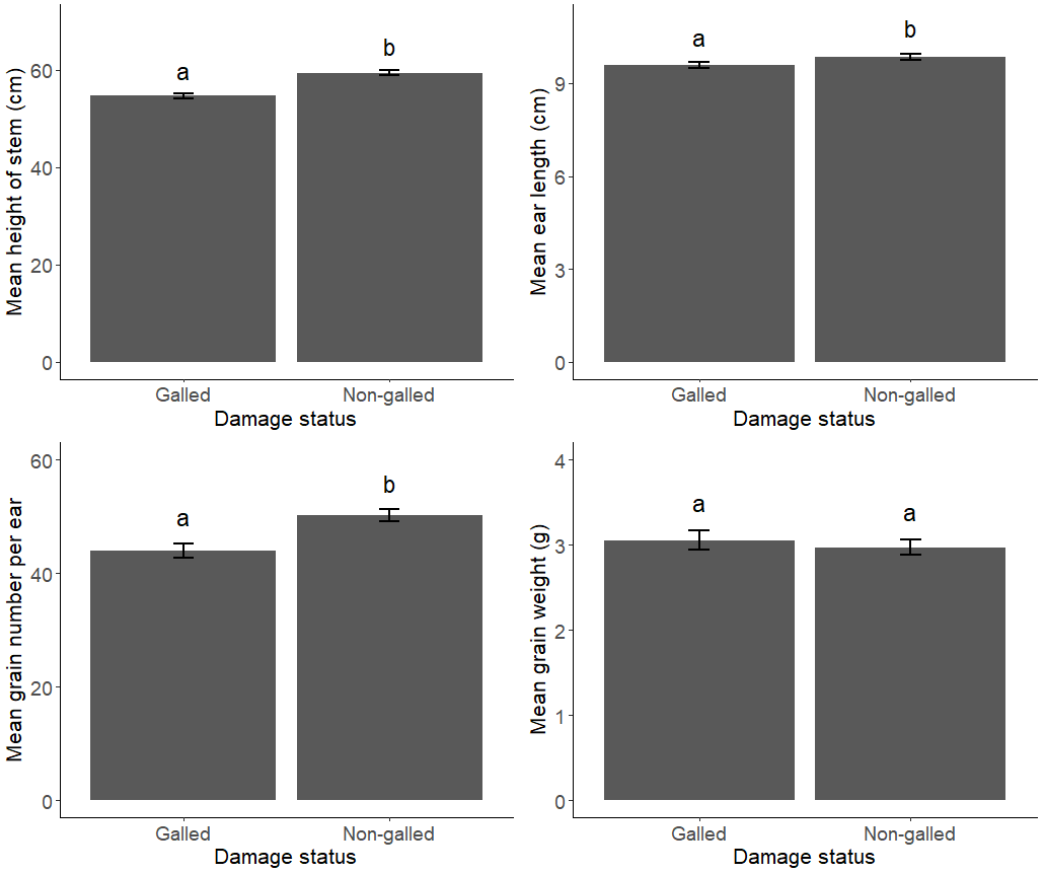

Figure 4. Crop damage assessment of stems sampled 2014 – 2016. Mean values (± SEM) show differences between galled and non-galled stems in **A.** height **B.** ear length **C.** grain number per ear **D.** grain weight per ear. Lowercase letters represent where these differences are statistically significant at the P = 0.05 level.

4.2 Objective 2 – Pheromone lure optimisation

4.2.1 Pheromone identification

Analyses of collections of volatiles from female *H. marginata* on the non-polar GC column with a male antenna EAG preparation indicated one strong EAG response and a weaker response to a compound eluting earlier (Figure 5). Analyses on the polar column showed a strong EAG response but the minor response was not so clear (data not shown). Retention data for the EAG responses and synthetic compounds are shown in Table 1.

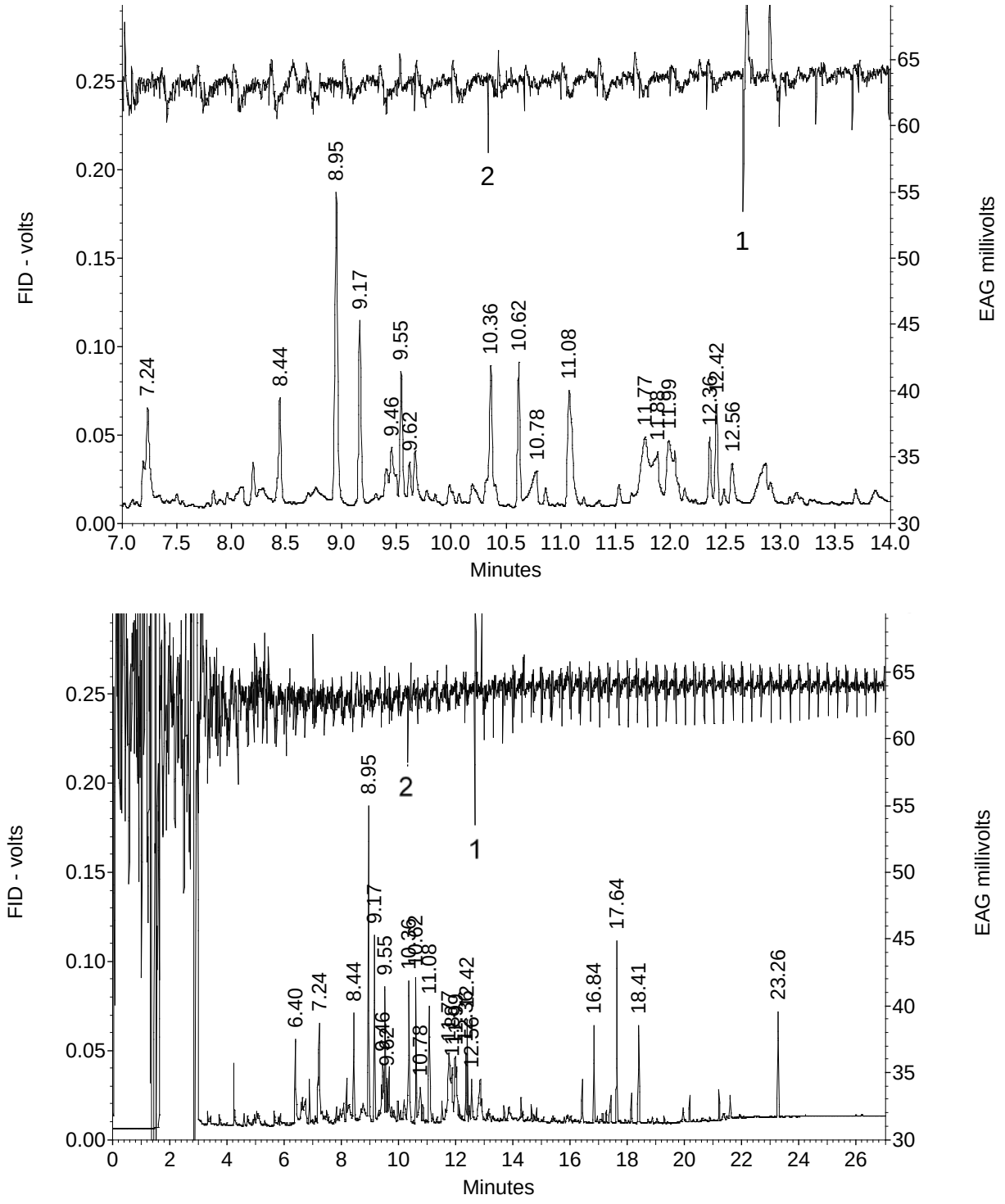

Figure 5. Coupled gas chromatography-electroantennography (GC-EAG) analysis of collection of volatiles from female *Haplodiplosis marginata* on non-polar column. Note that the lower panel is an expansion of the upper panel and FID signal is lower trace and EAG upper trace in each; major response (1) to 2-nonyl butyrate at 12.42 min, minor response (2) at 10.2 min; 2-heptyl butyrate at 10.00 min, 2-nonanol at 8.25 min.

Analyses of collections of volatiles from female and male *H. marginata* by GC-MS on both non-polar and polar GC columns (Figure 6) indicated a female-specific compound that was identified as 2-

nonyl butyrate by comparison of retention times (Table 7) and mass spectrum with those of the authentic synthetic compound, and the identification was confirmed by co-chromatography on both GC columns. Up to 50 ng per female of 2-nonyl butyrate was collected during 48 h. This compound had retention data consistent with that of the major response in the GC-EAG analyses (Table 1).

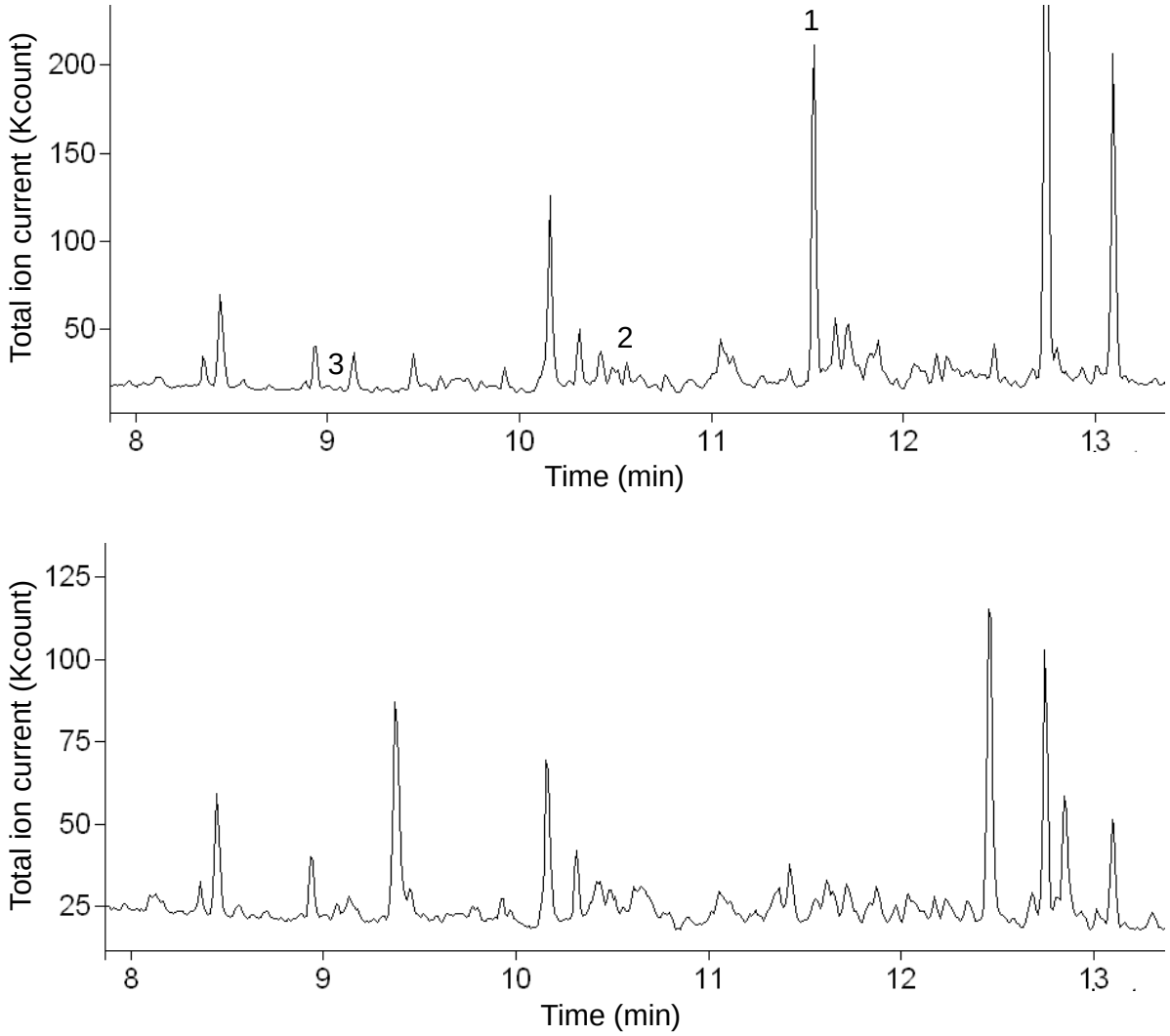

Figure 6. Coupled gas chromatography-mass spectrometry (GC-MS) analyses on polar GC column of volatiles from female *Haplodiplosis marginata* (upper panel) and volatiles from male *H. marginata* (lower panel). (1) 2-Nonylbutyrate, (2) 2-nonanol, (3) 2-heptyl butyrate.

2-Nonanol was detected in GC-MS analyses at approximately 2% of the 2-nonyl butyrate. Single ion scanning of the GC-MS analyses of volatiles from female *H. marginata* at *m/z* 71 and 89, characteristic of butyrate esters, showed the presence of 2-heptyl butyrate at approximately 1% of the 2-nonyl butyrate. 2-Undecyl butyrate, an analogue reported to be present by Censier *et al.* (2014b), could not be detected (<0.1% of major component). Similarly, 2,7-dibutyroxynonane, the female sex pheromone of the closely related orange wheat blossom midge, *S. mosellana* (Gries *et al.*, 2000), could not be detected by comparison with the authentic synthetic compound. Other potential minor pheromone components related to 2-nonyl butyrate, such as 2-nonanone and 2-

nonyl acetate, could not be detected (Table 1). In GC-EAG analyses of the synthetic compounds (10 ng injected), strong EAG responses were observed to 2-nonyl butyrate and 2-heptyl butyrate, but there was no detectable response to 2-nonanol (data not shown). The retention indices of 2-heptyl butyrate were consistent with those of the component responsible for the minor EAG responses in analyses of volatiles from female midges on both non-polar and GC columns in the GC-EAG system used (Table 7). Analysis of the volatiles from female *H. marginata* on the enantioselective cyclodextrin GC column indicated a peak at the retention time of (*R*)-2-nonyl butyrate (15.69 min), but no peak (<5%) at the retention time of the (*S*)-enantiomer (15.30 min).

Table 7. Retention indices relative to retention times of *n*-alkanes of electroantennography (EAG) responses in gas chromatography (GC)-EAG analyses of volatiles from virgin female *Haplodiplosis marginata* with male *H. marginata* EAG preparation, and of synthetic compounds

	Non-polar		Polar	
	GC-EAG (SPB1)[1]	GC-MS (VF5)[1]	GC-EAG (DBWax)[1]	GC-MS (DBWax)[1]
EAG major	1389		1601	
EAG minor	1235		1415	
2-Nonyl butyrate	1389	1403	1601	1591
2-Nonanol	1082	1104	1528	1513
2-Nonyl acetate	1218	1234	1460	1456
2-Heptyl butyrate	1201	1215	1400	1392
2,7-Dibutyroxy-nonane	1846	1861	2282	2245
2-Nonanone	1075	1092	1376	1378

1 GC column phase

4.2.2 Pheromone dispensers

Polyethylene vials were found to release 2-nonyl butyrate more uniformly than the rubber septa under laboratory conditions (Figure 7). The rubber septa released over 90% of the pheromone within the 1st week at 27 °C and 2.2 m s^{-1} wind speed. In contrast, 30% of the compound remained after 28 days in the polyethylene vials. Polyethylene vials containing an initial loading of 1 mg racemic 2-nonyl butyrate and returned from field tests after 2 weeks contained (mean ± SEM =) 0.72 ± 0.02 mg (n = 3). Polyethylene vials and rubber septa returned from the field after 6 weeks contained 0.41 ± 0.02 and 0.31 ± 0.02 mg, respectively.

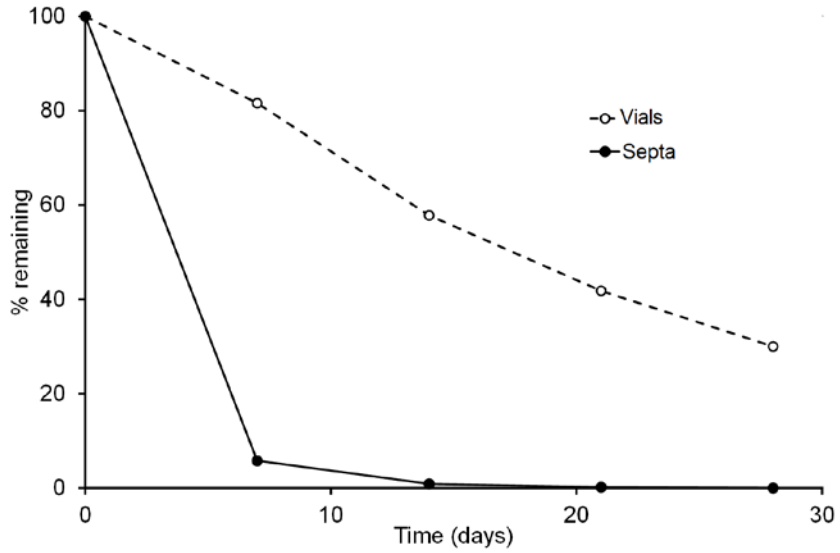

Figure 7. Release of 2-nonyl butyrate (1 mg) from rubber septa and polyethylene vials in laboratory wind tunnel at 27 °C and 2.2 m s^{-1} wind speed as measured by gas chromatography analyses of the amount remaining at intervals.

4.2.3 Field trapping experiments

Experiment 1 – pheromone dispensers

Traps baited with 1 mg racemic 2-nonyl butyrate dispensed from either rubber septa or polyethylene vials caught more male *H. marginata* than the unbaited traps at site 2 in winter wheat ($F_{2,9}$ = 21.33, P<0.001) during the 1st week of trapping. However, there was no difference in catches with the two dispenser types (Figure 8A). Catches during the next 2 weeks were too low for analysis but showed the same trend with mean catches per trap over the period of 4.3 ± 1.9 with vials, 5.3 ± 1.4 with septa, and no catches in unbaited traps.

Experiment 2 – pheromone chirality

Traps baited with (*R*)-2-nonyl butyrate caught significantly more male *H. marginata* compared to the other treatments ($F_{3,9}$ = 22.56, P<0.001). During the 14-day trapping period no adults were caught on the unbaited traps or the traps baited with (*S*)-2-nonyl butyrate, and the catch with racemic 2-nonyl butyrate was less than 5% of that with (*R*)-2-nonyl butyrate (Figure 8B).

Experiment 3 – effect of minor components

A total of 26 658 male *H. marginata* was caught during the 11-day trapping period. Traps baited with racemic 2-nonyl butyrate caught significantly more than unbaited traps but less than 10% of the number caught in traps baited with (*R*)-2-nonyl butyrate ($F_{5,45}$ =253.66, P<0.001; Figure 8C). Addition of the minor components, (*R*)-2-nonanol and/or (*R*)-2-heptyl butyrate, did not increase or decrease trap catches compared with catches with the major component, (*R*)-2-nonyl butyrate, alone. There was no interaction between treatment and block but the effect of block was significant

($F_{9,45}$ = 6.799, P<0.01).

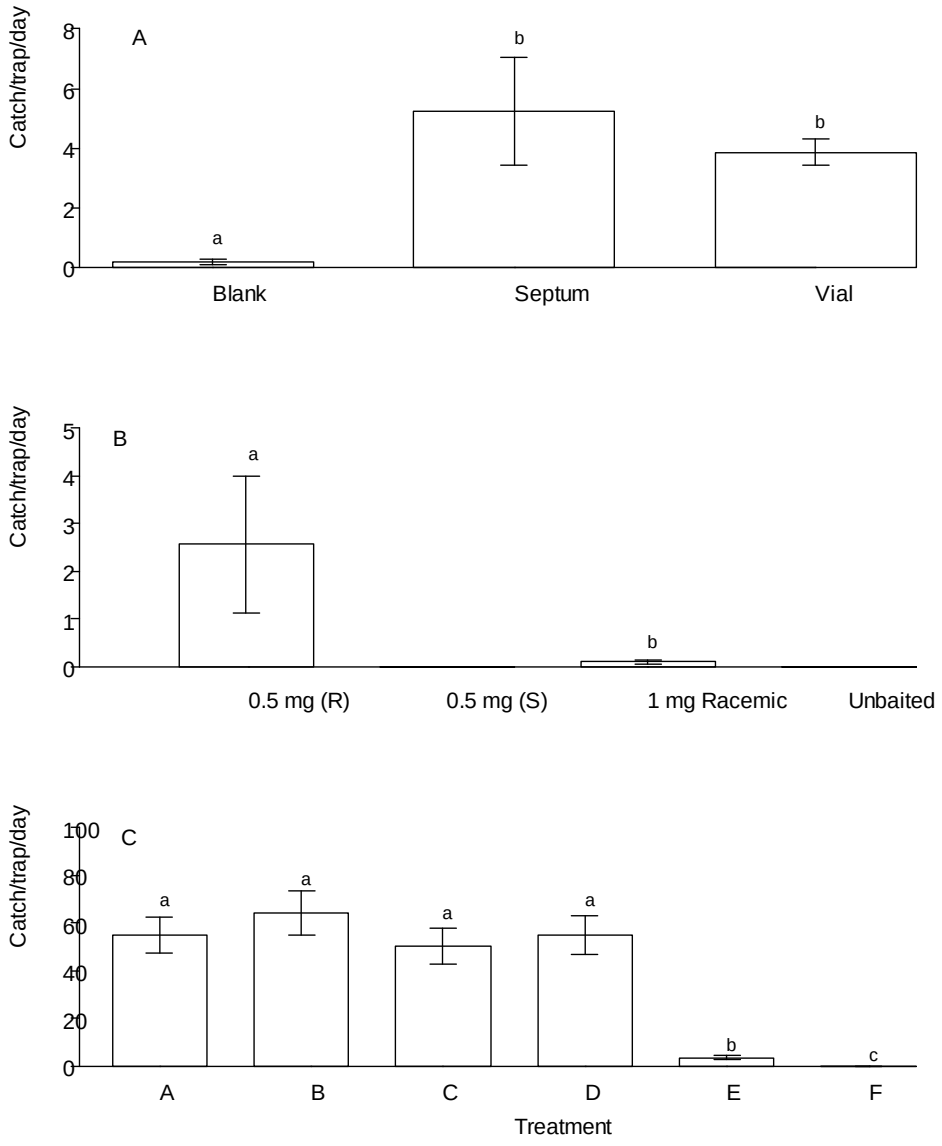

Figure 8. Mean (± SEM) daily catches of male *Haplodiplosis marginata* in traps baited with (A) racemic 2-nonyl butyrate (1 mg) dispensed from polyethylene vials or rubber septa (experiment 1, 15-21 May 2014); (B) racemic 2-nonyl butyrate (1 mg), (*R*)-2-nonyl butyrate (0.5 mg), (*S*)-2-nonyl butyrate (0.5 mg), and unbaited (experiment 2, 5-19 June 2014); and (C) a range of treatments (experiment 3, 18-29 May 2015): A, 0.5 mg (*R*)-2-nonyl butyrate; B, 0.5 mg (*R*)-2-nonyl butyrate + 2% (*R*)-2-nonanol; C, 0.5 mg (*R*)-2 nonyl butyrate + 2% (*R*)-2 heptyl-butyrate; D, 0.5 mg (*R*)-2-nonyl butyrate + 2% (*R*)-2-nonanol + 2% (*R*)-2 heptyl-butyrate; E, 1 mg racemic 2-nonyl butyrate; F, unbaited control. Bars show back-transformed means. Means within a panel capped with different letters are significantly different [LSD tests: P<0.001 (panels A and B), P<0.05 (panel C)].

Experiment 4 – pheromone loading

A total of 13 775 male *H. marginata* was caught during the 9-day trapping period. Significant differences in numbers caught were observed between all treatments ($F_{4,36}$ = 187.42, P<0.001) and trap catches were dose-dependent with more male *H. marginata* caught when higher pheromone loadings were used (Figure 9A). Log mean catch plotted against log pheromone loading indicated a linear association (Figure 9B).

Experiment 5 – comparison with other traps

Substantially more male *H. marginata* were caught in the pheromone trap compared with both the unbaited sticky and water traps. During the trapping period of 18 days with six replicates, over 6 500 *H. marginata* were caught using the pheromone traps compared with 26 and 27 in the sticky and water traps, respectively.

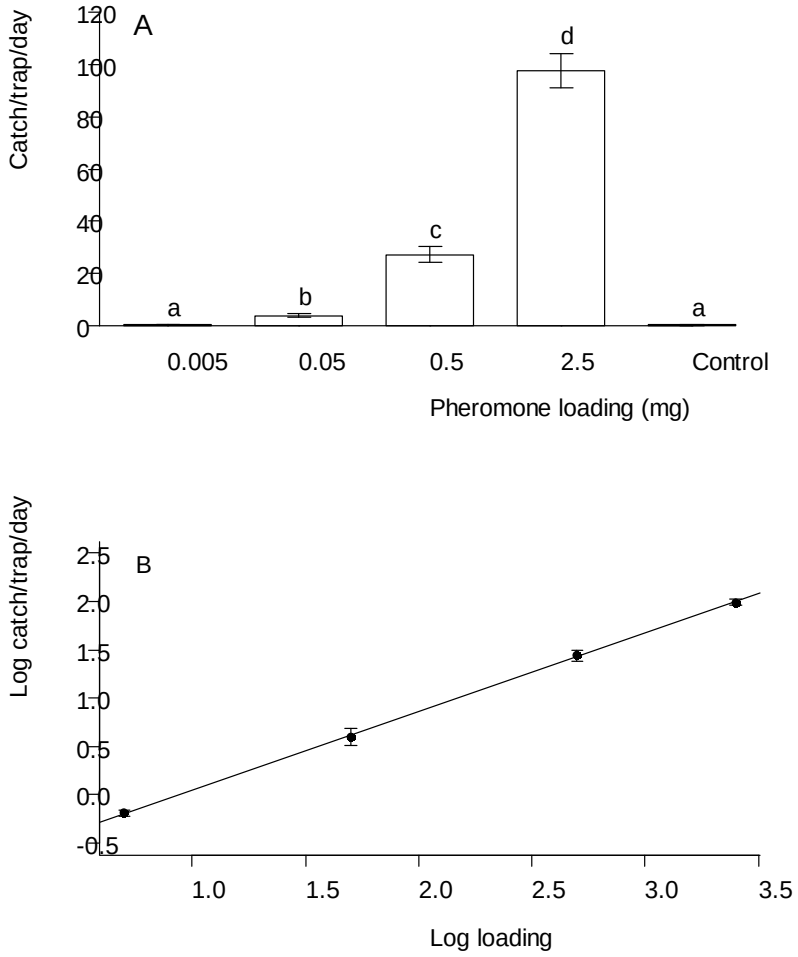

Figure 9. (A) Mean (± SEM) catches of male *Haplodiplosis marginata* in experiment 4 with different lure loadings of (*R*)-2-nonyl butyrate and unbaited control (2–11 June 2015; bars show back-transformed means). Means capped with different letters are significantly different (LSD test: P<0.05). (B) Log mean daily catch per trap of *H. marginata* against log pheromone loading in experiment 4.

4.3 Objective 3 – Pheromone trap development

4.3.1 Field experiments

Lure Longevity

For the duration of experimental period, fewer insects were caught with traps baited with the same lure than those with lures that were refreshed regularly ($F_{1,101}$=25.25, P<0.001) but this effect did not change significantly over time (Fig. 10). Clear differences were observed between the numbers of insects caught at each field site ($F_{1,6}$=88.04, P<0.001) and fewer insects were caught as the experiment progressed ($F_{1,101}$=194.65, P<0.001) (Fig.1).

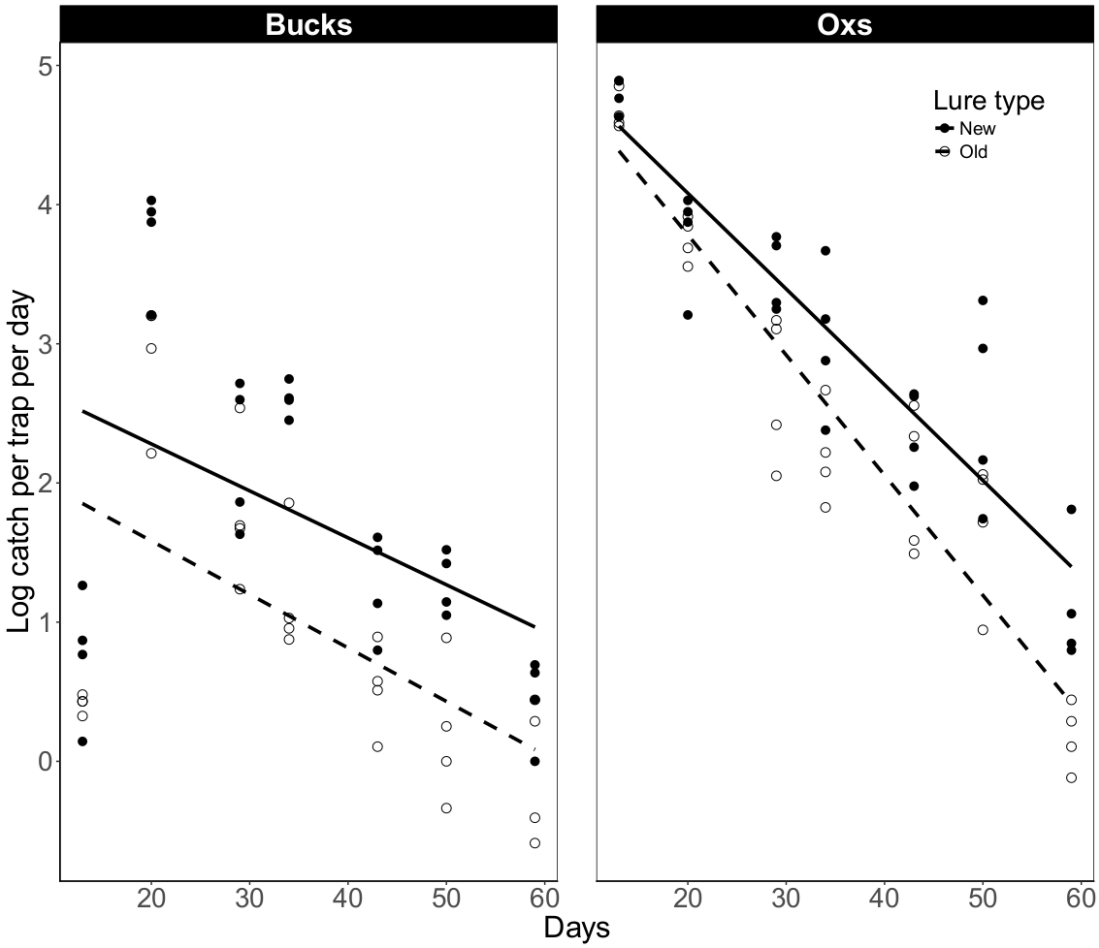

Figure 10. Catches of *Haplodiplosis marginata* males in traps baited with lures maintained continuously (old) or renewed at approximately weekly intervals (new) at two sites (3 May – 16 July 2016; *N* = 4 at each site; dots show log counts, lines show regressions)

The number of insects caught in traps with old lures expressed as a percentage of the catch in traps baited with new lures did not significantly decrease over time ($F_{1,5}$=4.536, P = 0.086) although there was clearly a negative trend (Fig. 11). In the old lures (*N* = 4), 39.4% ± 0.7 of the pheromone from site 2 (Bucks) and 36.1% ± 1.4 of the pheromone at site 3 (Oxon) remained after the 59-day trapping

period. Mean air temperatures during this time were 13.43 ± 0.10°C and 13.36 ± 0.11°C at sites 2 and 3 respectively. Maximum air temperature did not exceed 25°C at either site.

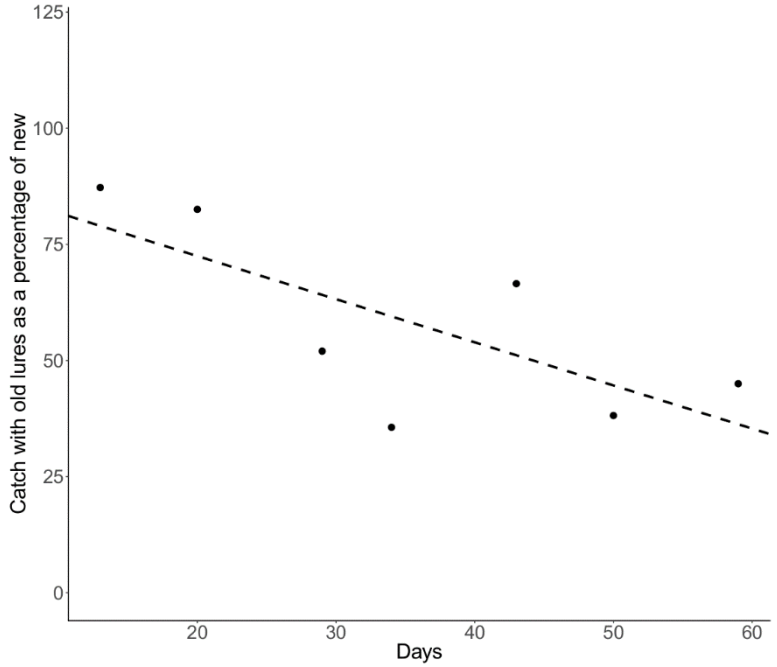

Figure 11. Total catches of *Haplodiplosis marginata* males in traps baited with lures maintained continuously expressed as a percentage of catches in traps baited with lures renewed for each trapping period at two sites (3 May – 16 July 2016; *N* = 4 at both sites).

Trap Height

Numbers of insects caught was very similar in each field: 49% of the total trapped were caught in field 1 and 51% in field 2. The lowest numbers of insects were caught at 80 cm and 120 cm; catches at 0 cm and 40 cm accounted for 98.3% of the total 3,100 trapped. Catch numbers were different a different heights ($F_{3,30}$ =110.33, P<0.001), and catch rates at 0 cm and 40 cm differed between fields ($F_{9,24}$ =5.78, P<0.001) (Fig. 3). This was accounted for by trap height in relation to crop height. Post hoc tests revealed that field 1 in spring wheat (crop height of approximately 10 cm) had far higher numbers of insects trapped at 0 cm than 40 cm (P<0.001). Field 2 in spring wheat (crop height of approximately 40 cm) had no difference in catches at 0 cm and 40 cm and had a higher number of insects caught at 40 cm compared to field 1 (P<0.001). There were therefore higher catches in traps positioned below crop height and lower catches in traps positioned above crop height (Fig. 12).

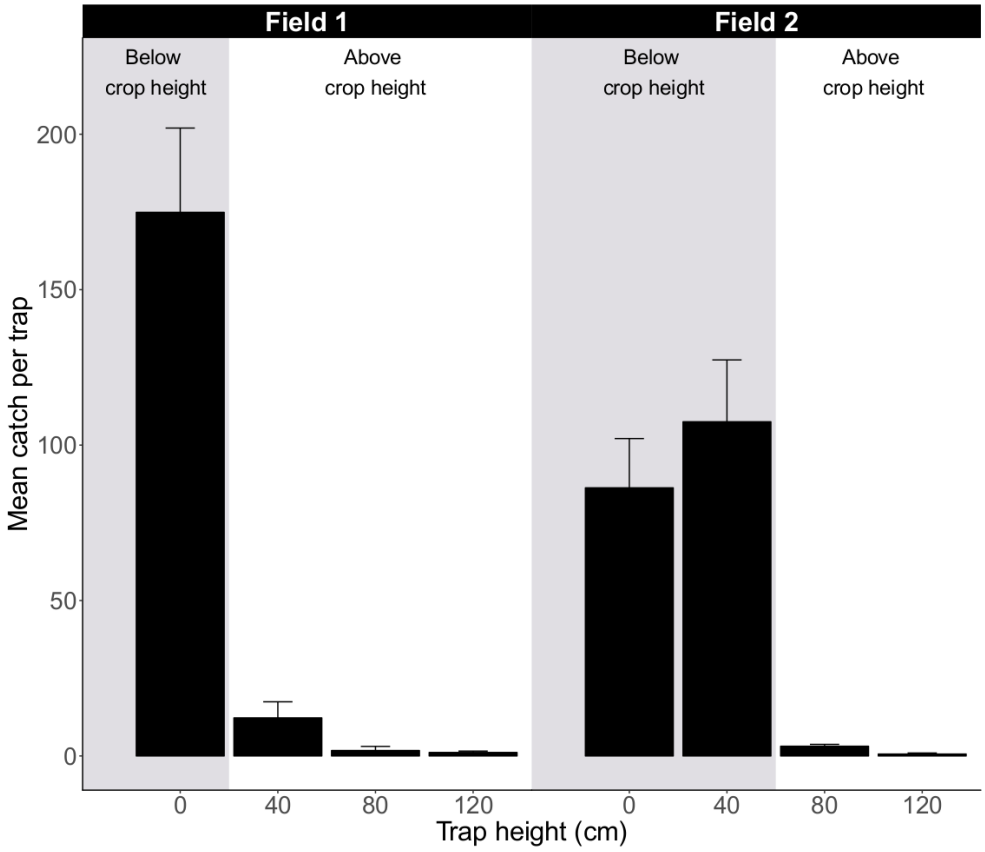

Figure 12. Mean catches (+SEM) of *Haplodiplosis marginata* males in traps positioned at different heights in fields of spring wheat (Field 1) and winter wheat (Field 2) at the Oxford field site (13-19 May 2016; *N* = 4 at each site and height; shaded areas represent traps at or below the height of the crop).

Distance from field margins

Catch rate was not affected by transect direction in relation to prevailing wind direction ($F_{2,9}$ = 0.29, P=0.75), but was affected by the distance of the trap from the field margin ($F_{2,22}$ =8.19, P<0.01) (Fig. 13). Post hoc testing revealed lower catches in traps in the field margin compared to those positioned 20 m (P<0.05) and 40 m into the crop (P<0.001). There was no difference in catch between the traps placed 20 and 40 m into the crop (P=0.54).

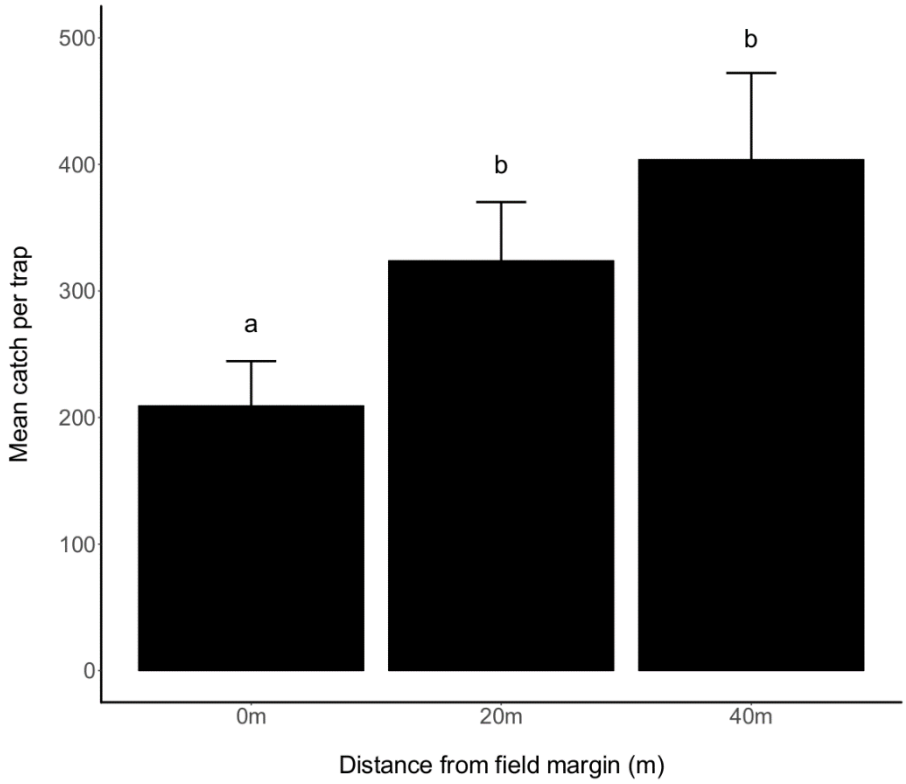

Figure 13. Mean catches (+SEM) of *Haplodiplosis marginata* males in traps positioned at increasing distance from the field margin (19 May – 1 June 2016; three sites, N = 4 at each site). Lowercase letters indicate significant differences between distances.

Range of interference

Number of insects caught per day in outer traps was higher than the number caught in inner traps ($F_{1,49}$=22.58, P<0.001) and was higher overall in arrays with a greater inter-trap distance ($F_{1,6}$=49.21, P<0.001). Differences between the catch rate of outer and inner traps reduced with increasing inter-trap distance ($F_{1,49}$=12.93, P<0.001) (Fig. 14).

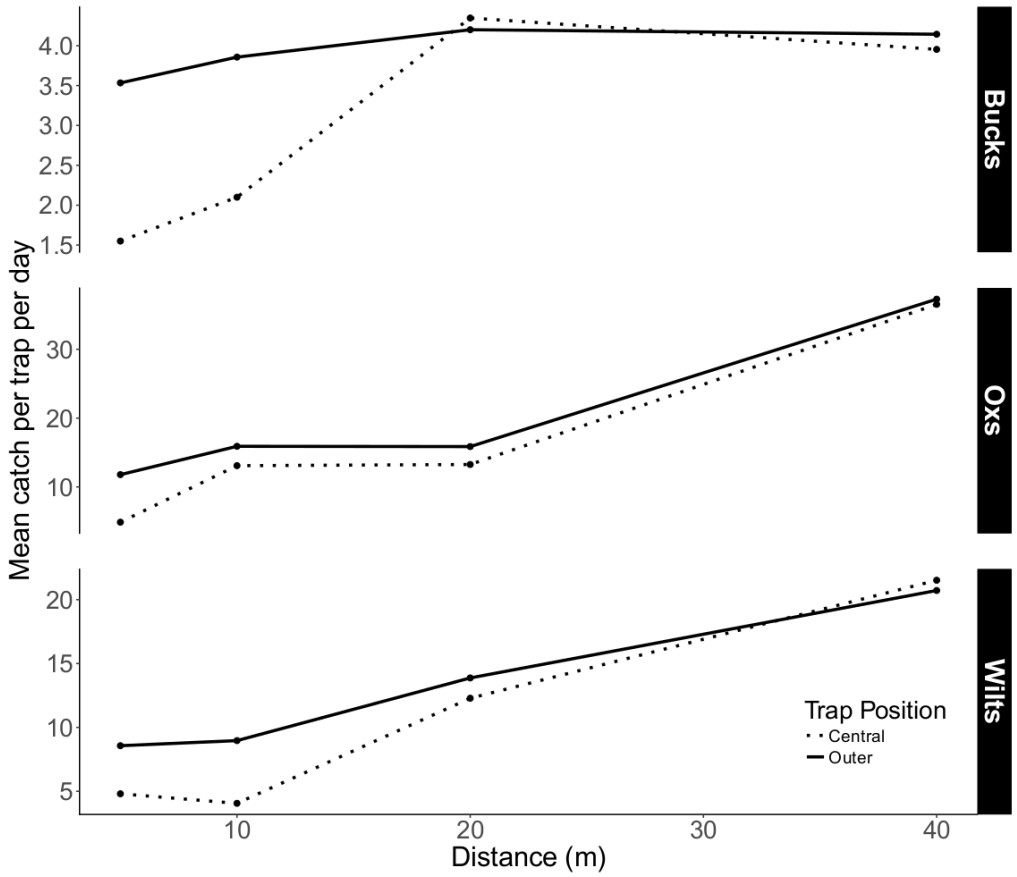

Figure 14. Mean numbers of *Haplodiplosis marginata* males caught in central and outer traps in hexagonal arrays of different inter-trap distances at three sites (1-22 June 2016; *N* = 9).

4.3 Objective 4 - Identification of natural enemies through gut analysis

4.3.1 Primer design and PCR assay development

The selected primer pair amplified a fragment of 348bp and had an optimum annealing temperature of 65°C which was used for all subsequent reactions. The sequences of the selected primers were as follows: F-COI-12 5'-GAGCACCAGATATAGCATTTCC and R-COI-360 5'-CCAGCCAATACTGGTAAAGAAAG. No cross-reactivity of the primers was observed with any of the non-target species tested, which included the Cecidomyiid *S. mosellana*. The new primers were able to detect pure *H. marginata* DNA at concentrations as low as 0.001 ng μL^{-1}.

4.3.2 Rate of digestion of *H. marginata* DNA in predator guts

There was a significant effect of digestion time on the probability of detecting *H. marginata* DNA from the guts of *N. brevicollis* ($F_{1,5}$=16.297, P<0.01). Detectability half-life of *H. marginata* DNA under these conditions was determined to be 31.07h (Figure 15). The assay achieved 100% positive results in individuals killed immediately after feeding, while the unfed beetles did not produce any positive results. The greatest decline in probability of detection occurred between 12h and 24h post-feeding.

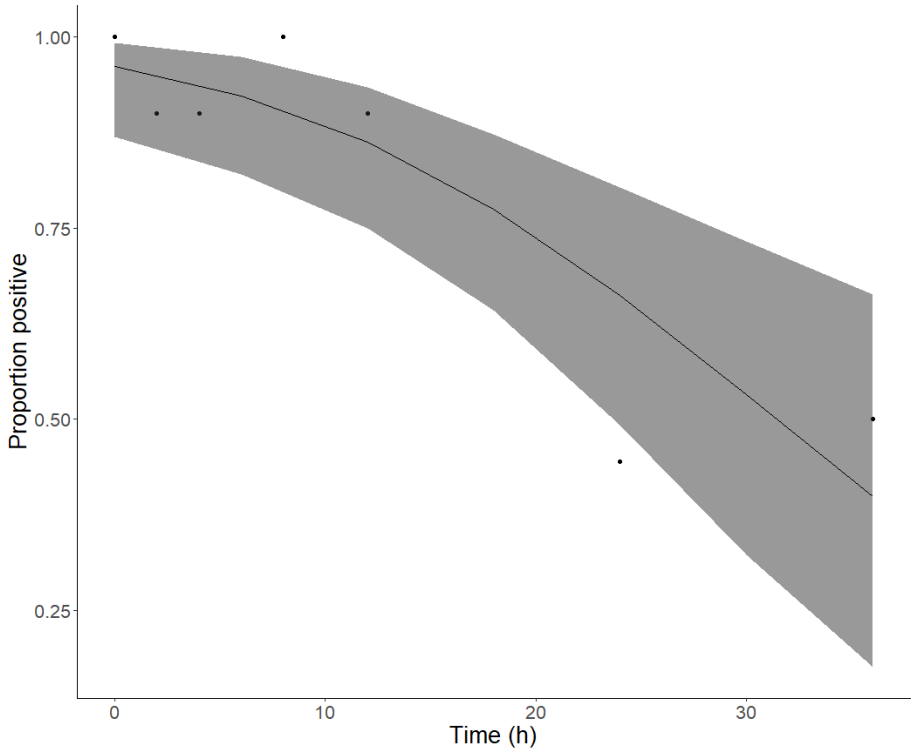

Figure 15. Proportion of positive assays for *Haplodiplosis marginata* DNA in the guts of *Nebria brevicollis* at time post-consumption of a single prey larvae. Fitted line represents probit model with 95% CI.

4.3.3 Field survey

In total 110 individual carabid specimens of 11 different species were trapped. The majority (47%) of beetles were caught in the central traps of the arrays. Positive results for the presence of *H. marginata* DNA were found in 7.2% of specimens and were obtained from 4 different species (Table 8). Beetles trapped late in the season (July) represented only 15% of all specimens tested, but had a much higher rate of positive results (23.5%) compared to beetles trapped in May (4.3%). This is despite the activity density of the beetles being almost identical in May and July (0.84 and 0.85 beetles per trap per day respectively).

Table 8. Number of individuals of each carabid species tested for the presence of *H. marginata* DNA during the field survey in Buckinghamshire, UK, and expressed as a percentage of the total carabids tested (in brackets). Number of individual assays testing positive for the presence of *H. marginata* for each carabid species tested and the percentage positive for that species (in brackets).

Species	**Number tested (*% of total carabids*)**	**Number positive (*% for species*)**
Poecilus versicolor	45 (*40.9*)	2 (*4.44*)
Poecilus cupreus	9 (*8.18*)	0 (*0*)

Nebria brevicollis	15 (*13.64*)	3 (*20*)
Pterostichus melanarius	6 (*5.45*)	0 (*0*)
Anchomenus dorsalis	1 (*0.91*)	0 (*0*)
Bembidion deletum	2 (*1.82*)	0 (*0*)
Bembidion tetracolum	1 (*0.91*)	0 (*0*)
Harpalus rufipes	19 (*17.27*)	2 (*10.53*)
Harpalus affinis	9 (*8.18*)	0 (*0*)
Abax parallelepipedus	1 (*0.91*)	0 (*0*)
Loricera pilicornis	2 (*1.82*)	1 (*50*)
Total	110 (*100*)	8 (*7.27*)

5 Discussion

5.1 Objective 1 – Phenological forecasting

Data on insect species development can be used to establish degree day models from which phenological events such as yearly adult emergence can be predicted. The life cycle of *Haplodiplosis marginata* has been relatively well described (Skuhravý *et al.*, 1983; Censier *et al.*, 2015), however there is little known about the developmental biology of this insect. Existing research into developmental thresholds only tested a limited number of temperatures meaning the posited lower developmental threshold of 10 °C is potentially inaccurate (Baier, 1963; Nijveldt & Hulshoff, 1968). This is demonstrated by observations of *H. marginata* pupation in soil temperatures below 10 °C in the UK (Pope & Ellis, 2012). Furthermore, laboratory studies so far completed have not fully recreate field conditions for *H. marginata* due to the variation that exists within soils, and potential differences in developmental time at constant and fluctuating temperatures (Hagstrum & Milliken, 1991). In addition to thermal thresholds for development, other environmental factors are important in the seasonal ecology of insects which are relevant when attempting to predict emergence (Tauber & Tauber 1976; Tauber *et al.*, 1998, Leather *et al.*, 1993; Koštál, 2006).

In the initial degree day model of emergence, the predictive ability was improved by incorporating rainfall into the selection of a date of biofix. In the model to predict peaks in emergence, the onset of a precipitation period followed by the accumulation of 512DD above 0 °C predicted an increase in *H. marginata* emergence to within 3 days of the midpoint of the observed peaks. In both cases, models incorporating rainfall were more accurate than those using soil temperature alone. The models proposed here, and that proposed by Jacquemin *et al.*, (2014), agree with theories of insect development which state that post-diapause, insects can remain in a state of 'readiness' until an environmental cue triggers the onset of pupation (Tauber & Tauber, 1976; Hodek, 1996; Koštál, 2006). The role of moisture in insect emergence has been studied in other insect species including

S. mosellana (Oakley *et al.*, 1998; Elliot *et al.*, 2009; Jacquemin *et al.*, 2014). It has been suggested that increased soil moisture might improve mobility (Menu, 1993; Ellis *et al.*, 2004) or act a behavioural stimulus (Tauber et al, 1994). More research needs to be done to understand the role moisture plays specifically in the development of *H. marginata* such as whether it is important for diapause termination or post-diapause development (Leather *et al.*, 1993). Soil textural analysis of the sites used in this experiment shows that the fields studied are either clays or dominant in clay particles. Organic matter comprised at least 6% of the soil at the study sites. The moisture retention capabilities and open structure associated with these soils (Davies *et al.*, 2001) may result in greater pupation success for this species, however this would require more extensive analysis of mortality rates in the soil. Nonetheless, the information shown here can be used to successfully predict the initiation of a degree day accumulation period, following which adult *H. marginata* will emerge. This is significant as for the first time, farmers could be provided with a window of time during which to inspect crops or deploy traps.

The two DD-based cumulative emergence models proposed here for *H. marginata* are comparable in terms of their reliability as determined by the r-squared and RSME values. The bimodal model however has a slightly better predictive power as shown by the standard deviation of the observed and predicted 10% cumulative emergence from previous years. The value for 10% emergence was deemed to be an appropriate proxy for the start of emergence given the error involved in trapping insects at very low densities; it is unlikely that the earliest onset of emergence will have been recorded particularly in 2014 when pheromone traps were not available. The probit model predicted that 10% emergence occurs at 550 DD post-biofix while the bimodal model estimated it to occur at 576DD. Both estimates fall well within the observed range of 538 – 621 DD. The bimodal model predicts a higher initial rate of emergence, which appears to fit the observed pattern of large initial peaks and smaller subsequent peaks of emergence. Over all sites and years, the probit model predicted the onset of emergence to within 4 days (± 4 days) which is on a par with the preliminary degree day-based model. The bimodal model improved on this by predicting emergence to within 3 days (± 4 days). The advantage of the new models is the ability to predict cumulative percentage emergence over the entire flight season, rather than just the start date. This will enable the midpoint and conclusion of flight periods to be estimated and aid in the assessment of the need for chemical controls or the effectiveness of insecticides applied earlier in the emergence period. The crop damage assessments presented here show that the effects of *H. marginata* damage are not always clear cut. Plant height, grain number and ear length were all compromised on damaged stems but total grain weight was not affected. The disparity between growth reduction and final grain weight may be due to a ‘pruning effect’ whereby the plant compensates for fewer grains through increased grain filling (Barnes, 1956). Ideally, numbers of insects emerging will be linked to potential crop damage to further aid the assessment of risk and inform pest management decisions. It is clear from the results here that more extensive, long term research is required before this becomes feasible.

Similar degree-day based models of varying complexity have long been used to monitor other insect pests (e.g. Riedl *et al.*, 1976) and a database of over 500 insect developmental requirements has been created to support the creation of further phenology models (Nietschke *et al.*, 2007). Advances in automated data collection and software programming have made it possible to set up national networks that incorporate models for multiple pests and provide monitoring alerts based on local conditions. Linking phenology models to geographical information systems (GIS) can aid the generation of regionally based risk assessments (Fand *et al.*, 2013). Multi-species networks are already in place such as the SOPRA system in Swiss orchards (Samietz, 2011), VIPS pest notifications in Norway (NIBIO, 2017); and NAPPFAST in North Carolina (Magarey *et al.*, 2007). Increased sophistication of basic degree-day phenology models could be achieved by simulating the effects of other relevant ecological or biological variables. For example, degree day accumulations also provide the basis for crop growth models (Miller *et al.*, 2001). This means that pest forecast models could be combined with growth models of the host crop to give a more detailed estimate of crop risk. For example, the CIPRA software based in Quebec combines models of insect pests, disease and crop phenology to provide real-time forecasting based on meteorological data (Bourgeois, 2005). In the case of *H. marginata*, cereal crops are most vulnerable during stem extension (Golightly & Woodville, 1974) therefore a forecast of adult emergence during this period would pose a greater risk to the crop than emergence occurring after the crop has booted. Combining forecasting models with decision support systems means that suitable control options can be presented to users based on their specific circumstances.

The observed waves of emergence of *H. marginata* mean that predictive models such as this are more important given that activity will need to be monitored throughout the flight season. Peaks in emergence could be an indication to survey the crop for eggs. No data yet has been collected on the extent of egg laying throughout the flight season, and even if the crop is no longer at a vulnerable life stage continued checking of eggs could give forewarning of future outbreak populations. Over a longer period, forecasts could also be used to reveal trends in the phenology of *H. marginata* relating to changes in climate which may not be evident from year to year. For example, milder overwintering conditions reduces fecundity and survival in the goldenrod gall fly (Irwin & Lee, 2000). Changes in climate are predicted to impact upon the phenology of insect pests in the future which may have implications for the severity of outbreaks (Cannon, 1998).

5.2 Objectives 2 & 3 – Pheromone lure optimisation and trap development

The use of pheromone traps in IPM has greatly improved the ease and reliability of insect monitoring (Witzgall *et al.*, 2010). In 2014, Censier *et al.* identified (*R*)-2-nonyl butyrate as the female sex pheromone of *H. marginata*. The work presented here confirms response of male *H. marginata* to this compound through EAG. Results from field experiments indicate that a polyethylene vial loaded with 0.5 mg of (*R*)-2-nonyl butyrate is a suitable lure for trapping adult *H. marginata* in the field, and would be equivalent in attractiveness to a lure containing 10 mg of the racemic compound. This

system will greatly improve detection in areas of low *H. marginata* populations, and will provide a greater degree of accuracy when monitoring for the start of adult activity. The optimum lure formulation maximises trap catch with a minimal amount of compound required. This is an important consideration in the development of a commercial lure. An interesting finding in this research was the inhibitory effect of the (*S*)-enatiomer of 2-nonyl buryrate, which is a chiral molecule. This is not a common finding in gall midges and has only been observed once before (Hillbur, 1999). It has been observed in other organisms however, and is thought to have evolved to further increase the specificity of pheromone communication (Mori, 2007). It highlights the need to test for antagonism in racemic mixtures of chiral compounds and may help to inform further research into the chemical ecology of other gall midge species.

Pheromone lures were still attracting male *H. marginata* adults after nearly nine weeks in the field which is comparable to data for commercially available lures for other pest species (Mcnally & Barnes, 1980; Vanaclocha et al, 2016) and longer than the recommended usage time of six weeks for *S. mosellana* lures (Bruce *et al.*, 2007; Bruce & Smart, 2009). Lures replaced each week consistently caught more midges than lures maintained continuously, even at the beginning of the experiment. The position of the trap in relation to the crop and other traps was also important in optimising the catch rate, as has been shown with other pheromone research (Kong *et al.*, 2014; Rhainds *et al.*, 2016). The height of the crop significantly affected trap catch. Based on these findings, it would be most practical for farmers to position pheromone traps at the height of the ear, as is recommended for pheromone traps of *S. mosellana* (AHDB, 2016). Catch rates of *H. marginata* declined when pheromone traps were situated in field margins but there were no differences in catch rates in traps positioned 20 m and 40 m into the crop. In practice, given that traps placed 40 m into the crop would increase maintenance time with no appreciable gain in catch rate, a position 20 m into the crop should be sufficient in most cases. In trap interference experiments, central traps caught fewer insects than the outer traps and this difference declined with increasing inter-trap distance. This indicates the occurrence of plume interactions, where the overlapping plumes from upwind lures divert the insect away from the central trap (Wall & Perry, 1978, 1980, 1987). On this basis, trap interference occurs at inter-trap distances below 20 m and that this should be considered the minimum trap spacing to avoid plumes from overlapping. The research presented here provides a basis for an effective pheromone lure and guidelines for best practice in the use of the trap.

Further innovations could concentrate on trap design to further increase catch rate (Edde *et al.*, 2005; Diaz-Gomez, 2010) or provide automated solutions for checking the traps. For example, 'Z-Traps' have been developed which count insects as they enter and upload the data instantly (Spensa Technologies, 2017). Another automated system has been developed known as Trapview which uploads pictures of trap captures at regular intervals. The software then uses auto-recognition to identify certain insect pests from the pictures, provide threshold-based alerts and has the capability to incorporate weather data into a database of insect activity (EFOS, 2017). The large amounts of data collected from automated pest monitoring systems could then be used to further

improve integrated pest management programmes (Okuyama *et al.*, 2011). The sex pheromones of other pests such as codling moth, *Cydia pomonella*, have successfully been exploited for the purposes of mass trapping in IPM programmes (El-Sayed *et al.*, 2006; Witzgall *et al.*, 2010). For mass trapping or pheromone disruption strategies involving *H. marginata*, at least 25 traps would need to be deployed per hectare to ensure coverage of the area at the current pheromone concentration. However, far higher catches can be obtained by increasing the pheromone loading to 2.5 mg or more (Rowley *et al.*, 2016). Further research would be required to determine the minimum distance between traps at a higher pheromone loading but it is likely to be large enough to offset the increased pheromone production costs in order to get complete coverage over an area.

The development of this simple monitoring tool means that the presence of *H. marginata* can be recognised with greater ease and accuracy than previously. The phenological forecasting system described previously could be used to identify high risk crops, and then pheromone traps could be deployed to act and an early warning system for the presence of this pest. If found to be present, farmers could employ a break crop rotation to prevent numbers building up to outbreak levels in subsequent years. This may lead to a much clearer understanding of the distribution of this insect and may lead to reports of its presence in location where it was previously unknown.

5.3 Objective 4 - Identification of natural enemies through gut analysis

The suppressive effect of natural enemies on pest populations through predation and parasitism is an important aspect of many IPM programmes. Previously, there was very little information on the natural enemies of *H. marginata* and no predators had been identified to species level (Rowley *et al.*, 2016). The difficulties of observing predator-prey interactions in the field are exacerbated in species such as *H. marginata*. The larval and pupal stages are the least mobile and therefore the most vulnerable to predation however being primarily belowground they are also the most difficult to observe. Advances in molecular biology have allowed the gut contents of predators to be screened for the DNA of target pest species. The work presented here describes the design of species specific primers which were successfully applied to a PCR-based assay to detect *H. marginata* DNA in gut contents. These primers performed well at a high annealing temperature of 65 °C which reduces the chance of erroneous base matching at the primer sites (King, 2008), but was not the highest temperature at which an amplicon was obtained to ensure the sensitivity of the assay (Sint *et al.*, 2011). The specificity of the assay was supported by the lack of cross reactivity with DNA from non-target species commonly found on agricultural land including the Cecidomyiid *S. mosellana*.

The assay was able to reliably detect *H. marginata* DNA at concentrations of 0.001 ng μL^{-1} which is comparable to other insect primers used in gut analysis (e.g. Ekbom *et al.*, 2014). The effects of digestion or inhibitors present in the guts of the predator may further reduce assay sensitivity in some instances. Nonetheless, the ability of the assay to detect the DNA from a single *H. marginata* larva in starved predator guts was repeatedly demonstrated in the feeding assay giving

confidence in the reliability of the test. The feeding assay further demonstrated that the half-life of detection for this assay was 31h post-consumption, which is comparable to assays for other predator-prey interactions (e.g. Juen & Traugott, 2004, Waldner *et al.*, 2013) and is well within the range so far reported for other carabids of 18 – 88.5h (Monzó *et al.*, 2011). A long detectability half-life is vital if the assay is to be used on field-caught specimens particularly when predators are mainly nocturnal, as with many carabids (Kromp, 1999). The results suggest the assay was more than adequate for the field survey described here where traps were in place for no more than 18h. From this survey, four Carabid species were identified as predators of *H. marginata* in the field for the first time. All of the species which tested positive are relatively common, highly generalist feeders of medium to large size (above 5 mm long). The proportion of positive assays was higher in July, despite the activity density being comparable between early and late season sampling. Drier soil in the late season may have prevented *H. marginata* from burrowing into the soil, or enabled carabids easier access to larvae belowground via the formation of fissures. This information is important in shaping IPM programmes for *H. marginata*. Several studies have shown the negative effects of insecticides, including the pyrethroids which are used in *H. marginata* control, on carabids (van Toor, 2006). This is an example of how current practices could be disrupting predator-induced suppression and contributing to the accumulation of pest populations (Matson *et al.*, 1997).

There are an estimated 50-250 natural enemy species for each agricultural pest (van Lenteren, 2000). Almost certainly there are more predators of *H. marginata* yet to be identified, for example spiders and carabid larvae which were not surveyed in this project. Additionally, faeces from vertebrates could be screened in a PCR-based assay as the bright orange larvae of *H. marginata* might well be a target for larger organisms when they move to the surface to pupate. There is also the opportunity to extend screening beyond predators. It may be possible to identify parasitoids of *H. marginata* using these primers (Rougerie *et al.*, 2011). Existing assays could be used to identify entomopathogenic nematodes and fungi that might be inducing lethal or sublethal effects in populations (Shapiro-Ilan, 2003). Even if the individual contribution to pest mortality of individual species is small, a diverse natural enemy complex could be sufficient to regulate *H. marginata* populations. Research suggests that natural enemy diversity has more of an impact on the pest status of concealed, endopterygote insects such as *H. marginata* (Wilby & Thomas, 2002).

The primers described here could be incorporated into a multiplex PCR reaction to screen for multiple pest species simultaneously which would give a clearer picture of the most important predators in a particular agri-ecosystem (King *et al.* 2010). This is essential if agricultural practices are to be modified to encourage certain species, as techniques that may be of benefit to one species may be detrimental to another. Additionally, augmentation of species may lead to increased levels of intraguild predation which may disrupt predation on the target species (Rosenheim *et al.*, 1995; Finke & Denno, 2005) although this effect may be lessened in more complex habitats (Finke & Denno, 2003).

5.4 Publications from this project

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