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# PCR-based gut content analysis to identify arthropod predators of *Haplodiplosis marginata*

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**Running Title** *PCR-based H. marginata gut content analysis*

**Abstract**

Saddle gall midge (*Haplodiplosis marginata*) is a cereal pest exhibiting sporadic outbreaks for which chemical control options are limited. Integrated Pest Management programs may offer a means of suppressing *H. marginata* outbreaks, reducing pesticide input. Many IPM programs benefit from the natural population suppression inflicted through predation and parasitism. The larval stage of *H. marginata* overwinters in the soil and may be preyed upon by ground-dwelling arthropods, however the natural enemies of *H. marginata* remain unrecognized. A PCR-based assay for detecting *H. marginata* in the guts of predators was designed using novel species-specific primers. Feeding trials involving *H. marginata* larvae showed a detectability half-life of 31.07 hours post-feeding in *Nebria brevicollis.* The guts of field-caught Carabidae were screened for *H. marginata* DNA. Four species: *Poecilus*

*versicolor, Nebria brevicollis, Harpalus rufipes* and *Loricera pilicornis* were identified as natural enemies of *H. marginata* for the first time. A higher proportion of positive results were obtained at the end of *H. marginata* emergence (July) compared to the beginning (May). The importance of understanding trophic interactions in the management of *H. marginata* is discussed in addition to the potential uses for the newly designed assay and primers.

## 1. Introduction

Saddle gall midge *Haplodiplosis marginata* (Diptera: Cecidomyiidae) (von Roser) is a pest of cereals that has been the focus of relatively little research in Europe due to the sporadic nature of outbreaks. Recent outbreaks in the United Kingdom and elsewhere have highlighted gaps in knowledge regarding the best options for its control and long-term management. Recent reviews have consolidated existing literature on the biology and ecology of this insect (Censier *et al.*, 2015; Rowley *et al.*, 2016). Briefly, *H. marginata* is a univoltine insect that overwinters in the larval stage. Adults emerge in late April through May and oviposit on the leaves of cereals and grasses (Censier *et al.*, 2015; Rowley *et al.* 2016). Newly hatched larvae then feed on the stem of the plant until maturity, forming saddle-shaped galls in the process (Golightly & Woodville, 1974). The larvae then drop from the plant in late July and burrow down into the soil to enter diapause, which can extend to more than one year when environmental conditions are not conducive for pupation to occur in spring (Nijveldt & Hulshoff, 1968). Gall formation on the stems of cereal plants can lead to inhibition of growth and yield loss, as well as increasing the risk of pathogen attack and stem breakage (Nijveldt & Hulshoff, 1968; Woodville, 1970; Golightly, 1979; Popov *et al.*, 1998). Application of pyrethroid insecticides, timed to coincide with adult emergence or the egg laying stage, can be effective against this pest (Ellis *et al.*, 2014). Later applications may be ineffective as larvae are protected from the insecticide by the leaf sheath whilst feeding

55 (Gratwick, 1992). It is widely accepted, however, that an over-reliance on chemical
56 pesticides is undesirable due to detrimental effects on human health and the environment
57 (Aktar *et al.*, 2009; Geiger *et al.*, 2010). In particular, pesticides such as pyrethroids can
58 have a negative impact on non-target organisms such as carabids (Holland & Luff, 2000; van
59 Toor, 2006). Integrated Pest Management (IPM) programmes aim to employ control
60 measures that minimise the impact on the wider environment (Kogan, 1998) and are
61 promoted by the EU Sustainable Use of Pesticides Directive as a means of minimising
62 chemical inputs in pest management (Directive 2009/128/EC). Such programs are based on
63 decision support systems that rely on knowledge of the biology and ecology of the target
64 organism, including interactions with other organisms in the crop environment (Kogan, 1998).
65 One strategy that may be adopted in IPM programs is to increase pest mortality from natural
66 enemies through conservation or augmentative biological control (Naranjo, 2001; Östman *et*
67 *al.*, 2003). Currently, the impact of predation on *H. marginata* population dynamics is poorly
68 understood and there is a clear lack of information on the natural enemies of this insect (see
69 below). Such knowledge would greatly benefit decision making in IPM programmes aimed at
70 this pest.
71
72 Predatory interactions involving invertebrates in the field can be difficult to study, often being
73 short-lived, inconspicuous, and unobservable without intervention (Stuart & Greenstone,
74 1990; Symondson, 2002). The problems are exacerbated with belowground interactions
75 (Juen & Traugott, 2004) which has led to a distinct lack of information on the arthropod
76 species that prey on primarily soil dwelling species such as *H. marginata*. An important
77 component of IPM programs is an understanding of the impact of natural enemies on pest
78 populations. In many cases, effective maintenance of natural enemy assemblages can help
79 to suppress pest populations (Symondson *et al.*, 2002; Wilby & Thomas, 2002; Cardinale *et*
80 *al.*, 2003). This is generally achieved by increasing numbers of existing predator populations
81 either artificially through introductions (augmentative biological control) or naturally through

beneficial environmental practices (conservation biological control). Generalist predators are potentially less effective against dipteran pests due to a large proportion of their life cycle being belowground or within the host plant (Symondson *et al.*, 2002). Nonetheless, the presence of natural enemies has been shown to impact dipteran pests such as brassica pod midge (Büchs & Nuss, 2000), onion maggot (Grafius & Warner, 1989) and cabbage root fly (Mowat & Martin, 1981).

Larvae of *Haplodiplosis marginata* are most vulnerable to predation in April and early May, when they move towards the soil surface to pupate, and in July and August, when mature larvae drop from the plant to the soil. Predation of the larvae of another Cecidomyiid, orange wheat blossom midge *Sitodiplosis mosellana* (Géhin), by Carabidae and Staphylinidae is thought to occur in the soil stage (Speyer & Waede, 1956), during pupation (Floate *et al.*, 1990) and on return to the soil to overwinter (Basedow, 1973; Holland & Thomas, 2000). Generalist arthropod predators active during these periods could therefore be exploited to enhance the suppressive effects of regular crop rotations as a means of reducing the frequency and severity of *H. marginata* outbreaks.

Current information on natural enemies of *H. marginata* or associated mortality at any life stage is limited. The parasitoids *Chrysocharis amyite* (Walker) and various *Platygaster spp*. are known to attack *H. marginata* larvae, but they have little impact on overall population size (Nijveldt & Hulshoff, 1968; Baier, 1963; Skuhravý, 1982; Rowley *et al.*, 2016). As with *S. mosellana*, Carabidae and Staphylinidae have been reported to prey on larvae of *H. marginata,* however, field observations are scarce and the exact species remain unidentified (Golightly & Woodville, 1974; Skuhravý *et al.*, 1993). Nothing is known about the species that prey on adults. A study in Canada identified 14 species of carabid preying on *S. mosellana* in the field (Floate *et al.*, 1990). This study utilized immunological markers to identify evidence of predation from gut content analysis. In the past two decades, PCR-

based molecular gut analysis has been developed as an alternative to immunological assays to identify predation through the detection of target organism DNA in the guts of predators (Chen et al, 2000; Symondson, 2002; Gariepy *et al.*, 2007). Given the relatively quick, cheap and easily reproducible nature of this technology it has become a widespread and reliable means of detecting trophic interactions in the field. PCR-based gut assays have been used extensively in agroecosystems to identify the natural enemies of pest species such as cereal aphids (Chen *et al.*, 2000), western corn rootworm (Lundgren *et al.*, 2009), cotton whitefly (Zhang *et al.*, 2007), slugs (Hatteland *et al.*, 2011), and pollen beetle (Öberg *et al.* 2011), including multiplex reactions with multiple target pest species (Harper *et al.*, 2005; King *et al.*, 2010). The method is highly suited to predator surveys such as this; where prey spend a large proportion of the time belowground, making observational studies impossible. Despite the potential for increased false negatives from soil contamination (Juen & Traugott, 2006) this technique has been used successfully to identify trophic interactions of belowground species in the field (Eitzinger *et al.*, 2013).

Here, we describe the development of species-specific primers for *H. marginata* for use in a PCR-based gut assay. A field survey of natural enemies of *H. marginata* in the UK using the assay identifies predators of this insect to species level for the first time. Knowledge of the key species that prey on the larval stage of this insect will help to inform decisions aimed at encouraging populations of beneficial insects as a means of aiding pest population suppression. This work may also lead to future applications of molecular techniques in further research efforts on this relatively understudied cereal pest.

## 2. Methods

### 2.1 *Insects*

135 *Haplodiplosis marginata* larvae were collected from fields in Oxfordshire (51°55"N, 1°10"W)
136 and Buckinghamshire (51°37"N, 0°48"W), UK, between April and June 2015. Larvae were
137 maintained in plastic containers of moist, sterilised compost at 4°C until use. Adult *Nebria*
138 *brevicollis* (Coleoptera: Carabidae) (Fabricius) beetles were collected in pitfall traps at Harper
139 Adams University, UK, in June 2015. Beetles were maintained in clear plastic containers at
140 20°C, 16:8 L:D, 60% RH and fed on *Tenebrio molitor* (Coleoptera: Tenebrionidae) (Linnaeus)
141 larvae prior to the feeding assay. Insect specimens used in cross-reactivity tests were
142 collected by hand (Harper Adams University), pitfall traps and pan traps (Oxfordshire) and
143 stored at -80°C prior to DNA extraction.
144
145 *2.2 DNA Extraction*
146 DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) in
147 accordance with the manufacturer's supplementary protocol for insect DNA extraction. Whole
148 insect specimens were washed in Tris-EDTA (TE) buffer prior to extraction, followed by
149 grinding with a sterile micro-pestle. Single whole *H. marginata* larvae and undissected
150 invertebrates were used for sequencing and assay cross-reactivity testing. For gut analyses,
151 the elytra of the beetles were removed and entire guts were dissected out, before being used
152 for DNA extraction. Following extraction, DNA was pelleted by centrifugation and
153 resuspended in 100 µL TE buffer before being stored at -20°C until use. One negative
154 control (no insect material) was included for every 20 extractions.
155
156 *2.3* PCR amplification and sequencing of *H. marginata* COI region
157 A 521bp fragment of *H. marginata* DNA from the mitochondrial cytochrome oxidase subunit I
158 (COI) gene was amplified using the universal insect primers C1-J-1718 and C1-N-2191
159 (Simon *et al.*, 1994; King *et al.*, 2010). Individual PCR reactions (25 µL) comprised of; 1X
160 PCR master mix (Invitrogen, Carlsbad, CA, USA), 0.625 U *Taq* polymerase (Invitrogen), 4
161 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).

### *2.4 Primer design and PCR assay development*

Primers specific to *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). Validation of the primer pairs consisted of specificity testing against *H. marginata* and 40 non-target organisms from orders Diptera, Coleoptera, Hymenoptera, Hemiptera, and Araneae (Table 1). PCR reactions proceeded as described in section 2.3. Following this the primer pairs showing no cross-reactivity were selected and the optimum PCR conditions examined by altering the annealing temperature across individual reactions (55 °C to 77 °C) observing for a strong single band. 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 $\mu L^{-1}$ to 0.0001 ng $\mu L^{-1}$, with 10 replicates of each dilution.

### *2.5 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 under controlled conditions using the carabid *N. brevicollis*. The half-life is the time at which *H. marginata* DNA can only be detected in 50% of the predators following feeding (Greenstone

189 & Hunt, 1993). Prior to feeding, *N. brevicollis* specimens were separated into individual clear
190 plastic containers (10 cm diameter x 6 cm height) with moist cotton wool and starved for 5
191 days to ensure guts were empty prior to the experiment. A single live larva of *H. marginata*
192 was placed into each container at time 0h and beetles were observed feeding. Beetles that
193 did not consume the larva within 15 minutes were excluded from the experiment. Beetles
194 were maintained at 20°C, 16:8 L:D, 60% RH for the duration of the trial. Groups of beetles
195 were killed by freezing at 0h, 2h, 4h, 8h, 12h, 24h and 36h post-feeding. All groups
196 comprised 10 beetles with the exception of the 24h group which had 9 beetles. Five beetles
197 were left unfed and killed at 0h. All specimens were stored at -80 °C and entire guts were
198 dissected from each beetle prior to DNA extraction (see section 2.2). PCR reactions
199 proceeded as described in section 2.3. Positive results were expressed as a percentage of
200 the total insects screened at each time point and a probit model was fitted to the data to
201 determine the time post-feeding at which the detection half-life occurred (Greenstone *et al.*,
202 2014). Statistical analysis was performed in R v.3.3.1 (R Core Team, 2016).
203
204 *2.6 Field survey*
205 Carabidae were collected using live pitfall traps from the field in Oxfordshire which was
206 planted with spring wheat. Five pitfall traps were positioned in a cross-shaped array
207 connected with barriers (10 cm h x 30 cm l) made from galvanised lawn edging to improve
208 the catch rate (Hansen & New, 2005). Each trap was comprised of a plastic beaker (8 cm
209 diameter x 10.6 cm height) with small rocks placed in the bottom as refugia (Sunderland *et*
210 *al.*, 2005). A corrugated plastic cover (12 cm x 12 cm) on wire supports was positioned 5 cm
211 above the trap. On each sampling date, six arrays were set up making 30 traps in total,
212 positioned in various field locations with at least 30 m between arrays. Traps were set in the
213 late afternoon or early evening and collected before noon on the following day. Live
214 specimens were immediately placed on ice at the point of collection, prior to storage at -80

°C. Trapping took place in early May 2016 on 2 occasions, 10 days apart, with an additional collection made in late July using just 20 traps (4 arrays).

## 3. Results

### *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 representative individuals from 8 different orders including the Cecidomyiid *S. mosellana*. Using the newly designed primers, it was possible to detect pure *H. marginata* DNA at concentrations as low as 0.001 ng $\mu L^{-1}$.

### *3.2 Rate of digestion of H. marginata DNA in predator guts*

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

### *3.3 Field survey*

From all trapping occasions, 110 individual carabid specimens of 11 different species were trapped. The majority of beetles (47%) 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 2). Beetles trapped late in the season (July) represented only 15% of all specimens tested, but had a much higher rate of positive results

242 (23.5%) compared to beetles trapped in May (4.3%). This is despite the activity density of
243 the beetles being almost identical in May and July (0.84 and 0.85 beetles per trap per day
244 respectively).
245
246 **4. Discussion**
247 The development of species-specific primers for *H. marginata*, as described here, increases
248 the potential for research on this cryptic insect at a molecular level. Here, we have applied
249 this to the development of a viable gut analysis assay, enabling highly specific and reliable
250 detection in the guts of predatory natural enemies. The field survey has identified four
251 carabid species feeding on this pest in the wild for the first time, with implications for its
252 effective management.
253
254 The COI region of the genome is commonly used for species-specific primer design as it is
255 less highly conserved than other regions (King, 2008). It is particularly appropriate for gut
256 analysis studies as it is located in the mitochondria, therefore each cell will have multiple
257 copies making the probability of detection greater than for nucleic DNA (Hoy, 1994). The
258 target amplicon is 348bp, which slightly exceeds the recommended maximum length of
259 300bp (King *et al.*, 2008) based on the idea that shorter fragments will be subject to less
260 digestion in the gut. The work done by Sint *et al.* (2011) however, suggests that this
261 recommendation might be too conservative. For example, Juen and Traugott (2006) found
262 no difference in the amplification success of 463bp and 127bp amplicons of *Amphimallon*
263 *solstitiale* (Linnaeus) DNA in the guts of *Poecilus versicolour* (Sturm) larvae. Furthermore,
264 no significant relationship was found between fragment length and the detectability half-life
265 taken from a range of studies (Greenstone *et al.*, 2014). The primers described here
266 performed well at a high annealing temperature of 65 °C which reduces the chance of
267 erroneous base matching at the primer sites (King, 2008), but was not the highest
268 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 $\mu L^{-1}$ 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 31 h 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 – 88.5 h (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 18 h. Additionally, the feeding trial was conducted at 20°C which is higher than typical field temperatures, and may reflect an underestimation of detection half-life in the field (Hoogendoorn & Heimpel, 2001). The carabid species used in this trial, *N. brevicollis*, is a common predator in arable environments (Luff, 2007) however detection half-life will vary depending on the predator species (Greenstone *et al.*, 2007). For example, the detectability of aphid DNA was higher in *N. brevicollis* compared to another common carabid, *Pterostichus melanarius* (Illiger), independent of the effects of ambient temperature or target amplicon size (von Berg *et al.*, 2008). Detectability appears to vary less between species of the same taxa than between taxa however (Waldner *et al.*, 2013), which suggests that the data shown here represent a reasonable benchmark for carabids of a similar size. Detection half-life can, however, vary

296 between life stages of the same species (Ingels *et al.*, 2013) and therefore further work will
297 need to be done to extend this assay to predatory carabid larvae.
298
299 This field survey shows for the first time the species of carabid beetle that are feeding on *H.*
300 *marginata*. Of the 12 species caught on the surveyed site, four tested positive for the
301 presence of *H. marginata* DNA. All of the species which tested positive are relatively
302 common, highly generalist feeders of medium to large size (above 5 mm long). A number of
303 these species are known to prey on dipteran adults and larvae (Penney, 1966; Allen &
304 Hagley, 1990; Lys, 1995; Sunderland *et al.*, 1995; Luff, 2002; King *et al.* 2010) and species
305 identified as predators of *S. mosellana* in Canada belong to two of the genera identified as
306 containing predators of *H. marginata* in this study (Floate *et al.*, 1990). Although many
307 species display burrowing behaviours, belowground predation by adult carabids has not
308 been well studied. Many carabid larvae are active belowground predators (Lövei &
309 Sunderland, 1996) and have been shown to feed on *S. mosellana* in the field (Floate *et al.*,
310 1990). While not surveyed here, they are potentially a significant source of predation for *H.*
311 *marginata* larvae. The proportion of positive assays was higher in July, despite the activity
312 density being comparable between early and late season sampling. Drier soil in the late
313 season may have prevented *H. marginata* from burrowing into the soil, or enabled carabids
314 easier access to larvae belowground via the formation of fissures. Basedow (1973) reported
315 from field observations of the Cecidomyiids *Dasineura brassicae* (Winnertz), *Contarinia tritici*
316 (Kirby) and *S. mosellana* mortalities of up to 65%, 58% and 43% respectively from predation
317 of larvae returning to the soil to overwinter. This was supported by the findings of Floate *et*
318 *al.*, (1990) and Holland & Thomas (2000) who found that larvae were more likely to be
319 preyed upon when returning to the soil to overwinter rather than during pupation. The
320 results presented here suggest that the same is true of *H. marginata* larvae.
321

As with other predator surveys using PCR-based gut analysis, there is the chance that a positive result could have resulted from scavenging or secondary predation of adult or larval *H. marginata* (Juen & Traugott, 2004; Foltan *et al.*, 2005; Sheppard *et al.*, 2005). Carabid beetles frequently exhibit intraguild predation (Snyder & Wise, 1999; Lang, 2002) and will feed on carrion, sometimes in preference to fresh prey (Mair & Port, 2001; Foltan *et al.*, 2005). In this scenario, the surveyed predators will not be affecting *H. marginata* populations directly, and may indirectly benefit them by consuming pest predators. Partially decayed organisms are harder to detect in the gut however (Foltan *et al.*, 2005), therefore it seems reasonable to assume the positive results obtained here are as a result of predation. This has implications for pest management, as these predatory arthropods could be contributing to suppression of *H. marginata* populations. As pitfall traps are only effective at sampling surface active arthropods, of which only carabids were surveyed here, the actual range of organisms preying on *H. marginata* could be much larger. Dipteran larvae are a primary food source of Staphylinidae (Good & Giller, 1991) and dipteran species are an important dietary component for many spiders (Harwood *et al.*, 2007; Schmidt *et al.*, 2012).

The primers developed for this study provide a useful resource for further molecular research on this insect. They could be used in the identification of this species in traps, which is particularly useful when specimens are partial or degraded (Frey *et al.*, 2004). This could be of value not only in monitoring tools, but also in expanding current knowledge on the distribution of *H. marginata* in the UK which at present is based on limited data (Rowley *et al.*, 2016). The assay described here could also be used as a tool in field-based predation experiments (Furlong, 2015) or included in multiplex PCRs to simultaneously screen for many pest species at once (King *et al.*, 2010). The detectability half-life of DNA in the guts of fluid feeders such as centipedes, heteropterans and spiders is generally much longer than that described in carabids (Harwood et al 2007; Greenstone et al 2007; Waldner *et al.*, 2013), therefore we are confident that this assay would be suitable for use in other predator

taxa. Such surveys could reveal further trophic links involving *H. marginata* in agroecosystems which are at present unknown. These primers could also be used to investigate parasitoid enemies of *H. marginata* (Rougerie *et al.*, 2011), providing information to further enhance pest management strategies. The field survey identifies for the first time, species which consume *H. marginata* in the field. Different rates of digestion and therefore prey DNA degradation between species means that further data are required to quantify rates of predation on *H. marginata*. The next step would be to obtain species-specific digestibility data under controlled conditions and conduct further field surveys to identify the most important predators of this pest. Quantification of predator density should be made alongside such surveys to further inform potential biological control strategies. Similarly, surveys should be extended over wider spatial and temporal scales to provide a more comprehensive assessment of *H. marginata* natural enemies. Nonetheless, the information presented here is vital in the management of this pest as it demonstrates that these and other species of arthropod predators are likely to be having an impact on *H. marginata* populations. This represents an important first step in understanding the predation pressures exerted on *H. marginata* populations, which may be a key aspect in the development of an effective IPM program for this insect.

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List of figure legends

Figure 1. Proportion of positive assays for *Haplodiplosis marginata* DNA in the guts of *Nebria brevicollis* at time post-consumption of a single prey larva. Fitted line represents probit model with 95% CI.

List of table headings

Table 1. Species tested using the *Haplodiplosis marginata* primers and general insect primers to assess for cross-reactivity with non-target taxa. All species tested negative.

Table 2. 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).

831 Table 1.

| Order | Family | Species |
|---|---|---|
| Coleoptera | Nitidulidae | *Meligethes sp.* |
| | Coccinellidae | *Harmonia axyridis* |
| | Carabidae | *Poecilus versicolor* |
| | | *Poecilus cupreus* |
| | | *Nebria brevicollis* |
| | | *Pterostichus melanarius* |
| | | *Anchomenus dorsalis* |
| | | *Bembidion deletum* |
| | | *Bembidion tetracolum* |
| | | *Harpalus rufipes* |
| | | *Harpalus affinis* |
| | | *Abax parallelepipedus* |
| | | *Loricera pilicornis* |
| Diptera | Cecidomyiidae | *Sitodiplosis mosellana* |
| | Dolichopodidae | Undetermined sp. 1 |
| | | Undetermined sp. 2 |
| | Tachinidae | Undetermined |
| | Syrphidae | Undetermined sp. 1 |
| | | Undetermined sp. 2 |
| | | Undetermined sp. 3 |
| | | Undetermined sp. 4 |
| | Tephritidae | Undetermined |
| | Calliphoridae | Undetermined |
| | Anthomyiidae | Undetermined |
| | Drosophilidae | Undetermined sp. 1 |
| | | Undetermined sp. 2 |
| | Muscidae | |
| | Undetermined | Undetermined |
| Hemiptera | Aphididae | *Sitobian avenae* |
| | | *Myzus persicae* |
| | | *Aphis fabae* |
| | | *Rhopalosiphum padi* |
| Hymenoptera | Undetermined | Undetermined sp. 1 |
| | | Undetermined sp. 2 |
| | | Undetermined sp.3 |
| | | Undetermined sp.4 |
| Symphypleona | Undetermined | Undetermined sp. 1 |
| | | Undetermined sp. 2 |
| Araneae | Undetermined | Undetermined sp. 1 |
| | | Undetermined sp. 2 |

832

833 Table 2.

834

| **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*) |

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