*This paper has been accepted for publication and will appear in a revised form, subsequent to editorial input by Cambridge University Press, in **Bulletin of Entomological Research** published by Cambridge University Press.*

This post-print is a PDF of a manuscript that has been accepted for publication and will appear in a revised form, subsequent to editorial input by Cambridge University Press, in **Bulletin of Entomological Research** published by Cambridge University Press.

Title: Development of specific ITS markers for plant DNA identification within herbivorous insects.

Running title: plant DNA detection in insects

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Keywords: gut-content analysis, specific primers, molecular markers, dispersion, ITS, ingested plant DNA.

Abstract

PREPRINT Bull. Entomol. Res.

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DNA based techniques have proved to be very useful methods to study trophic relationships between pests and their natural enemies. However, most predators are best defined as omnivores, and the identification of plant-specific DNA should also allow the identification of the plant species the predators have been feeding on. In this study, a PCR approach based on the development of specific primers was developed as a self-marking technique to detect plant DNA within the gut of one heteropteran omnivorous predator (*Macrolophus pygmaeus*) and two lepidopteran pest species (*Helicoverpa armigera* and *Tuta absoluta*). Specific tomato primers were designed from the ITS 1-2 region, which allowed the amplification of a tomato DNA fragment of 332 bp within the three insect species tested in all cases (100% of detection at t = 0) and did not detect DNA of other plants nor of the starved insects. Plant DNA half-lives at 25ºC ranged from 5.8h, to 27.7h and 28.7h within *M. pygmaeus*, *H. armigera* and *T. absoluta*, respectively. Tomato DNA detection within field collected *M. pygmaeus* suggests dietary mixing in this omnivorous predator and showed a higher detection of tomato DNA in females and nymphs than males. This study provides a useful tool to detect and to identify plant food sources of arthropods and to evaluate crop colonization from surrounding vegetation in conservation biological control programs.

Introduction

In nature, predators and parasitoids move over a broad range of spatial scales, and it is of vital importance to understand the dispersal characteristics of these beneficial insects for biological control of agricultural pests. Different marking and tracking techniques have been developed in order to evaluate the movement or identify the sources of several species of arthropods (Lavandero *et al*., 2004; Jones *et al*., 2006; Wanner *et al*., 2006; Goubault & Hardy 2007;

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Scarratt *et al*., 2008; Stephens *et al*., 2008). But those useful methods have a major inconvenience for field studies: either the insects or the plants need to be marked beforehand.

Many predators are omnivores, consuming plant provided foods at least during part of their life cycles (Albajes & Alomar, 2004; Wäckers *et al*., 2005). Enhancing the availability of such food sources within or close to crops provides resources which enhance populations of natural enemies, and adds to their colonization of the target crop, increasing the effectiveness of biological control (Landis *et al*., 2000; Gurr *et al*., 2004). Pollen grains present either on the exoskeleton or within the gut has been used to confirm feeding on certain plant species (Silberbauer *et al.*, 2004). However procedures for morphological pollen identification are too time consuming and not all predators feed on pollen.

In recent years, several studies have developed DNA-based techniques to analyse predator gut contents in arthropods, mainly in those where the feeding does not leave remains that can be morphologically identified. The first attempts developed specific SCAR *(sequence characterized amplified region)* markers (Agustí *et al*., 1999, 2000), but more recently, other regions like the *internal transcribed spacer region* 1 (ITS-1) (Hoogendoorn & Heimpel, 2001) or the *cytochrome c oxidase subunits* I and II (COI and COII) mitochondrial genes have been used to develop prey-specific primers (Agustí *et al.,* 2003a, b; Greenstone *et al*., 2007; Weber & Lundgren 2009). Based on this, an alternative way to track movement of omnivorous predators from their refuges would be the identification of ingested plant DNA within whole insects, as similarly done in predation gut contents analysis studies. Even if the COI region has been mainly used for primer design in gut analysis of predation, it is not clear which region would be most appropriate for the

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detection of ingested plant DNA. ITS 1-2 together with *trnH-psbA* region have been proposed to have a faster gene evolution rate than COI in plants (Chase *et al*., 2005).

Macrolophus pygmaeus (Rambur) (Heteroptera: Miridae) is a polyphagous predator that feeds on several arthropod species. Until recently *M. pygmaeus* on tomato has been misidentified as *M. melanotoma* (Costa) (= *M. caliginosus* Wagner) and is still named as *M. caliginosus* by commercial beneficial producers (Martinez-Cascales *et al*., 2006; Gemeno *et al*., 2010). This species spontaneously colonizes field and greenhouse crops from refuges present in the agricultural landscape of the Mediterranean basin (Alomar *et al.*, 2002; Castañe *et al.*, 2004; Gabarra *et al*., 2004). Like most mirids it is an omnivore that also feeds on plant tissues, therefore it was selected as a candidate for our study. Because *M. pygmaeus* is a small sucking insect and it is not known whether it feeds either on phloem or on leaf cells, we suspected that prohibitively small quantities of plant DNA would be present in its gut leading on a low detection of plant DNA. For this reason, we also tested two insects with chewing habits that would ingest a large amount of plant cells: *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) and *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) larvae, both important tomato pests.

Here, we show that molecular markers can be used to specifically identify plant DNA in herbivorous/omnivorous insects. We have developed a tomato-specific marker which allows the detection of tomato DNA in the gut of three different insect species with different feeding types (sucking or chewing insects) and showed the detection percentages of tomato DNA within their gut with digestion time. Finally, we have also shown that this marker allows the identification of plant DNA within field insects collected in tomato greenhouses with just a PCR reaction, avoiding the process of sequencing.

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Materials and Methods

Insects and plants

Macrolophus pygmaeus were reared at IRTA facilities as explained by Agustí & Gabarra (2009a,b). This colony is renewed every year with introductions of new field collected insects near Barcelona (NE Spain). They were maintained on tobacco plants (*Nicotiana tabacum* L.) and *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs. *Helicoverpa armigera* and *T. absoluta* larvae were collected in tomato fields near Barcelona and maintained on artificial diet and on tomato plants, respectively. All insects were maintained under controlled conditions of 25º ± 1ºC, 70±10% RH and L16:D8 photoperiod. *Ephestia kuehniella* eggs were provided by Biotop (Valbonne, France).

Tomato (*Solanum lycopersicum* L.), cabbage (*Brassica oleracea* L.) and tobacco (*Nicotiana tabacum* L.) plants were cultivated in greenhouses at IRTA. Potato (*Solanum tuberosum* L.), aubergine (*Solanum melongena* L.), pepper (*Capsicum annum* L.), zucchini (*Cucurbita pepo* L.) and cucumber (*Cucumis sativus* L.) plants were obtained from fields in the vicinity of our facilities). *Carlina corymbosa* L., *Ononis natrix* L., *Verbascum thapsus* L. and *Solanum nigrum* L. plants were obtained from the margins of the previously cited crops in the same area.

DNA extraction

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Whole individual insects were homogenized in clean microcentrifuge tubes to avoid possible contamination by its own faeces and DNA extractions were done using the DNeasy Tissue Kit **(**QIAGEN, Hilden**,** Germany; protocol for insects). Plant DNA was extracted from a 1 cm diameter leaf disc using the DNeasy Plant Mini Kit (QIAGEN) following the manufacturer's protocol. Total DNA was eluted with 100 mL in the AE buffer provided in the kit. All DNA extracts were stored at -20°C.

PCR amplification

Specific tomato primers were designed from the ITS 1-2 region by comparison with sequences of other solanaceous plants with CLUSTALW (Larkin *et al*., 2007). Sequences obtained from GenBank were: *S. lycopersicum* (AF244747), *S. tuberosum* (AY875827), *S. nigrum* (AJ300211) and *N. tabacum* (AJ300215). Guidelines proposed for the design of efficient and specific primers by Innis & Gelfand (1990) and Saiki (1990) were followed. Primers were synthesized by Roche Diagnostics, Basel, Switzerland. DNA amplifications were performed in a 10 µL reaction volume containing 1 µL of DNA extract, 5 µL of master mix of Multiplex Kit (QIAGEN) and 1 µL of primer mix (10 µM). Samples were amplified in a 2720 thermal cycler (Applied Biosystems, CA, USA) for 40 cycles at 94ºC for 30 s; 62ºC for 2 min and 72ºC for 90 s. A first cycle of denaturation at 95ºC for 15 min and a final extension at 72ºC for 10 min were carried out. Tomato DNA and water were always included as positive and negative controls respectively. PCR products were separated by electrophoresis in 1.5% agarose gels stained with ethidium bromide and visualized under UV light.

Species specificity

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The specificity of the tomato primers was tested by attempting to PCR-amplify DNA from leaf discs of 11 other cultivated and non-cultivated plant species belonging to six families (Table 1) (n= 2). These species were all selected as being present in the studied area and could potentially be fed on by the targeted insects. Starved *M. pygmaeus*, *H. armigera* and *T. absoluta* (n=10) were also tested.

Feeding trials and detection periods

Clean tomato leaves were cut in small discs (2.5 cm diameter) which included a fragment of the central leaf vein, where mirids usually feed. Each tomato leaf disc was put on a 0.5 cm thick layer of an agar solution (5‰) in small plastic boxes (2.6 cm diameter). A starved 48 h (at 25ºC) *M. pygmaeus* female was introduced in each plastic box for 3 hours at room temperature and was observed every 10 min. Only those individuals that had been seen with the stylet inserted into the leaf at least three times were considered to have fed and were frozen until tested by PCR. Lepidopteran feeding trials were prepared in the same way, but with a tomato leaf disc of 1 cm diameter and without the agar layer. In each of the plastic boxes, a third or fourth instar larva starved 48 h was individually confined for 3 h at room temperature. Lepidopteran larvae were only considered for the analysis if they had consumed at least 10 mm^2 of the tomato leaf.

Once the insects had been observed feeding, they were either immediately frozen at -20ºC (t= 0) or maintained at 25ºC for 2, 4, or 8h (*M. pygmaeus*); 6, 8 or 24 h (*H. armigera*) and 8 or 24h (*T. absoluta*). After that, they were frozen at -20°C. Twenty *M. pygmaeus* females were assayed at t=0 and 8h and 16 at t=4 h. Ten lepidopteran larvae were assayed for all times. Positive (tomato DNA) and negative (free DNA) control samples were included in all PCRs. Each sample was

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tested up to three times and considered positive if tomato DNA was detected in one of these three replicates. Negative exponential equations were fitted to describe the decay in the percentage of positive responses with time and R^2 was calculated (JMP 8.0.1; SAS Institute Inc.). From these equations, the half-lives (50% positive detection) were estimated (Greenstone *et al*., 2007).

Analysis of field collected Macrolophus pygmaeus

We analyzed *M. pygmaeus* collected from several tomato greenhouses in the studied area. Those predators were part of another study that has analyzed predation on two whitefly species and their parasitoids (Moreno-Ripoll *et al*., 2009; 2010). Once collected, those predators (25 males, 31 females and 83 nymphs) were frozen at -20ºC prior to DNA extraction. Each individual was analyzed by PCR using the tomato specific primers developed in this work.

Results

Development of ITS markers

Sequences of ITS-1, 5.8S and ITS-2 regions of *S. lycopersicum*, *S. tuberosum*, *S. nigrum* and *N. tabacum* were aligned and compared in order to design one pair of tomato-specific primers. These primers were named Le2F and Le1R and their sequence was 5'-CCGAGGCGCGCAAGCTCTTC-3' and 5'-TAAAGCCTTGCGGCGTGCGAG-3', respectively. They amplified a fragment of 332 bp for *S. lycopersicum* including part of ITS-1 and ITS-2, and the whole 5.8S region.

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Species specificity and detection periods

These primers were tomato-specific. No other plant species, neither cultivated nor weeds, amplified a band of the same length in the cross-reactivity test (Figure 1). Even if a band of a very high molecular weight was amplified in *N. tabacum*, this does not interfere with detection of the specific tomato band. Besides, a fragment of that size is unlikely to be detected after digestion. None of the starved insects tested (*M. pygmaeus, H. armigera* and *T. absoluta*) gave false positive results (Figure 2).

Tomato DNA was detected within all individuals of the three insect species tested after they had fed on tomato leaf discs with 100% detection in both chewing and sucking insects immediately after feeding (t = 0) (Figure 3). In all three species, tomato DNA detection decreased with time since t = 0 within *T. absoluta* and after 2h and 6h within *M. pygmaeus* and *H. armigera*, respectively (Figure 3). Detection curves were fitted to a negative exponential equation starting with the last detection time were a 100% of detection was obtained. Equations were: $y = 133.1\exp^{-0.17x}$, $R^2 = 0.98$; $y = 114.7\exp^{-0.03x}$, $R^2 = 0.95$ and $y = 88.7\exp^{-0.026x}$, $R^2 = 0.81$ for *M. pygmaeus, H. armigera* and *T. absoluta,* respectively. From these equations, half-lives of tomato DNA detection within their gut were estimated as 5.8h for *M. pygmaeus*, 27.7h for *H. armigera* and 28.7h for *T. absoluta*.

Analysis of field collected M. pygmaeus

Tomato DNA was found in 30.2% of field collected *M. pygmaeus* (n=139), being much higher in nymphs (36.1%) and females (32.3%) than in males (8%).

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Discussion

In this study, we show the detection of plant DNA within the gut of three insect species by the use of a specific molecular marker. Tomato-specific primers were highly specific, showing no cross-reactivity either with other closely-related plant species or with the insect species tested.

The COI region has been applied extensively in animal barcoding, but it is known that for most of the plant species it is not suitable due to its much slower rate of COI gene evolution in higher plants than in animals (Kress *et al.*, 2005). There is a lack of consensus on the most appropriate barcoding locus and criteria to be used in plants (Hollingsworth *et al.*, 2009; Valentini *et al.*, 2009a). Kress *et al.* (2005) proposed ITS and *trnH-psbA* as the best candidate regions for the design of plant-specific molecular markers and ITS has been shown to work on many plant groups and has been recommended (Chase *et al.*, 2005; Sass *et al.*, 2007; Chen *et al.*, 2010). According to these considerations we have designed a pair of primers from the ITS region that amplifies a fragment of 332 bp long that resulted very effective for the detection of tomato DNA within the gut of the insects tested. As previously suggested (Agustí *et al.*, 1999), those primers were designed to amplify relatively short fragments to make possible the detection of semi-digested DNA fragments.

In this study, tomato DNA was detected in both a small sucking insect (around 4 mm long) (*M. pygmaeus*) and two bigger chewing insects (*T. absoluta* and *H. armigera*). Even with this sucking insect, where the amount of ingested DNA was expected to be much lower than the bigger amount of leaf material (and then plant DNA) ingested by the chewers, the detection was possible in 100% of cases at t = 0. As we expected, a faster loss of detection was found within the sucking

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insect. Some other authors obtained longer detection periods within other sucking insects species compared with chewing ones (Greenstone *et al*, 2007; Hosseini *et al*., 2008), but as they also mention, detection depends not only on the size of the species analyzed, but on the species itself. Degradation of the plant DNA through digestion probably also depends on other biotic and abiotic factors, as happens with insect DNA (Lövei *et al*., 1990; Agustí *et al*., 1999; Weber & Lundgren, 2009).

Tomato DNA was identified in many field individuals of unknown age and feeding history which shows that even with a relatively quick digestion of tomato DNA within *M. pygmaeus*, this technique is useful to identify plant DNA in the gut contents of field collected insects. As with other predators (Agustí *et al*., 2003b; Harwood *et al*., 2007; Juen & Traugott, 2007), it is possible to analyze feeding events in the field and opens the possibility for more detailed studies to confirm the use of a range of food plants.

Such techniques can also be used to understand trophic interactions of omnivorous predators. In predatory Heteroptera, the functions of omnivory and the functional relationships between plant and prey feeding are still poorly understood, and it is not clear to what extent they depend on relative availability, amount or nutritional value of the food types (Gillespie & McGregor 2000; Sinia *et al*., 2004; Albajes *et al*., 2006). In some cases the digestive capabilities of these omnivorous predators may vary through their lives (Lundgren & Weber 2010). Comparing our data with those obtained by Moreno-Ripoll *et al*. (2009; 2010) using specific primers of two whiteflies and their parasitoids, 13.7% of all individuals were positive for both tomato and insect prey, whereas only-plant or only-prey remains were found in 16.6% and 25.9% of their guts. Simultaneous detection of both food sources was much higher on nymphs than on females and

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250 males (19.3%, 6.5% and 4% respectively). The fact that tomato DNA was detected in many field
251 collected *M. pygmaeus*, clearly shows that plant material was consumed within a few hours of
252 capture. Detection of both plant and prey within the same individual suggests dietary mixing,
253 mainly in nymphs, according to a model where plant feeding is essential for predation (Sinia *et*
254 *al*., 2004).
255
256 Recently, some studies (Miller *et al.*, 2006; Matheson *et al.*, 2007; Jurado-Rivera *et al.*, 2009;
257 Valentini *et al*. 2009b) have identified plant meal composition in insects by molecular methods.
258 In these studies plant DNA fragments from insect guts were sequenced and compared for
259 homologies in the BLAST database (http://blast.ncbi.nlm.nih.gov) in an attempt to identify the
260 ingested plant species. Such procedures, a very powerful tool when identifying unknown ingested
261 plants, are not very practical in field studies where the aim is to confirm the ingestion of a limited
262 number of host plants and a very high number of insects should be analysed (e.g. to confirm plant
263 sources of predators in crop colonization studies). That would not only require sequencing each
264 DNA fragment found in their gut, but even cloning each fragment when several DNA fragments
265 are present within the insect at the same time. In this case, it is much cheaper and more suitable to
266 develop specific plant primers, in order to identify plant DNA with just a PCR as it has been done
267 in most of the studies about molecular detection of predation and parasitism (King *et al.*, 2008;
268 Gariepy *et al.*, 2007; Agustí *et al.*, 2005). If several plant DNAs are expected, a multiplex PCR
269 can be used by developing one specific pair of primers for each of the plant species, avoiding the
270 cloning and sequencing needed in the previously cited studies.
271
272 This study shows the detection of tomato DNA within the gut of insects by using a specific
273 molecular marker. This marker allows knowing the percentage of insects which have been found

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to consume tomato plant in an insect population. This is a promising technique in conservation biological control because it can speed up the identification of food plants of colonizing species in the agricultural landscape surrounding target crops.

Acknowledgements

We thank Rafael Moreno-Ripoll and Rosa Gabarra to share their DNA extractions of field collected *M. pygmaeus* with us in order to analyze tomato DNA gut contents. We also thank Thaïs Aznar for her technical assistance and two anonymous reviewers for their comments. This work was funded by projects AGL2006-08726 and AGL2008-00546. NA was supported by the Ramon y Cajal Program and LP by a FPI doctorate studentship both from the Spanish Ministry of Science and Innovation (MICINN).

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Table 1. Plant species used in the specificity test (n=2). Species marked with * are non-crop plants.

Family	Species
Solanaceae	*Solanum lycopersicum* L., cv Bodar
	Solanum tuberosum L., cv. Red Pontiac
	Solanum melongena L., cv Cristal
	Capsicum annum L.,cv. Aristocrata
	Nicotiana tabacum L., cv. Brazillan Blend
	Solanum nigrum L. *
Cucurbitaceae	*Cucumis sativus* L., cv. Porto
	Cucurbita pepo L., cv. Mastil
Cruciferae	*Brassica oleracea* L., cv. Savoy
Compositae	*Carlina corymbosa* L. *
Papilionaceae	*Ononis natrix* L. *
Scrophulariaceae	*Verbascum thapsus* L. *

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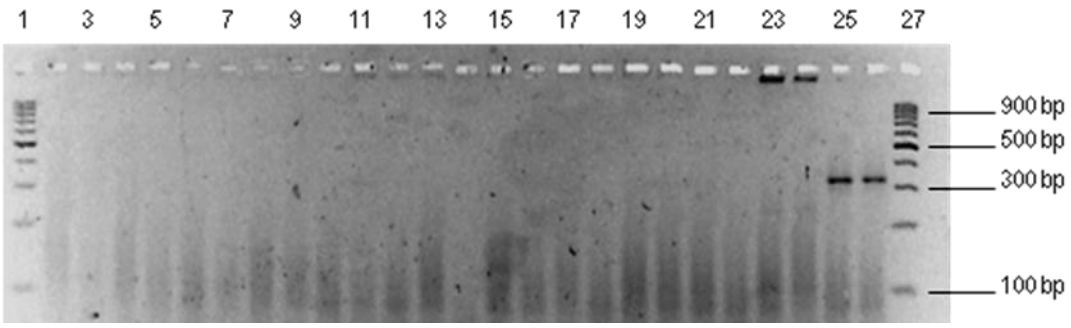

Figure 1: PCR products obtained using the tomato-specific ITS primers (332bp). Lanes 3-26 show different plant species: (3-4) *Verbascum thapsus*, (5-6) *Ononis natrix*, (7-8) *Carlina corymbosa*, (9-10) *Brassica oleracea*, (11-12) *Cucurbita pepo*, (13-14) *Cucumis sativus*, (15-16) *Capsicum anuun*, (17-18) *Solanum nigrum*, (19-20) *Solanum melongena*, (21-22) *Solanum tuberosum*, (23-24) *Nicotiana tabacum*, (25-26) *Solanum lycopersicum*. Lane 2, negative control. Lane 1 and 27, 100bp molecular-size marker.

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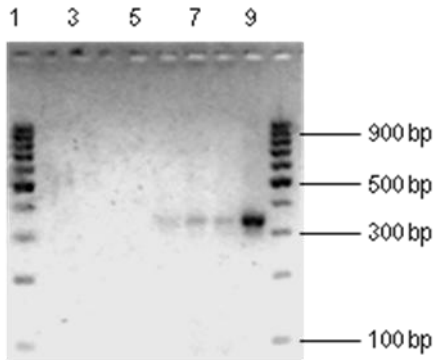

Figure 2: PCR products obtained using the tomato-specific ITS primers (332bp). Lane 3, starved *M. pygmaeus*. Lane 4, starved *T. absoluta*. Lane 5, starved *H. armigera*. Lane 6, *M. pygmaeus* fed on tomato. Lane 7, *T. absoluta* fed on tomato. Lane 8, *H. armigera* fed on tomato. Lane 9, tomato. Lane 2, negative control. Lane 1 and 10, 100bp molecular-size marker.

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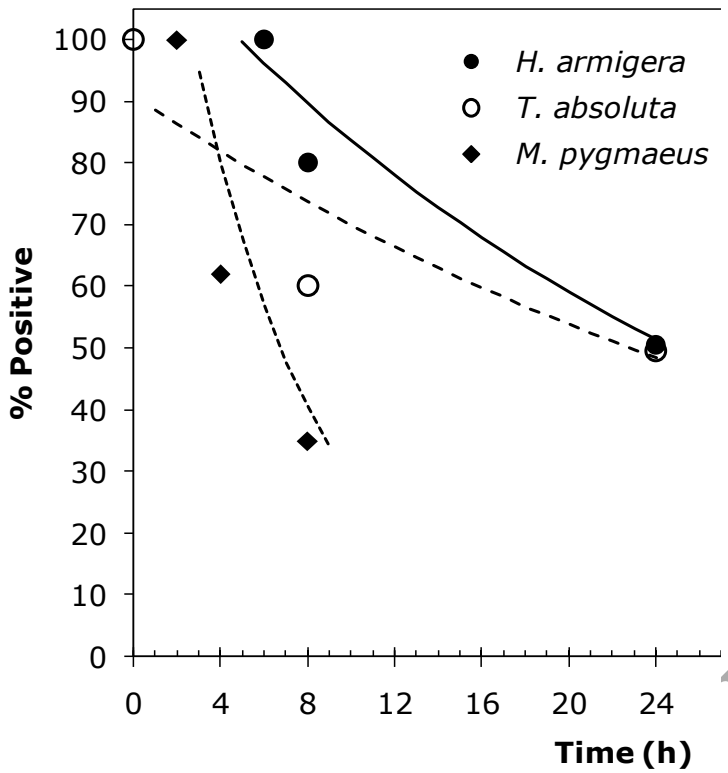

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506 Figure 3: Detectability of tomato DNA in the gut of *M. pygmaeus*, *T. absoluta* and *H. armigera* at

507 different times after ingestion. Equations and R^2 values are shown in the text.

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