



Integrating immunomarking with ecological and behavioural approaches to assess predation of *Helicoverpa* spp. larvae by wolf spiders in cotton



Dalila Rendon^{a,b,*}, James R. Hagler^c, Phillip W. Taylor^b, Mary E.A. Whitehouse^a

^a CSIRO Agriculture and Food, Australian Cotton Research Institute, 21888 Kamilaroi Hwy, Narrabri, NSW 2390, Australia

^b Department of Biological Sciences, Macquarie University, Sydney, NSW 2190, Australia

^c USDA-ARS Arid-Land Agricultural Research Center, 21881 North Cardon Lane, Maricopa, AZ 85138, USA

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ABSTRACT

Wolf spiders (Araneae: Lycosidae) are abundant ground predators in cotton fields that can provide important pest management services. These spiders can kill and consume larvae of the cotton bollworm *Helicoverpa* spp. (Lepidoptera: Noctuidae) that survive foraging on Bt cotton and descend from the plant to pupate in the soil. To determine predation frequency by wolf spiders in a Bt cotton field, we indirectly assessed predation using *Helicoverpa* spp. larvae marked with rabbit immunoglobulin G (IgG; “immunomarking”), and carried out capture-mark-recapture surveys to assess the likelihood of recapturing spiders. A laboratory feeding study with IgG-marked larva demonstrated that IgG is readily detected in spiders for up to 72 h after feeding. Following the release of IgG-marked larvae in a cotton field edge, 2.1% of spiders collected tested positive for the presence of IgG, providing indirect evidence of predation. A capture-mark-recapture survey revealed that spiders had opportunity to encounter IgG-marked larvae released along field edges, but only 6.7% of the spiders were recaptured, likely reflecting high spider mobility. In field feeding arenas, all three commonly encountered wolf spider species (*Tasmanicosa leuckartii*, *Hogna crispipes*, *Hogna kuyani*) ate *Helicoverpa* spp. larvae. These studies suggest that the low likelihood of spider recapture, and not prey rejection, is the most likely explanation for the low proportion of field-collected spiders testing positive for IgG marked prey remains, and that the frequency of IgG detection in spiders likely underestimated the frequency of predation events. We conclude that use of prey immunomarking together with capture-mark-recapture surveys can provide a powerful tool for assessing the effect of a predator on a prey species under field conditions.

1. Introduction

Predators in agroecosystems can serve as important biological control agents that maintain pest populations at low densities. To assess the efficacy of a predator as a biological control agent of an agricultural pest, it is important to understand how often the predator encounters and kills the pest in field settings. Studies of predator-prey interactions are commonly staged in confined arenas, where animals cannot disperse and are more likely to encounter each other than in natural settings (Macfadyen et al., 2015). Direct observations of predation events in the open field are challenging, because animals can be difficult to find, predation events are rarely witnessed, and the presence of an observer can disrupt predatory behaviour. To better understand the impact of a predator on a pest prey, molecular gut content analysis can complement observations of predator-prey interactions made in enclosures and in natural settings (González-Chang et al., 2016).

The cotton bollworm *Helicoverpa armigera* (Hübner) and its close relative the native budworm, *Helicoverpa punctigera* (Wallengren) (Lepidoptera: Noctuidae; together referred to here as ‘*Helicoverpa*’) are historically important pests in cotton, *Gossypium hirsutum* L. (Malvaceae) in Australia (Fitt et al., 2009). Since 1996, *Helicoverpa* has largely been controlled with plantings of genetically modified ‘Bt cotton’ (Whitehouse et al., 2009b; Wilson et al., 2013, 2018). As *H. armigera* has developed resistance to sprayed insecticides, there is ongoing concern about the capacity of *Helicoverpa* to develop resistance to Bt toxins (Downes and Mahon, 2012; Wilson et al., 2013). To inhibit the proliferation of Bt resistance, the few *Helicoverpa* that have succeeded in foraging and developing on Bt cotton must be prevented from surviving to reproduce and increasing the prevalence of traits conferring resistance in pest populations.

Final instar *Helicoverpa* larvae that survive foraging on Bt-cotton descend from the plant to pupate in the soil, where they are briefly

* Corresponding author at: Department of Horticulture, Oregon State University, Mid-Columbia Agricultural Research and Extension Center, 3005 Experiment Station Drive, Hood River, OR 97031, USA.

E-mail addresses: rendonda@gmail.com, dalila.rendon@oregonstate.edu (D. Rendon).

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exposed to a guild of ground dwelling predators. These predators are an important component of integrated pest management in cotton crops (Johnson et al., 2000; Naranjo et al., 2015; Perez-Guerrero et al., 2013), and may contribute to inhibiting emergence and proliferation of Bt resistance (Liu et al., 2014).

Wolf spiders (Araneae: Lycosidae) wander and hunt actively on the ground, and comprise an abundant group of predators in cotton crops (Rendon et al., 2015; Whitehouse et al., 2009a). Wolf spiders have been reported to feed on Lepidoptera larvae in cotton agroecosystems (Bishop, 1978; Hayes and Lockley, 1990; Johnson et al., 2000). Cotton fields in northern New South Wales (NSW), Australia, can harbour at least 12 different species of wolf spiders. Of these, *Tasmanicosa leuckartii* (Thorrell) and *Hogna crispipes* (Koch) can be very abundant (Rendon et al., 2015; Framenau and Baehr, 2016). Previous laboratory and greenhouse studies have shown that these spiders prey on fifth (final) instar *H. armigera* larvae as they descend from the plant to pupate in the soil (Rendon et al., 2016). Another common species, *Hogna kuyani* Framenau, has a body size large enough (cephalothorax width 4.7–5.4 mm; Framenau et al., 2006) to kill fifth instar *Helicoverpa* larvae. Together, this guild of ground-dwelling wolf spiders has the potential to reduce populations of *Helicoverpa* by attacking larvae as they descend from plants to pupate.

Biological control services provided by spiders on crop pests are challenging to assess. Many methods have been employed to examine the prey specificity, frequency, and timing of hunting by spiders in the field. Reliable data on prey choice and predation rates can be obtained by observing spiders directly in the field (Tahir and Butt, 2009). Even though field observations are the most direct way to assess predation by spiders, eyewitness accounts of arthropod predation events are generally rare. For example, studies relying on direct field observations of wolf spider predation have reported predation events only in 106 (4.2%) out of 2499 (Nyffeler and Benz, 1988), three (1.8%) out of 162 (Edgar, 1969), 13 (12.2%) out of 106 (Samu et al., 2003), and 162 (4.4%) out of 3704 (Hayes and Lockley, 1990) observed spiders. Moreover, fifth-instar *Helicoverpa* larvae only remain on the soil for a few hours before burrowing underground where they are safe from spiders (Rendon et al., 2016), limiting the opportunity for predation. Consequently, predation on *Helicoverpa* larvae by wolf spiders has not yet been observed or quantified in cotton fields.

As an alternative to field observations, many studies have manipulated spider and prey densities in enclosed arenas (e.g., field, greenhouse, or laboratory cages) and then estimated prey mortality over time by monitoring prey survivorship (Greenstone, 1999). Enclosure studies have the advantage of enabling observation of interactions between predators and prey, and manipulation of variables that might influence predation. However, enclosure size, predator and prey density, and lack of spatial complexity can influence predator behaviour, and this setting might not closely reflect outcomes in nature (Macfadyen et al., 2015; Naranjo and Hagler, 1998).

To overcome the limitations of direct field observations and enclosure experiments, predator gut content analysis offers a time-effective method to indirectly assess predation on a target prey species (Sunderland, 1988). The majority of field-based spider gut content evaluations have used PCR assays to detect prey remains (Ekbohm et al., 2014; Furlong et al., 2014; Hagler and Blackmer, 2013; Kobayashi et al., 2011; Kuusk and Ekbohm, 2010, 2012; Kuusk et al., 2008; Monzo et al., 2010; Schmidt et al., 2012), and a few have also used pest-specific monoclonal antibodies (MAb) in enzyme-linked immunosorbent assays (ELISA; Fournier et al., 2008; Hagler and Naranjo, 2005; Mansfield et al., 2008). However, there are drawbacks to both of these pest-specific approaches, such as short detectability periods and low reproducibility (see Fournier et al. (2008), Hagler et al. (2015a)).

To overcome these drawbacks, an alternative gut analysis technique that has recently gained attention entails strategically placing a specific protein mark (such as rabbit or chicken immunoglobulin G [IgG]) on a prey item of interest. These exogenous proteins can easily be applied

internally and/or externally to a target specimen (“immunomarking”, Hagler and Jones, 2010). The protein marks can then be detected in a predator’s gut using a suite of standardized IgG-specific enzyme-linked immunosorbent assays (ELISA; Hagler and Durand, 1994). The immunomarking procedure is better suited for mass throughput (e.g., less costly and labour intensive) and is more reliable (reproducible) than a prey-specific PCR assay (Hagler et al., 2015a). More importantly, the standardized ELISA immunomarking technique has proven to be more adaptable than PCR assays for studying predation. Specifically, the technique has been used in enclosure studies to pinpoint trophic level interactions (Hagler, 2006), to quantify predation rates on a key cotton pest (Hagler, 2011), and to quantify predator scavenging activity (Mansfield and Hagler, 2016; Zilnik and Hagler, 2013). Immunomarking has also proven useful in open field settings to study predation on eggs (Mansfield et al., 2008), larvae (Kelly et al., 2014), and pupae (Blubaugh et al., 2016), as well as granivory (Blubaugh et al., 2016; Lundgren et al., 2013). Despite its versatility, the immunomarking method has only been used in one study of mobile prey in an open field setting (Kelly et al., 2014). Here we used the immunomarking procedure to evaluate wolf spider predation of mobile *Helicoverpa* larvae in a cotton field.

To detect immunomarked prey in field-collected predators, it is necessary that the predators encounter and consume the marked prey and, in turn, that the predators are captured for analysis. Because prey and predator dispersal can lead to underestimation of predation rates, predation assessments using immunomarked prey are particularly informative when combined with predator surveys. As such, mark-recapture studies can be used both to estimate actual population size (Chao, 1989; Guillera-Aroita, 2017), to assess whether predators are sedentary or dispersive (and therefore unlikely to be recaptured; Thomas et al., 1998), as well as to estimate immigration rates (Hagler and Naranjo, 2004).

In the present study, we assess predation of *Helicoverpa* larvae by a guild of ground-dwelling wolf spiders by integrating immunomarking with ecological and behavioural studies. First, we evaluated the retention time of IgG-marked larvae in spiders under laboratory conditions. Then, using capture-mark-recapture surveys, we explored the likelihood of spiders encountering IgG-marked larvae in the field and being captured for ELISA. We then released IgG-marked larvae in a cotton field and tested for the presence of IgG in field-captured wolf spiders. Additionally, we also analyzed whether different species or life stages of wolf spiders captured in cotton fields consumed *Helicoverpa* larvae in field feeding arenas. Further, we tested the persistence of the IgG mark in the spiders in field feeding arenas after 24 h under hot field conditions. Finally, we discuss the advantages and disadvantages of the immunomarking technique for predation assessment of mobile prey in field settings, and how capture-mark-recapture assays can be used in concert with immunomarking to determine predation of *Helicoverpa* by wolf spiders in cotton fields.

2. Materials and methods

2.1. Study site

Field experiments were carried out in a Bt cotton plot located at the Australian Cotton Research Institute (ACRI) near Narrabri, NSW, Australia (30°S, 149°E). The three westernmost rows of a Bt cotton field were selected for this study (hereafter ‘cotton plot’, Fig. 1). The cotton plot (Bollgard II® cotton variety: Sicot 74 BRF®) was 3 m wide (= 3 rows: 1 cotton row/m) by 160 m long (Fig. 1). The plot was planted on 17 October 2014, and sprayed with glyphosate (Round-up®, Monsanto, Melbourne, Victoria, Australia) in November to control weeds. The field was flood irrigated every two weeks, weeds were removed by chipping, and no additional fertilizer was added. Being unfertilized, cotton plants in this plot were shorter than plants in adjacent fertilized plots (mean height ± SD on 18 February 2015 = 56.55 ± 9.0 cm, n = 30), and

Area surveyed for capture-mark-recapture and gut-content analysis (three transects per plot)

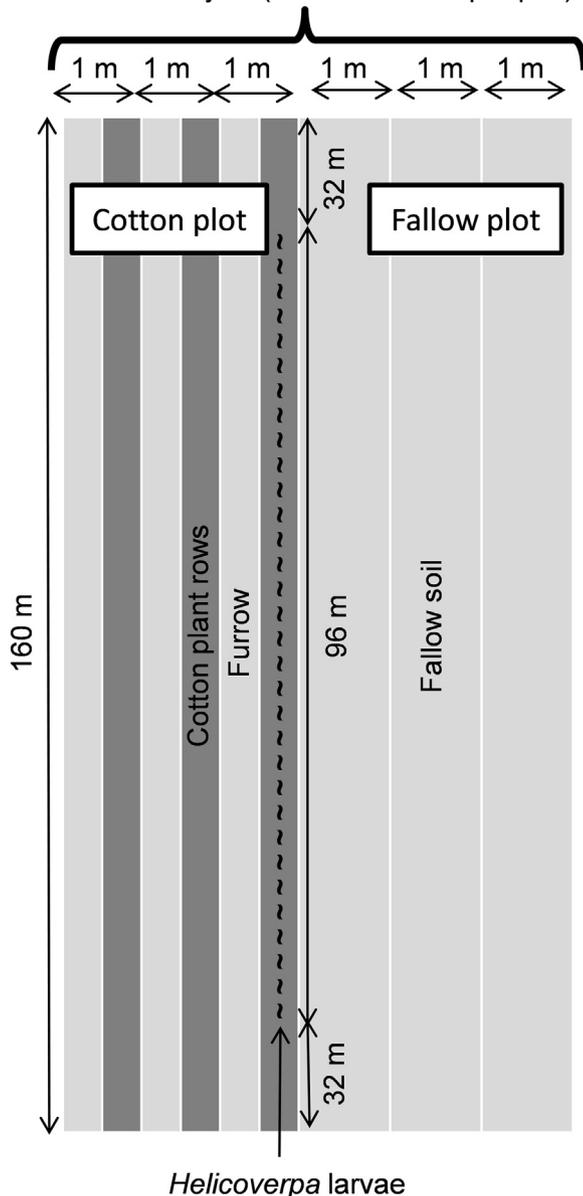


Fig. 1. Diagram of the cotton and fallow plots surveyed for spider capture-mark-recapture and gut content analysis.

this low canopy facilitated visual searches for wolf spiders between the plant rows. The cotton plot bordered a fallow field on its western edge, and the 3 m portion of this field adjacent to the cotton plot was used for this study as a ‘fallow plot’ (Fig. 1). In order to avoid removing spiders from the main cotton plot, spiders for the IgG detection study (Section 2.2.1), and *Helicoverpa* predation in field feeding arenas (Section 2.3.3) were collected on the edges of different Bt cotton fields, 100–200 m away from the main cotton plot.

2.2. Laboratory IgG detection study

2.2.1. Spider collection and maintenance

Male *T. leuckartii* wolf spiders for this trial were collected in and around Bt cotton fields during Nov and Dec 2014. Spiders were found by visual search after sunset using a headlamp (Petzl Tikka, 140 lm), and collected manually using a clear 70 mL cylindrical plastic container. All spiders collected for subsequent laboratory and field

experiments and surveys had a cephalothorax width greater than 3.5 mm, because smaller spiders do not attack fifth instar *Helicoverpa* larvae (D. Rendon, personal observation). After collection, all spiders were housed individually in clear plastic containers (228 mm height × 238 mm length × 238 mm width, 8.5 L, Décor Tellfresh Superstorer®, NSW, Australia, hereafter referred to as ‘feeding arena’) with 2 L of moist soil in a controlled environment room (24.4 ± 0.5 °C with a L14:D10 photoperiod). Spiders were kept in the feeding arenas for two to four days before being used in experiments. During this period each arena was sprayed with water daily to provide moisture, but no prey was provided.

2.2.2. Prey marking protocol

Helicoverpa armigera larvae were reared on an artificial diet (for protocol, see Downes et al. (2009), Teakle and Jensen (1985)) in a controlled environment room (24.4 ± 0.5 °C with a L14:D10 photoperiod) until they reached their fifth instar. A rabbit IgG-marked larval diet was prepared by pouring 2 mL of artificial diet into individual wells of a rearing tray and allowing it to solidify. Then, using a fine paintbrush, a layer of rabbit IgG solution (1.0 mg technical grade rabbit IgG/1.0 mL ultrapure water, Sigma-Aldrich #I5006, Castle Hill, NSW, Australia) was spread over the surface of the diet contained in each well (2 cm × 2 cm area) of the rearing tray. After placing a fifth instar larva in each well containing the rabbit IgG-marked diet, a topical application of rabbit IgG solution (10 µL) was placed on the larva’s exoskeleton and spread using a fine paintbrush. This ensured that each larva was marked internally and externally (Hagler, 2011). Larvae were allowed to feed freely on the rabbit IgG-marked diet for 24 h and were then transferred to wells of rearing trays containing unmarked (without IgG) conventional (non-Bt) cotton plant material (a mixture of leaves and green bolls) and allowed to feed freely for another 24 h (hereafter ‘IgG-marked larvae’). This was done to ensure that larvae retained the IgG mark for at least 24 h after feeding on IgG-marked diet, even if they then fed on unmarked plant material. A separate set of larvae, serving as a negative control treatment, were allowed to forage on unmarked (without IgG) artificial diet for 24 h, and then on cotton plant material for another 24 h. After larvae had been exposed to one of the two (IgG marked or unmarked) diet feeding treatments, each larva was weighed to the nearest 0.01 g (Sartorius Model A200S, Goettingen, Germany) and then randomly assigned to a spider feeding treatment as described below (Section 2.2.3).

2.2.3. IgG mark retention test

A single rabbit IgG-marked or unmarked larva was placed in each feeding arena 30–60 min after the dark phase in the controlled environment room began. Each arena contained a single male *T. leuckartii* wolf spider; spiders were checked every 10–15 mins to ascertain when they killed and consumed the larva. Once a spider finished feeding, it was held for 3, 12, 24, 48 or 72 h ($n = 9–11$ for each post-feeding time treatment). After each time interval, the spiders were killed by freezing at –20 °C. Spiders in the negative control treatment were frozen 12 h after feeding on an unmarked larva. A previous study showed that spiders do not reject larvae after delivering an initial bite, and all attacks result in consumption (Rendon et al., 2016). To test for the possibility of spiders picking up traces of IgG by contact with the soil or with the IgG-marked prey (false positives), those spiders that did not kill the larva after 24 h in the feeding arena were frozen at –20 °C ($n = 11$) and also examined for the presence of rabbit IgG by ELISA (see 2.2.4).

2.2.4. Rabbit IgG-specific ELISA

Spiders heavier than 0.40 g were sliced into sections using a razor blade, and then the whole body was crushed in a centrifuge tube containing 1.0 mL of tris buffered saline (TBS, pH 7.4; Sigma-Aldrich T1503). Spiders lighter than 0.40 g were crushed whole in a centrifuge tube containing 0.5 mL of TBS. We tested for the presence of rabbit IgG

in the bodies of spiders using a rabbit IgG-specific ELISA protocol modified from Hagler and Durand (1994). Each well of a 96 microtiter plate (Cellstar® #655-180, Greiner Bio-one, Kremsmunster, Austria) was coated with 100 μ L of goat anti-rabbit IgG (1 mg/mL stock solution diluted 1:500 in TBS; Sigma-Aldrich #R2004) and incubated overnight at 4 °C. The primary antibody was then discarded, and each microplate well was coated with 300 μ L of blocking solution for 30 min at room temperature. The blocking solution consisted of 1.0 mL of whole milk in 100 mL ultrapure H₂O. Blocking solution was discarded, and a 100 μ L aliquot of each spider-TBS sample was added to an individual well and incubated for 1 h at room temperature. Sample solution was discarded, and wells were washed three times with 300 μ L of TBS-Tween 20 (0.05%). A 50 μ L aliquot of peroxidase conjugate goat anti-rabbit IgG (secondary antibody, Sigma-Aldrich #A6154) diluted to 1:1000 in the milk protein-blocking solution was added to each well and incubated for 1 h at room temperature. The secondary antibody was discarded and the wells in each plate were washed again three times as described above. After washing, 50 μ L of TMB substrate (Sigma-Aldrich #T0440) was added to each well. After 10 min, the absorbance (optical density ELISA reading) of each well (spider sample) was measured using a microplate reader (Biotek EL808, Winooski, VT, USA) set at 655 nm. Spider samples were scored positive for presence of IgG if the absorbance was greater than three standard deviations above the average absorbance of unmarked larvae (Hagler and Miller, 2002). The mean ELISA reading (\pm 95% CI) was calculated for each feeding treatment and the percentage of individuals scoring positive in each treatment was determined.

2.3. Field studies

2.3.1. Wolf spider capture-mark-recapture field study

Capture-mark-recapture surveys were carried out at the cotton plot and fallow plot (see 2.1. Study site; Fig. 1) to estimate: (1) the abundance of wolf spiders large enough to kill fifth instar *Helicoverpa*, (2) recapture frequency of wolf spiders, and (3) whether wolf spiders crossed between the cotton plot and fallow plot.

The capture-mark-recapture survey was conducted every night except when raining between 29 January and 10 February 2015 during the ‘peak flower’ stage of cotton growth. This is also the period when adult wolf spiders are the most abundant in cotton fields (Rendon et al., 2015). The cotton plot and fallow plot were surveyed after sunset (2030 h) by one investigator walking along the 160 m rows (transects; Fig. 1). Each row was surveyed twice, once in each direction. Each wolf spider with a cephalothorax width greater than approximately 3.5 mm was marked by dusting its whole body with a colored dust (HCA Colours, Kingsgrove, NSW, Australia). Spiders found in the fallow plot were marked with yellow dust (VM317), and spiders found in the cotton plot were marked with blue dust (VM321). These dusts remain visible on spiders for more than three weeks even after irrigation and can only be discarded when moulting (D. Rendon, personal observation). Colour dust marking has some limitations; for instance, marked juvenile spiders that moulted could have been recorded as new spiders when recaptured leading to underestimation of recapture rates. Additionally, dust marking can influence spider habitation behaviour (Still et al., 2014), but in previous feeding trials, dust marking did not influence spider mobility or hunting behaviour (Rendon et al., 2016). We recorded whether wolf spiders found in each survey were observed inside the cotton plot or in the fallow plot, and if any dust marks were present. Spiders were classified to species (*T. leuckartii*, *H. crispipes*, *H. kuyani*) or family (Lycosidae; when juveniles still did not display a characteristic species pattern). To avoid counting the same spider twice in one night, spiders with a dust mark were dusted again to show a “fresh” mark (fresh marks were brighter and the dust was more concentrated than older marks). A Pearson Chi-square test on a contingency table was used to test for differences in the proportion of spider species captured in the fallow or cotton plots.

2.3.2. Open field assessment of *Helicoverpa* larvae predation by IgG detection

This experiment was undertaken at the cotton plot and fallow plot a week after the wolf spider capture-mark-recapture surveys (see 2.3.1). The aim of this study was to estimate consumption of fifth instar *H. armigera* and *H. punctigera* larvae by wolf spiders in a Bt cotton field. A combination of *H. armigera* (43%) and *H. punctigera* (57%) larvae were used for field predation studies. Larvae were reared and maintained on unmarked artificial diet (see Section 2.2.2), until they reached fifth instar. Then larvae were transferred individually to a well of IgG-marked diet and externally marked with IgG using a paintbrush. The marked larvae and diet were immediately transferred to a cool room (11.84 ± 0.91 °C, mean \pm SD; L24:D0 photoperiod) to prevent larvae from developing into pupae. Under these conditions, the larvae still consumed the protein-marked diet.

Because the cotton plot was Bt cotton, very few *Helicoverpa* spp. larvae would survive to pupation (Whitehouse et al., 2014). Consequently, we simulated a scenario in which ‘resistant’ larvae survived foraging on Bt cotton by releasing fifth instar IgG-marked larvae on the soil of the cotton plot. Approximately 28 h after placing larvae on IgG-marked diet, larvae were released in the cotton plot at sunset (approximately 20:00 h). Larvae were placed on the western outermost row of the cotton plot (edge bordering fallow plot), and distributed every 1 m on the soil on top of the ground bed, next to cotton plant stems. Larvae were only placed in the middle 96 m of the row, excluding the 32 m at each end (Fig. 1). Ninety six IgG-marked larvae were released in the plot on 13, 17, 19, 21 and 23 February 2015 for a total of 480 released larvae, and wolf spiders were collected on 14, 18, 20, 22, and 24 February 2015. The cotton plot and the fallow plot were searched for wolf spiders with cephalothorax width greater than 3.5 mm after sunset (2030 h) by one investigator walking back and forth along three transects in each plot (each 1 m by 160 m) on each of the survey nights. The transects were searched for spiders for 2 h each night; fewer than three spiders were found during the last 30 min of every search, suggesting that a longer survey time would have yielded few additional spiders. All wolf spiders were collected manually using a 70 mL clear plastic container. Wolf spiders with distinctive species cephalothorax patterns were identified to species (*Tasmanicosa leuckartii*, *Hogna crispipes*, or *Hogna kuyani*); all other spiders were classified as ‘Lycosidae’. Field-collected spiders were brought to the laboratory within 2 h after collection and killed by freezing at -20 °C. All the field-collected spiders ($n = 93$) were subsequently assayed for the presence of rabbit IgG by ELISA (see 2.2.4).

2.3.3. Larva predation in field feeding arenas

A study was conducted in field feeding arenas to assess predation and immunomarking efficiency on rabbit IgG-marked larvae by different wolf spider species, life stages, and sexes. Wolf spiders with a cephalothorax width greater than 3.5 mm ($n = 79$) were collected from the edges of cotton fields 100–200 m away from the cotton plot, (no individuals with coloured dust marks were found at this location) and assigned to a predation study conducted in feeding arenas. The number of spiders tested from each species (*T. leuckartii*, *H. crispipes*, *H. kuyani*, or ‘Lycosidae’) was dependent on the individuals collected during 14–24 February 2015. Immediately after collection, a single spider was placed in a feeding arena (described in 2.2.1) and placed between rows of the cotton plot. Cotton branches were placed on top of each feeding arena to provide shade for the larvae and spiders. An IgG-marked *Helicoverpa* larva was placed inside each field enclosure approximately 5 min after the spider. Due to *Helicoverpa* species availability, spiders randomly received either a *H. armigera* ($n = 39$) or *H. punctigera* ($n = 40$) as prey; preliminary tests have shown that spiders do not discriminate between these species. Spiders were left in the field feeding arenas at ambient conditions for 24 h (average daily high temperature = 37.1 °C). After 24 h, each feeding arena was searched for larva remains as evidence of predation, and the spiders were placed

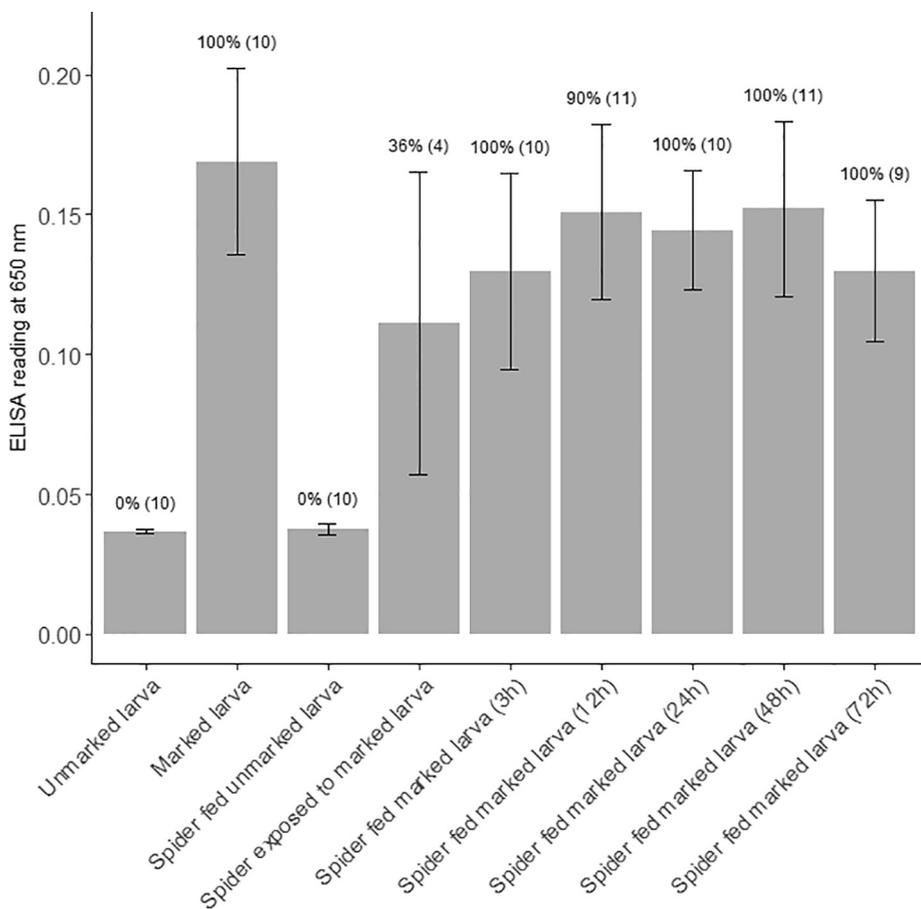


Fig. 2. Mean (\pm 95% C.I.) ELISA values yielded by the various treatments in the rabbit IgG-marked prey retention test. Numbers above the error bars represent percentage of individuals that scored positive for presence of rabbit IgG prey remains for each treatment. Numbers in parentheses represent the sample size for each treatment. For the “spider exposed to marked larva” treatment, only positive absorbances are graphed ($n = 4$ of the 11 samples examined).

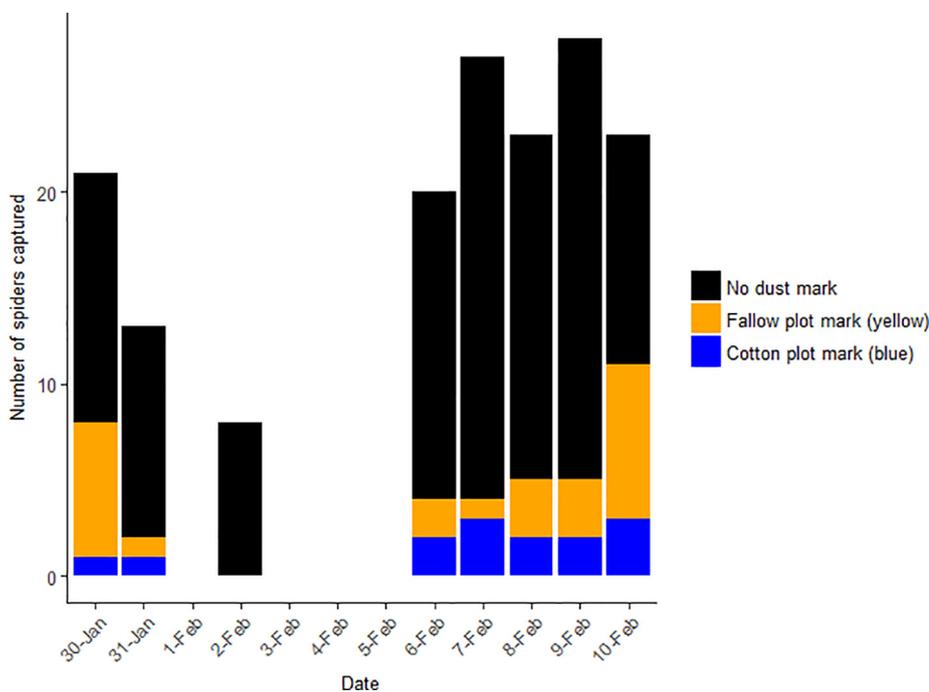


Fig. 3. Total spiders found in capture-mark-recapture survey in the cotton plot and fallow plot from 30 January to 10 February 2015. All the spiders that were found without a dust mark were subsequently marked for potential recapture on following nights with either yellow or blue dust if they were captured in the fallow or cotton plot; respectively. Those dates with no spiders captured represent nights where surveys were cancelled due to inclement weather (total eight survey nights). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

individually in clear 70 mL containers and frozen at -20°C .

All spiders were tested for the presence of rabbit-IgG-marked larva remains by ELISA (see 2.2.4) to further validate the efficacy of the immunomarking procedure under field conditions. A pairwise proportion comparison test with a Hoch correction was used to test for

significant differences in the proportion of spiders that killed the larva between species (*T. leuckartii*, *H. kuyani*, *H. crispipes*, Lycosidae sp.) or lifestages (male, female, juvenile). All analyses were carried out using SPSS v. 20 (IBM, 2011).

3. Results

3.1. IgG mark retention test

Most spiders that consumed a rabbit IgG-marked larva tested positive for the presence of IgG over the 72 h duration of the laboratory study (Fig. 2). Only one spider tested negative for the presence of IgG 12 h after consuming an IgG-marked larva (false negative). Out of the 82 total spiders offered a *Helicoverpa* larva and assigned to different time treatments, 11 did not kill the larvae and were further tested for the presence of an IgG mark. Of these, 36% (4 of 11) of the spiders that occupied an arena for 24 h but did not kill the larva also tested positive (falsely) for the presence of IgG. None of the spiders that fed on an unmarked larva (negative control, $n = 10$) tested positive for the presence of IgG (Fig. 2).

3.2. Wolf spider capture-mark-recapture field study

A total of 163 wolf spiders were captured and marked with a coloured dust throughout the course of the eight night survey. Of these, 93 were collected in the fallow plot and marked with yellow dust and released, and 70 were captured in the cotton plot and marked with blue dust and released. The majority of spiders captured on each survey night were unmarked (Fig. 3). On the last day of the survey (10 February), 11 spiders were recaptured with a blue or yellow dust mark, representing 6.7% of the total number of spiders (163) marked over the course of the survey (Fig. 3). Overall (pooling survey data from all nights), a total of 93 spiders were captured in the fallow plot; of these, 72 were unmarked (and subsequently marked with yellow dust and released), 16 had a ‘fallow plot’ yellow dust mark, and five had a ‘cotton plot’ blue dust mark. A total of 70 spiders were captured in the cotton plot; of these, 52 were unmarked (and subsequently marked with blue dust and released), nine had a ‘fallow plot’ mark, and nine had a ‘cotton plot’ mark (Fig. 4). There was no difference in the proportion of *Tasmanicosa leuckartii*, *Hogna kuyani*, *Hogna crispipes*, or Lycosidae sp. captured in fallow or cotton plots (pairwise proportion test, all $p > 0.05$). Likewise, there was no difference in the proportion of spiders captured and marked in the fallow or cotton plot being recaptured in the fallow or cotton plot (Fig. 4).

3.3. Open field assessment of *Helicoverpa* larvae predation by IgG detection

A total of 93 wolf spiders that were large enough to kill fifth instar larvae (41 males, 29 females, and 23 juveniles; 39*H. kuyani*, 30*H. crispipes*, 20 *T. leuckartii*, and 4 Lycosidae) were collected in the cotton and fallow plots on the five nights following each release of rabbit-IgG-marked larvae (14, 18, 20, 22, and 24 February 2015). Of these, only two (2.1%), collected on the first sampling date of the study (14 February 2015) tested positive for the presence of rabbit IgG (one female *T. leuckartii* with a ‘fallow plot’ yellow dust mark, and one female *H. kuyani*, with no dust mark). All other field-collected spiders examined over the duration of the study tested negative for the presence of rabbit IgG.

3.4. Predation in field feeding arenas

Fifty-two out of 79 (65.8%) of the spiders placed in the field feeding arenas consumed the rabbit IgG-marked prey larvae within the 24 h exposure period. Female spiders were more likely to kill the larva than were males or juveniles, and there was no difference in predation rates between spider species (Table 1). Every spider that consumed an IgG-marked larva tested positive for the presence of IgG. Also, seven out of the 27 spiders (25.9%) that did not eat an IgG-marked larva tested positive (falsely) for the presence of IgG.

4. Discussion

This study is one of only two to date to have used immunomarking procedures to assess predation on a mobile prey in an open field setting (Kelly et al., 2014). Previously, the technique has been used mostly to assess predation of enclosed predators and prey, in field cages and in greenhouse cages (Hagler, 2006, 2011; Mansfield and Hagler, 2016). When assessing predation in an open field, the frequency of negative ELISA results can be influenced by the number of marked prey released, number and proportion of predators captured, and mobility of the marked prey and predators in a sampling area (Sivakoff et al., 2012). To overcome these constraints, we adopted a multi-tactic approach to assess predation in an open-field setting. Specifically, we used prey immunomarking methodology in combination with a conventional marking technique to monitor predator dispersal, and field enclosures to confirm wolf spider predation on *Helicoverpa* larvae.

The immunomarking technique was very effective. Rabbit IgG was

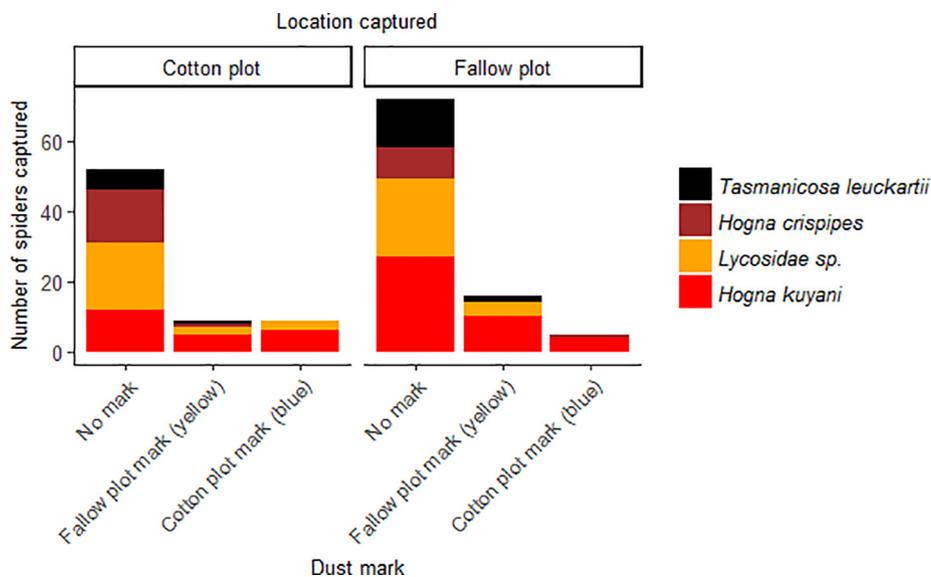


Fig. 4. Total (pooling all survey data) capture-mark-recaptures from the wolf spider survey in the fallow plot and cotton plot from 30 January to 10 February 2015. Boxes indicate how many spiders of each species were found in total in each plot location with or without a dust mark.

Table 1

Predation outcomes of males, females and juveniles of different wolf spider species on *Helicoverpa* in field enclosures. Different boldface lowercase letters in parentheses indicate significant differences between species and different boldface uppercase letters in parentheses indicate significant differences between lifestage feeding activity (pairwise proportion test with Hoch correction, $p < 0.05$).

Lifestage	Female		Male		Juvenile		Total	
	Sample size	Predation (%)						
Wolf spider species								
<i>Hogna kuyani</i>	8	100	13	53.8	16	56.2	37	64.9 (a)
<i>Tasmanicosa leuckartii</i>	3	100	8	62.5	9	66.7	20	70 (a)
<i>Hogna crispipes</i>	5	80	5	60	2	50	12	66.7 (a)
Lycosidae	2	100	3	33.3	5	60	10	60 (a)
Total	18	94.4 (A)	29	55.2 (B)	32	59.4 (B)	79	65.8

reliably detected in spiders for at least 72 h under laboratory conditions. Likewise, all the spiders tested positive for the presence of IgG after consuming an IgG-marked larva within the previous 24 h in field arenas, even when field temperatures exceeded 35 °C. These results concur with the previous findings that spiders can retain food in their gut for long periods (Sopp and Sunderland, 1989) and that protein marks are known to be heat tolerant (Hagler, 1997). Given that we do not know how much time passed since field-collected spiders consumed larvae, the long retention interval coupled with the sampling interval employed (every other day) proved to be a good fit for our study protocol.

Predation studies revealed that all wolf spider species tested readily fed on *Helicoverpa*. In the field feeding arenas, 65.8% of spiders fed on the IgG-marked prey within a 24 h period. Additionally, the field feeding study revealed that *H. kuyani*, *H. crispipes* and *T. leuckartii* were similarly likely to kill the larvae, but that adult females were more likely to kill the larvae (94%) than were adult males and juveniles (< 60%). Therefore, the relative proportion of male and female wolf spiders encountering *Helicoverpa* could influence the wolf spider predation of *Helicoverpa*. In artificial settings such as these feeding arenas, handling and the un-natural environment might decrease spider foraging behaviour. However, in a natural setting such an open cotton field, predation rates by wolf spiders on *Helicoverpa* larvae upon encounter might be higher.

Two out of 93 spiders (2.1%) collected in the cotton and fallow plots tested positive for the presence of rabbit IgG. This low rate was expected, given that it depends on the likelihood of capturing a spider that has encountered and consumed a marked larva. Marked *Helicoverpa* larvae are less likely to be eaten if wolf spiders are absent or scarce where the prey are active. For this reason, the capture-mark-recapture survey was useful for estimating the density and movement of wolf spiders in cotton fields, and to assess whether spiders were likely to encounter an IgG-marked larva. We found that spiders originally marked in the cotton or fallow plot could be recaptured in either plot, confirming that spiders are highly mobile and do routinely cross the field edges, as was also reported by Pearce (2004). As spiders regularly crossed the edge between the cotton plot and the fallow plot, they had therefore the opportunity to encounter and kill IgG-marked larvae that were released along the interface between these plots.

Predation is often less frequently detected in field settings than in enclosure settings. For example, Northam et al. (2012) found that despite wolf spiders readily feeding on mayflies in cages, gut content analysis did not show evidence of mayfly predation by wolf spiders in a natural creek setting. Similarly, Kelly et al. (2014) reported a low capture rate (4%) of predators containing IgG in their guts after releasing IgG-marked larval prey in an open field setting. Also, low recapture rates are very common in mark-recapture research conducted in agricultural settings. For example, Pearce (2004) reported a 4.5% recapture rate of wolf spiders in soybean fields, Hagler and Naranjo (2004) reported < 1% recapture rate of marked *Hippodamia convergens* in cotton fields; while Lefevbre et al. (2017) reported a recapture rate of 13%–27% for ground hunting spiders. Our recapture rate of 6.7% of the spiders captured on the last day of the survey fits into the range of these previous studies.

The probability of capturing a spider that had encountered and then consumed an IgG-marked larva also needs to be considered in open field studies of predation. This depends on the number of spiders in the field, the probability that a spider will capture a marked prey, and the probability that this spider will then be captured. Low recapture rates can indicate that the wolf spider population is so large that only a small proportion of the population was sampled, that some marked juveniles lost their marks, and/or that the population is open, with many individuals moving in and out of the study site.

Previous studies indicate that open communities are commonplace for wolf spiders. For example, Samu et al. (2003) found that the wolf spider *Pardosa agrestis* walked on average 4 m per day, which is a distance similar to that covered by spiders crossing the cotton and fallow plot interface in the present study. Moreover, Seer et al. (2015) found that the wolf spiders *Pardosa Agricola* and *Arctosa cinerea* have on average home ranges that span up to 75 m for males and 58 m for females, which is a substantially longer distance than our study plot. Consequently, the population in our study most likely experienced high levels of emigration and immigration, and spiders that ate an IgG-marked larva may not have been recaptured for ELISA because they left the plot soon after hunting.

Prey mobility can also limit encounters between spiders and IgG-marked larvae. Other studies have marked immobile prey items in open field settings to detect predation (e.g., protein marked eggs, pupae, and seeds). These studies have reported capturing proportionally more predators with an IgG mark (22%–36%; Blubaugh et al., 2016; Lundgren et al., 2013; Mansfield et al., 2008) than was the case in the present study (2.1%) or a previous study using mobile prey (4.2%; Kelly et al., 2014). *Helicoverpa armigera* larvae typically remain exposed to potential predators for less than five hours on the soil before burrowing underground to pupate (Rendon et al., 2016). To counter the limited exposure period, we conducted releases of IgG-marked larvae over multiple nights. Therefore, the low capture rate of spiders testing positive for IgG is not likely to be caused primarily by limited exposure to prey.

Ultimately it would be advantageous to estimate the impact of wolf spiders on the survival of fifth instar *Helicoverpa* (as represented by IgG-marked prey). This can be deduced by estimating the proportion of marked *Helicoverpa* larvae consumed by wolf spiders. Consequently, we need to calculate the population size of wolf spiders in this community, the proportion of that population that were marked, and the proportion of the marked population that captured marked prey. While a number of capture-mark-recapture models have been used to calculate population size, their assumptions are problematic for the present study. For example, the Lincoln-Petersen model assumes that the population is closed (no deaths, emigration, or immigration; Link, 2003), but the wolf spider population in our study was open. The Jolly-Seber stochastic model (Jolly, 1965; Seber, 1965) and its derivatives (Carothers, 1973; Chao, 1989; Pledger et al., 2003) require animals to be individually marked, and assume that individuals are equally likely to be caught and re-caught (Carothers, 1973; Guillera-Arroita, 2017). This assumption may not hold with spiders where males usually range a lot further than females (e.g., Seer et al., 2015, Whitehouse and Jackson, 1993). Future work combining mark-recapture and immunomarking could involve

measuring these differences so that the impact of wolf spiders on *Helicoverpa* could be more accurately gauged. Nevertheless, in our study, the low proportion of wolf spiders recaptured (with a dust mark, 6.7%) indicates that the proportion of spiders testing positive for IgG (2.1%) is likely to an underestimation of the true proportion of the spider population feeding on *Helicoverpa* larvae.

Immunomarking has many attributes that make it a useful technique for analysis of predation. However, our studies in laboratory and field feeding arenas revealed that there was a relatively high incidence (36.3% and 25.9%, respectively) of false positives for the presence of prey remains. That is, some of the spiders yielded a positive reaction for IgG even though they had not killed or eaten an IgG-marked larva. Similarly, Mansfield and Hagler (2016) reported that 46% of predators that did not consume but were exposed to IgG-marked *Lygus* prey tested positive for the presence of an IgG mark. False-positive results are also associated with DNA-based gut content analyses (Greenstone et al., 2011). Removing an external mark and only using an internal IgG mark (Hagler et al., 2015b) could reduce the incidence of false positives with *Helicoverpa* prey, however, because larvae burrow through their diet as they feed, traces of the IgG in the diet could still transfer onto the larval exoskeleton and thus the risk may not be completely removed. The high false positive rate in feeding arenas likely reflects contamination from a confined enclosure, as the wolf spiders had to continuously walk around on the same surface over the duration of the study. There are several possible reasons to assume that this would be unlikely to occur in an open field setting. First, *Helicoverpa* larvae do not travel far before they bury to pupate (Rendon et al., 2016). As such, it is unlikely that a larva would leave IgG remnants far away from its release site. Second, there were no evident wolf spider burrows near the *Helicoverpa* larvae release sites, which suggests that the same wolf spider would not continuously walk in the vicinity. Third, Mansfield and Hagler (2016) suggest that the high false positive rate reported in their study was possibly due to an unsuccessful predation attempt on an externally-marked prey item. However, wolf spiders that initiate an attack (lunge or bite) always consume their prey (Rendon et al., 2016), so protein transfer due to an unsuccessful attack is unlikely. Lastly, there is always a potential human error associated when moving spiders from the feeding arenas to sample containers for ELISA testing. Specifically, small traces of soil containing IgG could have been transferred to the sampling container too. However, for the open field study, it was unlikely that the soil where the spiders were standing when caught was contaminated with IgG.

In summary, using the prey immunomarking method with capture-mark-recapture surveys and predation trials in enclosed arenas can be a powerful tool for estimating the effect of a predator on a prey species under field conditions. This study, as an initial step in this direction, indicated that while only 2.1% of field-collected wolf spiders tested positive for IgG (an indirect indication of *Helicoverpa* predation in the field), the low likelihood of catching wolf spiders suggests that a higher number of the larvae could have been consumed by the predators. While there is room for improvement of the immunomarking method (e.g., mark the prey internally), the field protocol (e.g., increase the number of marked prey released in the field and increase the search area for the predators), and the capture-mark-recapture techniques (e.g., individual marking; Jolly, 1965; Seber, 1965), estimates for unequal likelihood of capture (Carothers, 1973; Pledger et al., 2003) for future studies, the capture of spiders testing positive for the presence of IgG provides indirect evidence for predation of *Helicoverpa* larva in an open field setting. Predation trials in feeding arenas demonstrated that *H. kuyani*, *H. crispipes* and *T. leuckartii* wolf spiders all can kill *Helicoverpa* larvae. This suggests that, despite being difficult to detect in field settings, these wolf spiders do kill fifth instar *Helicoverpa* larvae on the soil. As such, the diverse guild of wolf spiders found in cotton fields can be an important biological control agent for *Helicoverpa* larvae that descend from plants to pupate in the soil.

5. Conflicts of interest

None.

6. Author contributions

DR, MEAW and PWT conceived and designed the field experiments. JRH designed the immunomarking protocol and the laboratory retention experiment. DR collected and analysed the data. DR wrote the manuscript, all authors edited the manuscript.

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