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Development and application of a glassy-winged and smoke-tree sharpshooter egg-specific predator gut content ELISA ☆

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Abstract

The recent invasion of southern California by the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* (Say) (Hemiptera: Cicadellidae), has triggered a statewide control effort. Management of GWSS will include biological control using resident and imported natural enemies. Currently, very little information is available on the role of generalist predators in suppression of GWSS eggs, nymphs or adults. We have developed a sharpshooter egg-specific monoclonal antibody (MAb) for use as a diagnostic tool for predator gut content analysis. The MAb was tested by an indirect enzyme-linked immunosorbent assay (ELISA) for specificity to the different life stages of GWSS, smoke-tree sharpshooter (STSS), *Homalodisca liturata* Ball (Hemiptera: Cicadellidae), and various life stages of 27 other arthropod species. We found that the MAb only reacted to the egg stage of both sharpshooters and, to a lesser extent, to the adult stage of gravid GWSS and STSS females. Moreover, the ELISA was more responsive to younger GWSS eggs than older ones. Laboratory trials were conducted to determine how long GWSS egg antigen remained detectable in the guts of the green lacewing, *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae) and the ladybird beetle, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) using both an indirect and sandwich ELISA format. We found that GWSS egg antigen was detectable for up to 30 and 12 h in the guts of *C. carnea* and *H. axyridis*; respectively, and that the sandwich ELISA was much more sensitive than the indirect ELISA. Finally, 98 field-collected lacewings were examined for sharpshooter remains using our sharpshooter-specific sandwich ELISA. The assay detected sharpshooter egg antigen in 8.2% of the lacewings examined. This work represents a first step towards identifying the GWSS predator complex.

Keywords: ELISA; *Homalodisca coagulata*; *Homalodisca liturata*; *Harmonia axyridis*; *Chrysoperla carnea*; Leafhopper; Pierce's disease; Biological control; Predator–prey interactions; Gut content analysis; Monoclonal antibody

1. Introduction

The glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* (Say) (Hemiptera: Cicadellidae), is a polyphagous pest native to the southeastern region of the United States. It was first reported in California in 1989 (Sorenson and Gill, 1996) and has since spread throughout southern California (Blua et al., 2001). GWSS feeds on the plant's xylem fluid and can acquire and transmit *Xylella fastidiosa* Wells (Xanthomonadales: Xanthomonadaceae)

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(Redak et al., 2004). This xylem-limited bacterial pathogen is responsible for several devastating plant diseases in California such as Pierce's disease in grape (Almeida and Purcell, 2003a), almond leaf scorch (Almeida and Purcell, 2003b), and oleander leaf scorch (Costa et al., 2000). Another vector of *X. fastidiosa* is the smoke-tree sharpshooter (STSS), *Homalodisca liturata* Ball (Hemiptera: Cicadellidae). The STSS is native to California and a close relative to GWSS (Burks and Redak, 2003). Both species partially overlap in the host plants they share and in geographic ranges (Blua et al., 2001). However, while GWSS has been associated with epidemic outbreaks of Pierce's disease (Redak et al., 2004), STSS and other native leafhopper species that are known vectors of *X. fastidiosa* have only been associated with sporadic disease outbreaks (Purcell and Frazier, 1985). Moreover, Pierce's disease occurrence associated with the native leafhopper vectors have rarely been of economic significance because they are usually limited to localized portions, especially the edges of fields (Purcell and Frazier, 1985).

A multi-disciplinary research program is currently underway to develop new management techniques for *X. fastidiosa* epidemics in California (Tariq et al., 2004). A key component to this area-wide program will be to identify key natural enemies of the GWSS [National Research Council (NRC, 2004)]. To date, egg parasitoids, particularly *Gonatocerus* spp. (Hymenoptera: Mymaridae), are considered the most effective GWSS natural enemies and have been the subject of numerous studies (de León et al., 2004; de León and Jones, 2005; Irvin and Hoddle, 2005; Triapitsyn et al., 1998, 2003; Vickerman et al., 2004). However, very little effort has been expended on identifying key predators of GWSS (NRC, 2004).

Identifying the impact of insect predators can be challenging as they are usually small, elusive, nocturnal or cryptic. Direct visual field observations of predation are rare and often difficult to obtain. While predation studies using enclosures can provide some indication of predator impact, it fails to recreate natural conditions and can result in an overestimation of predation. This may be especially true for GWSS generalist predators because sharpshooter adults and nymphs are highly mobile and may easily escape predation in the field. Furthermore, GWSS may be unpalatable to some spider species, which would still catch GWSS in their webs or kill them under enclosed conditions (K. Daane, pers. obs). A more valid method to qualitatively identify predators of key pests in nature is by the molecular analysis of predator gut contents for pest remains (reviewed in Sheppard and Harwood, 2005; Symondson, 2002). The state-of-the-art predator stomach content analyses include both monoclonal antibody (MAb)-based enzyme-linked immunosorbant assays (ELISAs), which detect prey-specific proteins (Agustí et al., 1999; Greenstone and Morgan, 1989; Greenstone, 1996; Hagler et al., 1991, 1993, 1994; Schenk and Bacher, 2004; Symondson and Liddell, 1993), and polymerase chain reaction (PCR)-based assays, which detect prey-specific DNA (Agustí et al., 2003a,b; Chen et al., 2000; Hoogendoorn and Heimpel, 2001; Kasper et al., 2004; Zaidi et al., 1999). While pest-specific ELISAs have been used for over a decade to identify predators of agricultural pests (Hagler and Naranjo, 1994a,b, 2005; Hagler et al., 1992; Symondson et al., 1999), PCR-based techniques have only recently been implemented for gut content analysis of predators (reviewed in Sheppard and Harwood, 2005; Symondson, 2002). ELISA-based gut content assays possess important advantages over PCR-based assays. First, MAb-based ELISAs can be species and life stage-specific, which provides a higher level of precision to document predation (Hagler and Naranjo, 1996). Second, ELISA-based gut content assays are more suitable for screening large numbers of predators because they are less tedious, time consuming, and expensive than PCR-based assays once the pest-specific MAb has been developed (Chen et al., 2000).

Efficient molecular gut content analyses have important applications to the field of biological control. Because these methods are highly sensitive and rapid, they are powerful tools for acquiring crucial information needed to develop biological control programs targeting arthropod pests (e.g. Morris et al., 1999) or weeds (Bacher et al., 1999; Schenk and Bacher, 2004).

The main objectives of this study were to: (1) develop a MAb-based ELISA specific to GWSS egg protein, (2) determine how long GWSS egg antigen can be detected in the gut of green lacewings, *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae) and ladybird beetles, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae), and (3) examine a small number ($n = 98$) of field-collected lacewings by ELISA to determine the proportion of individuals feeding on GWSS eggs in nature.

2. Methods and materials

2.1. Antibody production

Hybridoma development was contracted out with Maine Biotechnology Services (Portland, ME). Five female mice (BALB/c, 10–12 weeks old) were immunized by intraperitoneal injections with crude GWSS egg protein (2.0 μg/ml). Each mouse received three booster injections every three weeks. The titer of each mouse sera was assayed by indirect ELISA (Hagler et al., 1991) to determine their response to GWSS egg antigen (ca. 2.0 μg/ml egg protein). The mouse yielding the highest immuno-response (1:2000-fold dilution) was selected for hybridoma fusion. Techniques leading to the production of hybridoma cell lines secreting antigen-specific MAbs were identical to those described by Hagler et al. (1991, 1994).

The screening of clones was conducted at the USDA-ARS, Western Cotton Research Laboratory (WCRL), Phoenix, Arizona, USA. A total of 50 supernatants of parental fused hybrid cells were examined by indirect ELISA. Single GWSS eggs were homogenized in 500 μl TBS buffer (Tris-buffered saline; pH 7.4). Fifty microliters of GWSS egg homogenate were placed in each well of a

96-well assay plate (Falcon Pro-Bind 3915) and incubated at 4 °C overnight. After removal of the unbound antigen from the assay plate, 330 µl of 1.0% nonfat dry milk (NFDM) in distilled H_2O was added for 30 min at 37 °C to block any remaining unoccupied sites in the wells. Wells were rinsed three times with TBS–Tween 20 (0.05%) and twice with TBS. Duplicate samples from individual supernatants (96 total) from each hybridoma culture were dispensed (50 µl) into wells of the 96-well assay plates. Each plate included a TBS blank, a positive control (polyclonal antiserum from the immunized mouse diluted 1:800 in TBS), and a negative control (pre-immune normal mouse serum diluted 1:800 in TBS). Plates were incubated for 1 h at 37 °C, then rinsed as above. Aliquots (50 µl) of goat anti-mouse IgG/lgM conjugated to alkaline phosphatase (TAGO, Burlingame, CA) diluted 1:500 in 1% NFDM were added to the wells and incubated for 1 h at 37 °C. Plates were again rinsed as above, and 50 µl of 1.0 mg/ml *p*-nitrophenyl phosphate substrate (Sigma Chemical, St. Louis, MO) in 1 M diethanolamine and 0.5 mM $MgCl_2$ (pH 9.8) was added to each well. After 30 min, the absorbance of each well was measured using a SpectraMax 250 microplate reader (Molecular Devices, Sunnyvale, CA) set at 405 nm.

We selected the 13 parental cell lines that yielded the highest response to GWSS egg antigen, and tested them further for cross-reactivity to the various life stages [egg, nymph, adult female, gravid female (such specimens were collected from colonies that were actively laying eggs) and adult male] of GWSS and STSS by the ELISA described above. Three parental cell lines were chosen for subcloning, and two subclone lines, 1D4-1D8 and 6D5-2H1, were mass produced. The two MAbs that were mass produced had very similar characteristics (e.g., they showed very similar patterns of reactivity and specificity to GWSS, STSS, and the other insects examined for cross-reactivity). Hence, only the results yielded by 6D5-2H1 are presented here. MAb 1D4-1D8 was used to develop the GWSS secondary antibody used in the sandwich ELISA (see below). The use of this MAb subclone for conjugation to the enzyme was based on random choice. Mean ELISA absorbance values yielded by each sharpshooter life stage were analyzed for differences using one-way analysis of variance (ANOVA) ($\alpha = 0.05$) followed by Tukey–Kramer multiple comparison test (SAS Institute, 2002).

2.2. Monoclonal antibody cross-reactivity tests

MAb 6D5-2H1 was screened for cross-reactivity to other arthropod species by the indirect ELISA described above with the following modifications: (1) the goat anti-mouse IgG/lgM antibody was conjugated to horseradish peroxidase (HRP) (Biosource International, Camarillo, CA, #AMI0704, diluted 1:3000 in phosphate buffered saline–Tween with 1.0% NFDM and 0.05% bovine serum albumin); (2) the substrate was TMB 1 (tetramethylbenzidine) component HRP (BioFX Laboratories, Owings Mills, MD); (3) the plates were read 10 min after substrate application; and (4) the microplate reader was set at 650 nm.

The arthropod species used for the cross-reactivity tests were selected because we considered them to be either potential predators of GWSS or possible prey for generalist predators. Arthropods were obtained from Rincon-Vitova Insectaries (Ventura, CA), obtained from colonies reared at the WCRL, or collected from fields near Phoenix, AZ. Arthropod samples ($n = 20$ per species) were prepared by grinding individuals in 500 µl TBS. Each well of the 96-well assay plate was coated separately with a 50-µl aliquot of each sample. Each plate also included a single positive control (a single GWSS egg homogenized in 500 µl TBS) and seven negative controls (TBS only). One-way analysis of variance (ANOVA) ($\alpha = 0.05$) followed by a Tukey–Kramer multiple comparison test was conducted to compare differences in the mean ELISA absorbance values for each arthropod species (SAS Institute, 2002).

2.3. GWSS egg antigen decay rates

GWSS adult females ($n = 60$) were collected from citrus orchards in Riverside, CA and placed in four $30 \times 30 \times 30$ cm cages (15 individuals/cage) containing four chrysanthemums (*Dendranthema* × *grandiflorum*). Each plant was inspected each day for new egg masses. Leaves containing eggs that were either 1, 3, 5, 7, 9, or 11 days old were frozen at −20 °C. Individual eggs of known ages were then homogenized in 500 µl TBS and assayed with MAb 6D5-2H1 using the modified indirect ELISAs described above. Single eggs were an experimental unit. One-way analysis of variance (ANOVA) ($\alpha = 0.05$) followed by a Tukey–Kramer multiple comparison test was conducted to compare differences in the mean ELISA absorbance values yielded for each egg treatment (SAS Institute, 2002).

GWSS eggs (approximately 5-day-old) were also examined by sandwich ELISA. The wells of a 96-well Costar microplate (#9017; Corning; Corning, NY) were first coated with 80 µl of the primary antibody, MAb 6D5-2H1, diluted 1:1000 in TBS. After 60 min at 27 °C, the primary antibody was discarded and 400 µl of 1.0% NFDM was added to the wells for 30 min. The NFDM was then discarded and wells were coated with either, 80-µl aliquot of GWSS egg homogenate (1 egg ground in 500 µl TBS; $n = 20$), or 80 µl TBS (negative controls, $n = 20$). After 60 min at 27 °C, the egg and TBS samples were discarded and wells were rinsed three times with TBS–Tween 20 (0.05%) and twice with TBS. Aliquots (80 µl) of the GWSS-specific HRP-conjugated secondary MAb (1D4-1D8), diluted 1:500 in 1% NFDM, was added to each well and incubated for 60 min at room temperature (see Section 2.4 for details on the secondary antibody used in the sandwich ELISA). Plates were then rinsed as above, and 80 µl of TMB 1 component HRP substrate was added to each well. After 10 min, the absorbance of each well was measured as described above.

2.4. Predator feeding trials

Feeding trials were conducted to determine how long GWSS egg antigen can be detected by ELISA in a predator's gut after consumption, and if consumption of alternative prey items affects the sensitivity of the ELISA for detecting GWSS. The predators tested were third-instar *C. carnea* and adult *H. axyridis*. Both species were purchased from Rincon-Vitova Insectaries (Ventura, CA). The gender of *H. axyridis* individuals was not determined but the sex ratio was approximately 1:1 (Rincon-Vitova Insectaries, pers. comm.). We selected these two species because they represent small (*C. carnea*) and large (*H. axyridis*) predator species, they are commonly found in California (Koch, 2004; Rosenheim, 2001); they are voracious predators (Koch, 2004; Zheng et al., 1993); and lacewings have been observed feeding on GWSS eggs in the wild (K. Daane, pers. obs.).

Prior to the feeding trials, individual predators were placed in 4.0-cm diameter petri dishes and starved (with ad libitum access to water via a $0.5 \times 0.5 \times 05$-cm cube of sponge saturated with water) for 36 h. We determined the number of GWSS eggs offered to each predator species by conducting preliminary trials to examine their typical level of hunger (data not shown). GWSS eggs used the trials were 3- to 5-day-old. Lacewings were fed three GWSS eggs over a 45-min period and were isolated from food (with ad libitum access to water) or given ad libitum access to pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) eggs after eating the GWSS eggs. The post-feeding intervals tested were 0, 6, 9, 12, 24, and 30 h ($n = 20$ individuals per interval and 20 negative controls). For the 6, 9, and 12 h post-feeding intervals, the predators were held under constant light at 25 °C. For the 24 and 30 h intervals, the predators were maintained under a 16:8 h (L:D) photoperiod at 25 °C. After each post-feeding interval, the individuals were frozen at −80 °C. Similarly, *H. axyridis* were fed five GWSS eggs over a 45-min period, and isolated from food (except water) for 0, 3, 6, 9, and 12 h. The whole body of each individual predator was homogenized in 500 μl TBS buffer and assayed initially by an indirect ELISA using MAb 6D5-2H1 as described above. The poor results yielded from the indirect gut content ELISA prompted us to develop a sandwich gut content ELISA. The 1D4-1D8 MAb was conjugated to horseradish peroxidase by Lampire Biological Laboratories (Pipersville, PA) using their proprietary protocol. Predators were scored positive for prey remains if they yielded a sandwich ELISA response three standard deviations above the mean of their respective negative control (Sutula et al., 1986). Student's *t* test was conducted on these data to determine if there were significant differences between the diet treatments (i.e., no alternative prey vs. alternative prey available following the consumption of GWSS eggs) (SAS Institute, 2002). Finally, we calculated the prey detection half-life for each predator species using Logit regression (STATA, 2003).

2.5. Gut content evaluation of field-collected lacewings

The gut contents of 98 field-collected *Chrysoperla* spp. (approximately 90% *C. carnea*; 10% C. *Comanche* Bank, *C. rufilabris* Burmeister, and *Chrysopa nigricormis* Burmeister) larvae were analyzed by the sandwich ELISA as described above. The larvae were collected from various shrubs and ornamental trees that harbored GWSS from October 2002 to October 2004 in Bakersfield, CA. Predators were collected by beating the foliage and branches of the plants with a wooden stick over a sweep net placed under the host plant for ca. 30 s. All collections were made between 08:30 and 14:00. Specimens were immediately placed in a cooler containing dry ice and then stored at −80 °C upon arrival at the laboratory.

Each individual lacewing larva was first homogenized in 180 μl of phosphate buffered saline (PBS, pH 7.4). A 20-μl aliquot from the homogenized sample was then pipetted into a 1.5-ml microtube containing 120 μl PBS. The remaining 160-μl from the original sample was stored at −80 °C for future analysis. Negative control lacewings ($n = 24$) used for this study were only fed pink bollworm eggs until they reached their 3rd instar. Field-collected lacewings were scored positive for GWSS egg antigen if they yielded an ELISA response three standard deviations above the mean of the negative controls (Sutula et al., 1986).

3. Results

3.1. Specificity and cross-reactivity tests

Subclones 1D4-1D8 and 6D5-2H1 were selected for mass production because of their high ELISA reactivity to GWSS egg antigen and lack of cross-reactivity to the other arthropod species examined. Both MAbs yielded equally clear results, showing similar patterns of sensitivity and species specificity. For brevity, only the results obtained for MAb 6D5-2H1 are presented here.

Specificity tests revealed that the MAb only reacted to the egg, and to a lesser extent, to the adult gravid female stage of GWSS and STSS (Fig. 1). None of the other 27 species of arthropods tested reacted to 6D5-2H1 (Fig. 2). The positive control, a single GWSS egg, yielded a significantly higher ELISA response (approximately 25 times higher) than all the other arthropod treatments (Fig. 2).

3.2. GWSS egg antigen decay rates

The indirect ELISA results indicate that younger GWSS eggs are significantly more immuno-reactive than older eggs (Fig. 3). For instance, 1- and 3-day-old eggs yielded ELISA absorbance values two times greater than eggs that were over 7 days old. Despite the reduction in reactivity of the MAb to older eggs, all egg treatments still yielded ELISA values at least 9-fold higher than the TBS control, suggesting that GWSS egg antigens remain detectable until eggs hatch.

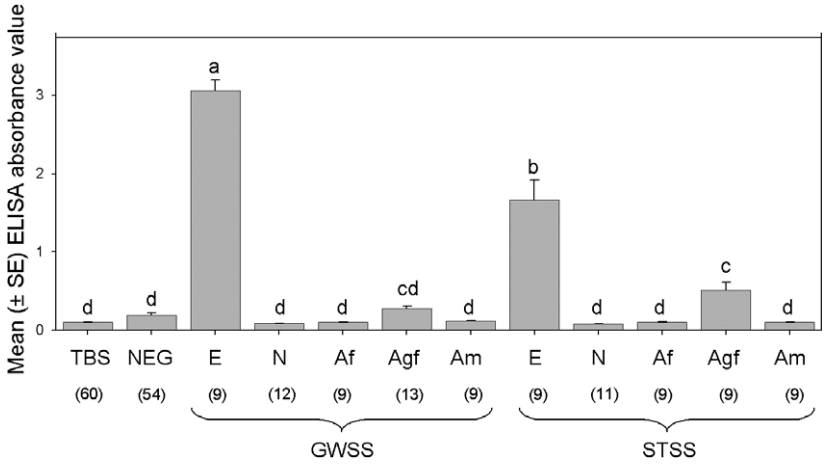

Fig. 1. Mean (±SE) ELISA response of egg-specific monoclonal antibody 6D5-2H1 to tris-buffered saline (TBS) blanks, normal mouse serum negative controls (NEG), egg (E), nymph (N), adult female (Af), adult gravid female (Agf), and adult male (Am) life stages of the glassy-winged sharpshooter (GWSS), and the smoke-tree sharpshooter (STSS) ($F_{11,205} = 170.9$, $P = 0.0001$). The numbers in parenthesis below each life stage examined are the sample size. Bars with different letters are significantly different (Tukey–Kramer test; $\alpha = 0.05$).

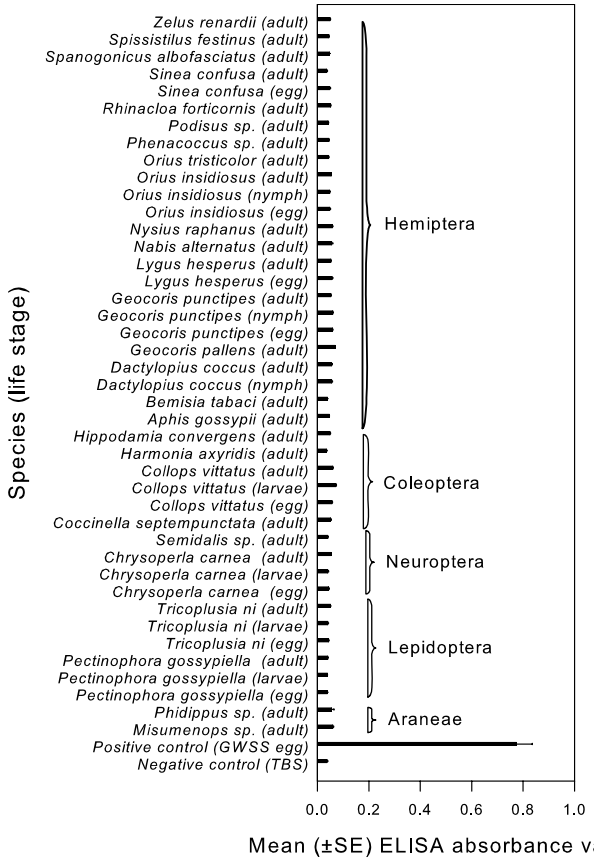

Fig. 2. Mean (±SE) ELISA response of egg-specific monoclonal antibody 6D5-2H1 to different life stages of several arthropod species ($F_{43,822} = 61.3$, $P = 0.0001$; $n = 20$ per species).

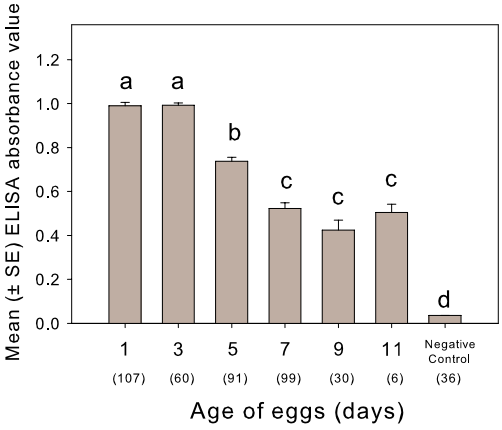

Fig. 3. Mean (±SE) ELISA response of egg-specific monoclonal antibody 6D5-2H1 to GWSS eggs ranging in age from 1 to 11 days old and TBS negative controls ($F_{6414} = 174.5$, $P = 0.0001$). The numbers in parenthesis are the sample size for each treatment. Bars with different letters are significantly different (Tukey–Kramer test; $\alpha = 0.05$).

3.3. Predator feeding trials

The results of the lacewing feeding trial are given in Fig. 4. Generally, the percentage of lacewings scoring positive by ELISA decreased as the time after feeding increased. The indirect ELISA format was unreliable for the detection of GWSS egg antigen in lacewing guts beyond the 0 h post-meal feeding interval (Fig. 4A). In comparison, the sandwich ELISA was much more effective at detecting GWSS egg remains in the lacewing guts, particularly in the guts of those lacewings that were provided additional prey after consuming GWSS eggs (Fig. 4B). For example, over 80% of the individuals yielded a positive ELISA response for GWSS egg remains up to 24 h after feeding (Fig. 4B). We found that the presence of GWSS egg protein remained detectable by sandwich ELISA for up to 30 h in lacewing guts when alternative prey was available following the ingestion of GWSS eggs (Fig. 4B). We also found that the sandwich ELISA format can detect GWSS egg antigen significantly longer in lacewing individuals that were provided with alternate prey after feeding on GWSS eggs (Fig. 4B). The predicted half-life that GWSS eggs could be detected in

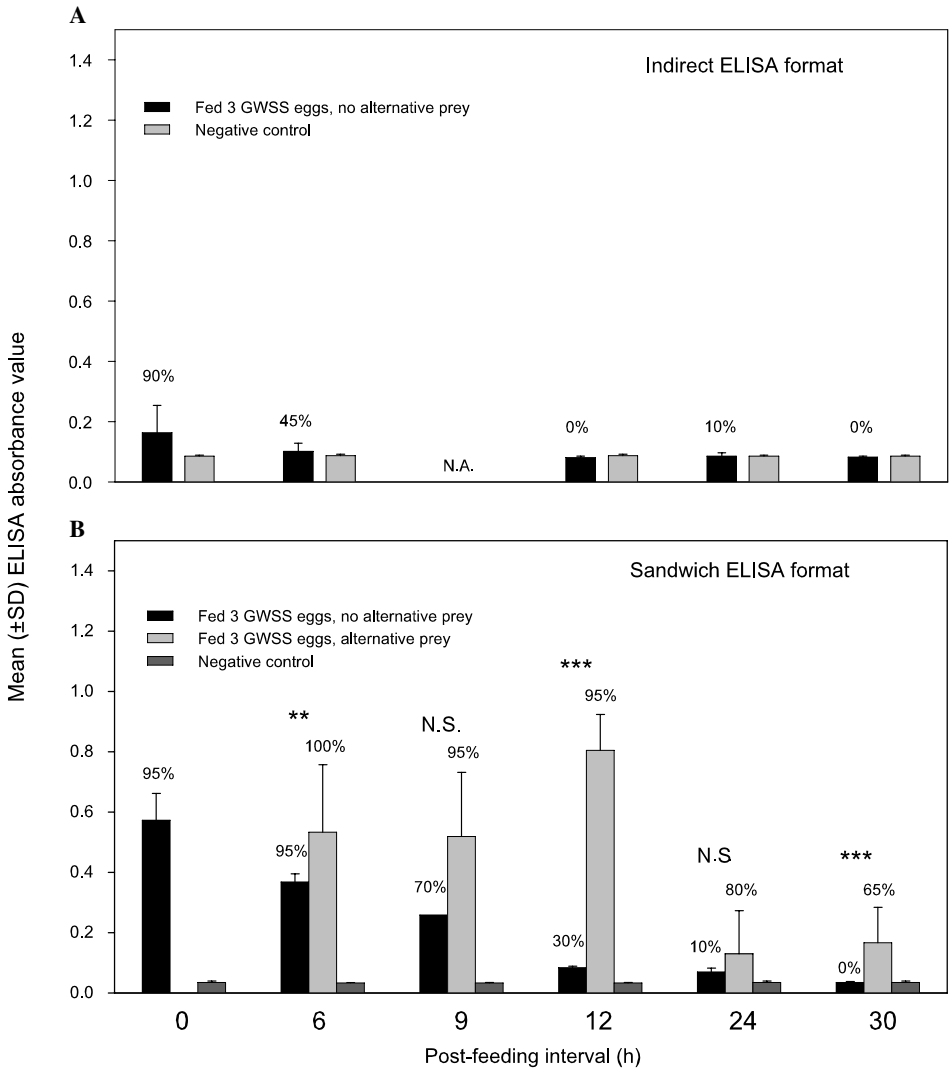

Fig. 4. Mean (±SE) ELISA response of egg-specific monoclonal antibody 6D5-2H1 to individual *Chrysoperla carnea* fed three GWSS eggs, held for various time intervals, and tested by indirect ELISA (A), or sandwich ELISA (B). Following the ingestion of the target prey, lacewings were either given a diet of water only (no alternative prey available), or ad libitum access to pink bollworm eggs (alternative prey available). For the 6, 9, and 12 h post-feeding intervals, the predators were held under constant light at 25 °C. For the 24 and 30 h intervals, the predators were maintained under a 16:8 h (L:D) photoperiod at 25 °C. The number above each error bar represent the percentage of individuals scoring positive for each treatment ($n = 20$ per treatment). For the sandwich ELISA format (B), the symbols represent significant differences between the absorbance values of lacewings under both diet regimes (Student's t test): $^{**}P < 0.01$; $^{***}P < 0.001$; NS, not significant. None of the negative controls scored positive for GWSS remains.

the gut of those *C. carnea* that were not provided with additional prey after feeding was 5.5 h by the indirect ELISA and 11.8 h by the sandwich ELISA. When *C. carnea* were provided with additional prey after feeding on GWSS eggs the predicted half-life increased to 34.4 h by the sandwich ELISA.

The results of the ladybird beetle feeding trial are given in Fig. 5. The indirect ELISA was unreliable at detecting GWSS egg remains at all of the post-meal retention intervals tested (Fig. 5A). With the sandwich format, however, 100% of the ladybird beetles scored positive immediately (0 h) after consuming GWSS eggs (Fig. 5B). But only 5% of the individuals were positive 12 h after ingesting the target prey (Fig. 5B). Due to low detection rates, we did not undertake the evaluation of longer time intervals (>12 h) with *H. axyridis*. Poor detection rates prevented us from predicting the prey retention half-life for *H. axyridis* individuals assayed with the indirect ELISA format. The GWSS prey detection half-life was 2.2 h when the beetles were assayed by the sandwich ELISA format.

3.4. Gut content evaluation of field-collected lacewings

The gut content analyses of field-collected lacewings revealed that 8 of the 98 (8.2%) individuals examined contained sharpshooter egg antigen in their guts (Fig. 6). Moreover, 7 out of the 8 positive ELISA reactions yielded ELISA values 10–47 times higher than that of the unfed *C. carnea* (negative controls).

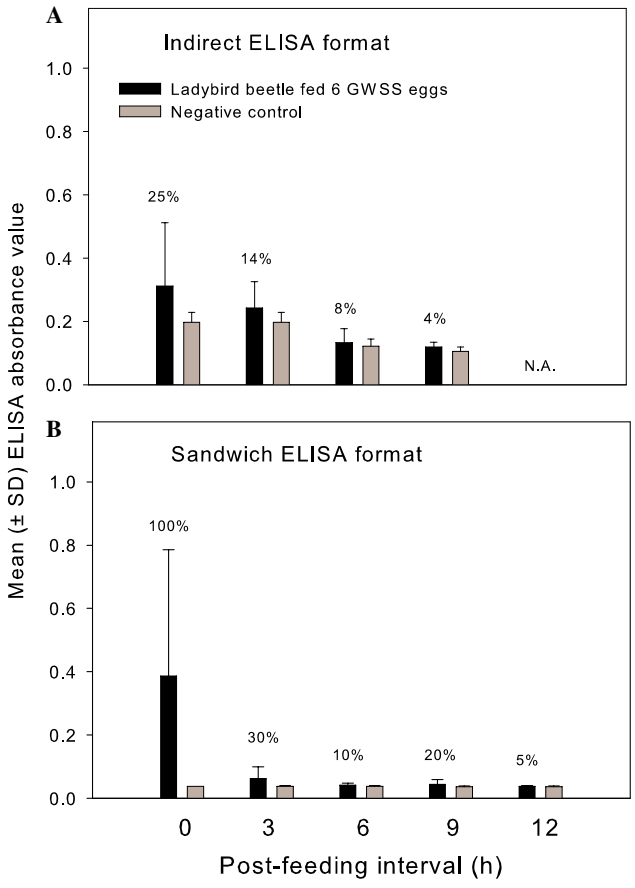

Fig. 5. Mean (±SE) ELISA response of egg-specific monoclonal antibody 6D5-2H1 to individual *Harmonia axyridis* fed five GWSS eggs, held for various time intervals under constant light at 25 °C and tested by indirect (A), or sandwich (B) ELISA format. The number above each error bar is the percentage of individuals scoring positive for each treatment ($n = 20$–25 per treatment). None of the negative controls scored positive for GWSS remains.

4. Discussion

4.1. MAb specificity, cross-reactivity, and antigen decay

Our original goal was to develop a GWSS egg-specific MAb. However, our results showed that our MAb is specific to both GWSS and STSS egg antigens (Figs. 1 and 2). The cross-reactivity with STSS does not necessarily reduce the usefulness of this MAb as a tool for identifying key predators of GWSS. For instance, while both sharpshooter species co-occur in some geographical areas of southern California, the sole or predominant sharpshooter species found on commercially important host plants such as citrus and grapes is GWSS (Blua et al., 2001). Therefore, we are reasonably confident that predators collected in citrus and grapes yielding a positive ELISA reaction can be attributed to predation on a GWSS egg or a gravid female. If predators are collected in regions inhabited by other closely related leafhopper species, additional cross-reactivity tests will be warranted.

In addition to the strong immuno-reactivity to GWSS and STSS eggs, our results revealed that the MAb reacted to gravid sharpshooter females (Fig. 1). We speculate that this is most likely explained by a high concentration of vitellin in the ovaries of gravid females (Raikhel and Dhadialla, 1992). Similarly, we found that younger GWSS eggs were more reactive than older eggs (Fig. 3), which supports the observation that vitellin is depleted as the embryo develops and reaches maturity (Chen, 1978). The age related decay rate of GWSS egg antigen may adversely affect the prey detection half-life of our gut content ELISA. This is an area of research that deserves further investigation.

We targeted the egg stage for the development of our MAbs rather than the other sharpshooter life stages for three reasons. First, while GWSS adults and nymphs are highly mobile and can easily escape attack by generalist predators, the sessile eggs are more likely to be vulnerable to natural enemies. Second, higher success rates have been achieved in developing MAbs that recognize egg proteins of insects compared to proteins from other life stages (Agustí et al., 1999; Greenstone, 1995; Hagler et al., 1991, 1993, 1994; but see Bacher et al., 1999). In insects, vitellogenin-derived proteins constitute 60–90% of all yolk proteins (Chapman, 1998). Following their internalization (or endocytosis) in the oocytes, some yolk proteins undergo transformations (e.g., phosphorylation, sulfation, or glycosylation) which give rise to high molecular weight species-specific proteins (Raikhel and Dhadialla, 1992). Injecting a relatively simple mixture of high molecular weight proteins (>5000 Da) into a mouse is generally more immunogenic than a complex mixture of low molecular weight proteins (Harlow and Lane, 1988). Finally, we have developed a PCR-based assay that can detect GWSS DNA in predator guts (de León et al., 2006) and we are currently combining the attributes of ELISA and PCR assays to identify key predators of GWSS (Fournier et al., in preparation). Therefore, we can differentiate egg predation from adult and nymphal predation. Specifically, we first assay field-collected predators for sharpshooter DNA using the GWSS-specific PCR assay to determine if a predator has ingested GWSS. A positive response in the PCR assay indicates that the predator has fed on a GWSS egg, nymph or adult. Predators scoring positive in the PCR assay are then assayed by the GWSS egg-specific ELISA described here to determine if they contain GWSS egg antigen.

4.2. Predator feeding trials

The sandwich ELISA format consistently yielded higher percentages of positive individuals and longer detection periods than the indirect ELISA format (Figs. 4 and 5). This finding corroborates results from other studies in which different ELISA formats were compared (Hagler, 1998). In an indirect ELISA, predator samples are first added to the ELISA microplate. Predator samples containing a large quantity of nontarget protein (e.g., *C. carnea* and

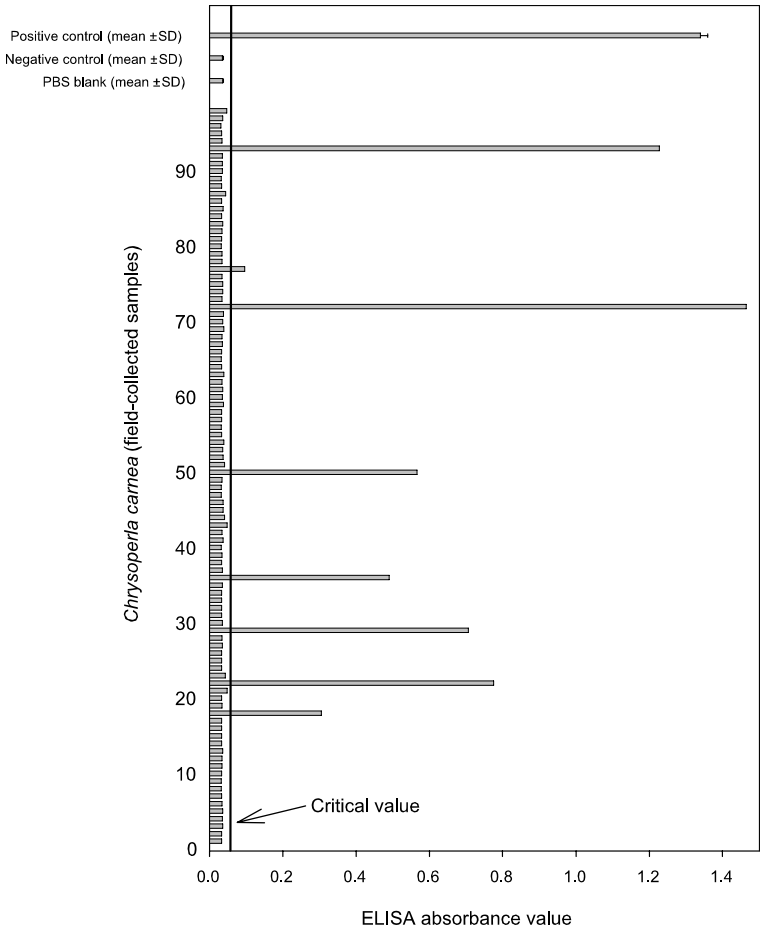

Fig. 6. ELISA response of egg-specific monoclonal antibody 6D5-2H1 to field-collected lacewing *Chrysoperla carnea* ($n = 98$), PBS blanks ($n = 44$), negative controls (*C. carnea* not fed GWSS; $n = 48$), and positive controls (GWSS egg, $n = 2$) using the sandwich ELISA. Lacewing larvae scored positive for GWSS egg remains when they yielded an ELISA response three standard deviations above the mean of the negative controls (critical value = 0.046).

H. axyridis) can potentially saturate the limited number of competitive binding sites on an ELISA microplate matrix. Consequently, the probability of minute traces of GWSS egg antigen reaching one or more of the binding sites on the indirect ELISA plate is greatly reduced (Hagler et al., 1997). The net outcome is high incidence of false-negative ELISA reactions. This problem inherent to an indirect ELISA, can be overcome by developing a sandwich ELISA. A sandwich ELISA is designed to "capture" a rare antigen from a complex mixture. In a sandwich ELISA, each microplate well is first coated with the pest-specific MAb. After blocking, the homogenized predator is then added. Since the ELISA microplate was first coated with GWSS-specific MAb, the only antigen that can attach to the well is the protein that the MAb was developed to detect (the targeted prey remains). The "sandwich" is completed by adding a conjugated pest-specific antibody. Our results and the results of others suggest that the extra effort needed to conjugate a MAb to an enzyme is essential for the development of a reliable predator gut content immunoassay (Hagler, 1998; Hagler et al., 1997).

Overall, our results on GWSS egg antigen retention in predator guts (Figs. 4 and 5) are comparable to detection periods reported in other studies using prey-specific MAbs under similar temperatures (Agustí et al., 1999; Hagler and Naranjo, 1997; Symondson and Liddell, 1996). Several factors, including predator species, digestive rate, physiological state of the predator and prey (e.g., age of the prey), predator size (e.g., protein concentration), meal size, and temperature have been found to impact detection periods of prey antigen in predator guts (Agustí et al., 1999; Hagler, 1998; Hagler and Naranjo, 1997; Hagler et al., 1992, 1997; Schenk and Bacher, 2004; Sunderland, 1996). Detection period of prey remains is an important factor to consider when collecting predators for gut content analyses (Schenk and Bacher, 2004). For example, if predators are assumed to feed equally on a prey, predators exhibiting longer detection intervals will score positive more frequently than predators with shorter detection intervals. Such a scenario could lead to an over estimation of predation for predators with a long prey retention interval and an underestimation of predation for predators with a short prey retention interval. Moreover, some predators might feed strictly during the day or night (Pfannenstiel and Yeargan, 2002). Thus, it is critical to collect sufficiently large numbers of predators (see Hagler and Naranjo, 2005), and the collections should

be made frequently (e.g., every 1 or 2 h) through the day and night.

Finally, we found that *C. carnea* individuals that were provided with alternative prey after feeding on GWSS eggs showed significantly longer detection periods than individuals that were not (Fig. 4B). Only a few studies have compared the decay of target prey antigens in predators exposed to both feeding regimes (alternative prey vs. no alternative prey). These studies yielded contradicting results (Fichter and Stephen, 1984; Lövei et al., 1990; Symondson and Liddell, 1995). For instance, Fichter and Stephen (1984) found fewer positive ELISA reactions for pest moth remains when arachnid predators were fed alternate prey. Similarly, Lövei et al. (1990) monitored prey antigen decay rates among three species of predaceous beetles and observed detection periods to be consistently shorter (albeit the differences were not statistically different) for the predators provided with *ad libitum* access to alternative food. In contrast, Symondson and Liddell (1995) reported significantly longer detection periods when carabid beetles were fed with alternative prey after consuming the target prey.

4.3. Gut content evaluation of field-collected lacewings

The gut content analysis of field-collected lacewings revealed that 8.2% of the 98 individuals examined contained sharpshooter egg antigen in their guts. This is the first study that provides proof of in-field predation of GWSS eggs by lacewings. Although the sample size of tested specimens was relatively small ($n = 98$), our finding suggest that *C. carnea* is a potential biological control candidate for GWSS. Further predator gut content examinations are being conducted on lacewings and a wide variety of other predator species collected en masse from various host plants inhabited by GWSS (Fournier et al., in preparation.).

Predator gut content analyses by ELISA offer a unique means for studying several aspects of predator–prey interactions. First, because it involves no disturbance of the studied organisms prior to the collection of predators, it yields unequivocal information on predators' prey choice under natural, open-field conditions. Second, ELISA gut content analyses provide a precise and cost-effective way for rapid screening of large number of predators. For example, Hagler and Naranjo (2005) examined the gut contents of over 32,000 field-collected predators, representing a total of nine taxa, for the presence of *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae) remains. The key predators identified in that study are now being exploited for a conservation biological control program targeting whitefly pests in cotton agro-ecosystems (Hagler and Naranjo, 2005; Naranjo et al., 2004).

While molecular gut content assays are invaluable for the qualitative assessment of predation, they have limitations. First, ELISA gut assays are not quantifiable (see Hagler and Naranjo, 1996; and Naranjo and Hagler, 1998 for a review). Second, false positive ELISA reactions can sometimes occur if a higher-order predator feeds on a predator that had previously preyed upon a GWSS (e.g., secondary predation) (Harwood et al., 2001). Finally, ELISA false positive reactions for direct predation can also occur if a predator consumes a dead GWSS (e.g., scavenging) (Calder et al., 2005). Molecular techniques designed specifically to overcome these limitations are currently being developed (JRH, submitted).

The development of a GWSS/STSS egg-specific ELISA provides a tool to complement PCR-based assays in identifying prey remains in predator stomachs. While DNA markers can effectively detect prey remains (Agustí et al., 1999, 2003a,b; Chen et al., 2000; Hoogendoorn and Heimpel, 2001; Zaidi et al., 1999), they give no indication of which prey stage is consumed. Combining ELISA and PCR assays can reveal which life stage(s) is most vulnerable to predation by any given predator species. Furthermore, using two methods of predator gut analysis greatly increases the reliability of the data. To this end, we have developed a PCR-based assay that detects GWSS DNA in predator guts (de León et al., 2006), and we are currently assaying field-collected predators using both methods (Fournier et al., in preparation).

5. Conclusion

There has been increasing awareness over the past decade of the importance of generalist predators for biological control of insect pests (reviewed in Symondson et al., 2002). Moreover, the exploitation of indigenous predators greatly limits the risk of nontarget effects in biological control programs aimed towards invasive pests such as GWSS in California (Follett and Duan, 2000). Once the key predators of the various life stages of GWSS are identified, this information can be used to better implement effective conservation or inundative biological control programs.

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