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Incidence of microbial infections revealed by assessing nodulation in field-collected insects from Adana Province

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Abstract: Results of a field study designed to assess the extent of natural microbial infections in insects collected from agrarian fields surrounding Adana, Turkey, are reported. We identified and dissected specimens to assess the numbers of nodules. Formation of darkened melanotic nodules is the predominant cellular immune reaction to microbial and parasitic infection, and once formed, the nodules are permanently attached to internal surfaces. At least some nodules were found in 99% of 1200 examined specimens that were healthy in appearance. The number of nodules ranged approximately from 1 nodule/individual to >120 nodules/individual. We inferred that insects are regularly challenged by microbial and parasitic infections. The key implication of these data is that insect immune systems can limit the host range and effectiveness of microbial agents deployed in biological control programs. Future advances in the efficacy and use of biopesticides will depend on understanding and attenuating insect innate immune effector systems. Some insect pathogens have already evolved effective mechanisms to achieve this advance.

Key words: Biological control, insect immunology, naturally occurring infections, nodulation

1. Introduction

Entomopathogenic microbes, occurring naturally, are virulent insect pathogens that include viruses, fungi, and bacteria. A wide range of lethal parasites also infect insects. Some of these organisms serve as important natural regulators of insect populations (Lacey et al., 2001). Appreciation of insect diseases and the possibilities of using insect disease agents in biocontrol programs have a long history (Steinhaus, 1957; Tanada, 1959). Commercially useful agents include viruses, fungi, bacteria, protozoans, parasitoids, nematodes, and predators, all deployed in the biocontrol of insect pests, weeds, and plant diseases. The following example illustrates this point.

The control of the rhinoceros beetle, *Oryctes rhinoceros*, is among the successes in microbial control (Caltagirone, 1981). This insect was responsible for severe damage to oil palms in Asia, including Malaysia, Fiji, and Western Samoa. After considerable efforts with parasites and predators, a search for *O. rhinoceros* diseases led to the discovery of a new virus called *Rhabdionvirus oryctes* (Hüger, 1966). The virus was introduced into Western Samoa and several other islands, where it became established. Oil palm losses were very effectively reduced (Hüger, 1966). Other biocontrol programs were not as successful. The use of *Bacillus thuringiensis* var. *kurstaki* (*Btk*) against the diamondback moth in cole crops (cabbage, broccoli, etc.) enjoyed a large, albeit short-lived success. *Btk* was developed into commercial products that competed with traditional chemical control throughout the 1980s. However, the overuse of these products created field resistance, the first field resistance to a *Bt* product to be recorded (Tabashnik et al., 1990).

Many factors affect the relative success and failures of biocontrol of insect pests, including costs, the context of comprehensive integrated pest management programs, education of users, government activities, and political and environmental concerns (Lomer, 1999). Viewed from a technical perspective, however, successful biocontrol depends on biological issues. These issues span a range of biological organization from the ecological level of microbe–host population dynamics to the molecular and cell biology of host defense mechanisms.

One of the most important barriers to successful deployments of microbial control agents may lie in insects' robust and complex innate immune effectors. Insect innate immunity comprises a number of host defense effector systems. The insect integument and alimentary canal are formidable physical barriers to microbial invasion. Once past these barriers, invading microbes are confronted with fast-acting cellular defense actions,

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including phagocytosis and nodule formation (Lavine and Strand, 2002; Stanley and Miller, 2006). These cellular defense reactions begin immediately after an infection is detected within an insect. Some hours after an infection is detected, the insects unleash an array of antimicrobial peptides that constitute the humoral immune system (Lemaitre and Hoffmann, 2007). The combined arsenal of immune effector mechanisms allows insects to either stifle infections at their onset or overcome invasions, infections, and wounds. These mechanisms can limit the effectiveness of microbes deployed for biocontrol of insect pest populations. Tunaz and Stanley (2009) showed that most insects in agrarian habitats of Kahramanmaraş, Turkey, experience naturally occurring infections. The insects recover from invading microbes with fast-acting cellular defense actions, including nodule formation.

Until Tunaz and Stanley's study (2009), it was unclear which insect immunity functions protected insects from microbial/parasitic infections in nature. However, now we know that at least one of the insect immunity functions is nodulation, which protects insects from infections in nature. It is also not known how insect immunity can influence biocontrol programs, but laboratory and field experiment results indicate that insect febrile reactions alone may limit the effectiveness of fungal biocontrol agents (Ouedraogo et al., 2004). We are investigating the hypothesis that most insects living in agrarian fields experience infections and recover from them. If supported, the significance of our hypothesis is that insect immunity can impose limitations on the effectiveness of microbial-based biocontrol programs. This study reports the results of a field investigation designed to test the hypothesis.

2. Materials and methods

2.1. Insects

We collected the insects from fields surrounding the city of Adana, Turkey, in 2010–2013, using either hand collection or routine sweep net procedures. The collected species, collection sites, site altitudes, and biological stages are indicated in Section 3. The specimens were transferred to the laboratory (20 ± 1 °C, 60 ± 5% RH) at Kahramanmaraş Sütçü İmam University. Most insects were identified to the species level. We placed voucher specimens in the entomology collection of Kahramanmaraş Sütçü İmam University.

2.2. Nodulation assay

After identification of insect specimens, the extent of nodulation was assessed. Insects were anesthetized by chilling on ice and their hemocoels were then exposed. Melanized brownish-black nodules were counted under a stereomicroscope at 45×. The nodules were distinct, and direct counting reliably reflected the extent of the nodulation response to infections (Miller and Stanley, 1998). After the first count the alimentary canal was removed. Nodules in previously unexposed areas and remaining internal tissues were then counted.

2.3. Statistical analysis

We analyzed the data on nodulation using the general linear models procedure, and mean comparisons were made using least significant difference test ($P \leq 0.0001$) (SAS Institute, 1989).

3. Results

We assessed nodulation in a total of 120 insect species collected during winter, spring, summer, and fall of 2011, 2012, and 2013 (Tables 1–3). In the broadest description, nodules were recorded in 99% of the 1200 specimens examined, although there was a very wide range of nodules/specimen from 1 nodule/insect to >120 nodules/insect.

We recorded a significantly higher number of nodules from insects associated with soil than from insects collected from plants (Table 4). This is true, for example, in sunn pest adults collected in April 2011 and 2013 (Tables 1 and 3). We also noted that the new generation of sunn pests had very few nodules (approximately 10/adult) compared to older, overwintered adults (>105/individual) (Table 3). The 3-year averages for insect orders are shown in Table 5. We recorded significantly more nodules in the orthopteran species than in the lepidopteran, hemipteran, and coleopteran species (Table 5), which is due to orthopteran species mostly being collected from soil. We recorded statistically similar numbers of nodules in larvae, nymphs, and adults of insect species (Table 6). In general, insect orders in contact with soil are probably the main associations with a higher number of nodules. While the actual occurrence of natural infection may be a random event with no predominant patterns, the data indicate that virtually all insects had experienced infection(s).

4. Discussion

The data reported in this paper support our hypothesis that most insects in agrarian fields experience microbial infections, from which they may recover and continue their lives. Several points support this idea. First, we recorded nodules in virtually all examined insect specimens. Second, nodules occurred in species representing major insect orders, including Coleoptera, Lepidoptera, Hemiptera, and Orthoptera. Third, we recorded more nodules from insects found in the soil, a site of significant microbial challenge, than other sites. We infer that insects are generally exposed to microbial challenges throughout their lives and in a great number of cases they probably survive the infections.

The nodulation process is the predominant insect cellular defense action. In their study of tobacco hornworm

Table 1. Average numbers of nodules in insects collected from fields in the Adana region in 2011. Values indicate numbers of discrete nodules ± SEM. Collection dates are in dd/mm/yy .

	Nodules/insect	Collection site, biological stages	Number of individuals	Collection date, altitude
Lepidoptera				
Pieris brassicae	54.1 ± 22.8	Weeds, larvae	10	23/04/11, 50 m
Heliothis armigera	24.9 ± 15.0	Cotton, larvae	10	12/08/11, 25 m
Ostrinia nubilalis	50.2 ± 18.9	Corn stalk, larvae	10	16/09/11, 15 m
Heliothis armigera	20.5 ± 7.3	Alfalfa, larvae	10	07/10/11, 40 m
Spodoptera littoralis	17.2 ± 4.3	Alfalfa, larvae	10	07/10/11, 25 m
Sesamia nonagrioides	8.9 ± 2.5	Corn stalk, larvae	10	15/10/11, 10 m
Hemiptera				
Eurygaster integriceps	120.3 ± 28.2	Soil, wintered adults	10	24/04/11, 70 m
Nezara viridula	13.2 ± 1.2	Alfalfa, adults	10	24/04/11, 70 m

Table 2. Average numbers of nodules in insects collected from fields in the Adana region in 2012. Values indicate numbers of discrete nodules ± SEM. Collection dates are in dd/mm/yy.

	Nodules/insect	Collection place, biological stages	Number of individuals	Collection date, altitude
Lepidoptera				
Pieris brassicae	52.3 ± 18.2	Weeds, larvae	10	29/04/12, 50 m
Helicoverpa armigera	6.1 ± 1.8	Alfalfa, larvae	10	30/06/12, 100 m
Helicoverpa armigera	4.2 ± 0.9	Alfalfa, larvae	10	04/07/12, 100 m
Helicoverpa armigera	5.6 ± 1.2	Alfalfa, larvae	10	15/10/12, 100 m
Helicoverpa armigera	13.9 ± 1.2	Cabbage, larvae	10	12/11/12, 29 m
Spodoptera exigua	25.8 ± 6.7	Alfalfa, larvae	10	20/04/12, 80 m
Spodoptera exiqua	6.7 ± 1.5	Alfalfa, larvae	10	15/10/12, 56 m
Aspitates ochrearia	18.5 ± 6.5	Weeds, larvae	10	30/05/12, 1100 m
Scopula sp.	30.7 ± 8.3	Weeds, adults	10	11/06/12, 1000 m
Amata sp.	36.3 ± 2.6	Weeds, larvae	10	11/06/12, 1050 m
Colias croceus	2.4 ± 0.8	Alfalfa, larvae	10	04/07/12, 100 m
Geometridae	8 ± 1.7	Alfalfa, larvae	10	21/06/12, 100 m
Ostrinia nubilalis	5 ± 1	Corn stalk, larvae	10	12/11/12, 29 m
Sesamia nonagrioides	9.6 ± 5.1	Corn stalk, larvae	10	15/10/12, 50 m
Sesamia nonagrioides	22.1 ± 3.1	Corn stalk, wintered larvae	10	12/11/12, 29 m
Sesamia nonagrioides	21.8 ± 2.3	Corn stalk, wintered larvae	10	03/12/12, 58 m
Spodoptera littoralis	14.8 ± 1.8	Alfalfa, larvae	10	03/12/12, 60 m
Autographa gamma	6.3 ± 2.02	Alfalfa, larvae	10	03/12/12, 56 m
Coleoptera				
Agriotes sp.	3.5 ± 0.6	Alfalfa, adults	10	08/04/12, 80 m
Agriotes sp.	3.3 ± 0.3	Soil, adults	10	21/06/12, 100 m
Gonioctena fornicata	0.8 ± 0.3	Alfalfa, adults	10	15/04/12, 80 m
Gonioctena fornicata	6.6 ± 1.4	Alfalfa, larvae	10	08/04/12, 80 m
Gonioctena fornicata	1.1 ± 0.4	Weeds, adults	10	11/05/12, 100 m
Coccinella semptempunctata	2.5 ± 0.6	Weeds, larvae	10	29/04/12, 80 m
Coccinella semptempunctata	1.9 ± 0.5	Weeds, adults	10	11/05/12, 100 m
Coccinella semptempunctata	1 ± 0.4	Weeds, adults	10	11/06/12, 1000 m
Coccinella semptempunctata	1.7 ± 0.6	Alfalfa, adults	10	15/10/12, 50 m
Coccinella semptempunctata	1.3 ± 0.4	Weeds, adults	10	12/11/12, 30 m
Coccinella semptempunctata	1.1 ± 0.4	Weeds, adults	10	03/12/12, 55 m
Coccinella undecipunctata	7.5 ± 1.3	Alfalfa, adults	10	21/06/12, 100 m
Anisoplia spp.	6.6 ± 3.1	Weeds, adults	10	11/05/12, 100 m

Table 2. (Continued).

Anisoplia austriaca	9.2 ± 1.4	Weeds, larvae	10	11/06/12, 1030 m
Psylliodes sp.	1.2 ± 0.3	Weeds, larvae	10	11/05/12, 100 m
Larinus latus	34.3 ± 6.2	Weeds, adults	10	20/05/12, 90 m
Larinus latus	16.4 ± 3.6	Weeds, adults	10	11/06/12, 1100 m
Mylabris variabilis	10.2 ± 1.9	Weeds, adults	10	30/05/12, 1100 m
Triponita hirta	9.3 ± 1.5	Weeds, adults	10	06/05/12, 100 m
Triponita hirta	6.9 ± 1.6	Weeds, adults	10	21/06/12, 100 m
Lixus sp.	8.6 ± 6.2	Weeds, adults	10	11/06/12, 1100 m
Clytra quadripunctata	16.0 ± 2.0	Weeds, adults	10	11/06/12, 1000 m
Scarabaeus sp.	6.2 ± 1.0	Weeds, adults	10	11/06/12, 1100 m
Agapanthia kirby	116.0 ± 1.8	Weeds, adults	10	11/06/12, 1000 m
Myriochile meloncholica	116.0 ± 1.8	Soil, adults	10	21/06/12, 100 m
Myriochile melancholica	18.7 ± 2.5	Soil, adults	10	15/10/12, 50 m
Carabidae	21.0 ± 4.0	Soil, adults	10	21/06/12, 100 m
Hypera variabilis	2.4 ± 0.5	Alfalfa, adults	10	21/06/12, 100 m
Hypera variabilis	1.0 ± 0.3	Alfalfa, adults	10	03/12/12, 55 m
Hemiptera				
Dolycoris baccarum	28.4 ± 9.0	Alfalfa, adults	10	20/04/12, 80 m
Dolycoris baccarum	60.0 ± 19.8	Weeds, nymphs	10	06/05/12, 100 m
Carpocoris mediterranus	10.5 ± 2.6	Weeds, nymphs	10	06/05/12, 100 m
Carpocoris mediterranus	9.5 ± 2.5	Weeds, nymphs	10	21/06/12, 100 m
Carpocoris sp.	8.3 ± 1.6	Weeds, nymphs	10	30/05/12, 1100 m
Rhynocoris sp.	20.7 ± 5.3	Weeds, adults	10	20/05/12, 1100 m
Rhynocoris sp.	21.0 ± 5.3	Weeds, adults	10	11/06/12, 1100 m
Rhynocoris annulatus	12.6 ± 5.8	Weeds, adults	10	11/06/12, 1100 m
Eurygaster integriceps	64.3 ± 11.1	Wheat, wintered adults	10	11/06/12, 1100 m
Aneyrosoma leucogrammes	3.7 ± 1.2	Weeds, adults	10	30/05/12, 1100 m
Notonecta spp.	8.1 ± 2.5	Water, adults	10	11/06/12, 1100 m
Nezara viridula	26.4 ± 9.3	Weeds, nymphs	10	20/05/12, 100 m
Nezara viridula	7.4 ± 1.3	Alfalfa, adults	10	21/06/12, 100 m
Nezara viridula	11.8 ± 3.9	Alfalfa, adults	10	12/11/12, 30 m
Aelia rostrata	5.2 ± 1.9	Wheat, wintered adults	10	21/03/12, 150 m
Lygaidae	15.2 ± 2.9	Soil, adults	10	21/06/12, 100 m
Apodiphus amygdali	1.8 ± 1.6	Cherry, adults	10	15/07/12, 870 m
Graphasoma lineatum	8.4 ± 1.6	*Salvia*, adults	10	15/07/12, 870 m
Aelia rostrata	9.6 ± 6.0	Soil, wintered adults	10	04/27/04, 650 m
Orthoptera				
Acrididae	55.2 ± 5.2	Soil, wintered adults	10	21/06/12, 100 m
Acrididae	50.1 ± 3.7	Weeds, adults	10	15/10/12, 110 m
Acrididae	37.5 ± 3.1	Weeds, adults	10	12/11/12, 30 m
Acrididae	44.8 ± 4.7	Weeds, adults	10	03/12/12, 56 m
Acrididae	77.5 ± 5.8	Alfalfa, adults	10	04/07/12, 100 m
Poecilimon spp. (Tettigoniidae)	27.3 ± 8.5	Weeds, nymphs	10	11/06/12, 1100 m
Poecilimon spp. (Tettigoniidae)	28.5 ± 5.5	Weeds, adults	10	12/11/12, 30 m
Gryllus assimilis	79.5 ± 5.5	Soil, adults	10	11/06/12, 1000 m
Gryllus bimaculatus	16.0 ± 1.5	Soil, adults	10	12/11/12, 55 m
Diptera				
Asilidae	52.0 ± 3.0	Weeds, adults	10	11/06/12, 1000 m
Calliphoridae	0.6 ± 0.3	Alfalfa, adults	6	03/12/12, 55 m
Hymenoptera				
Formicidae	1.1 ± 0.4	Soil, adults	10	11/06/12, 1000 m
Neodiprion sertifer	7.8 ± 1.1	Pine, larvae	10	11/06/12, 1100 m
Vespula spp.	3.8 ± 1.2	Weeds, adults	10	15/10/12, 56 m
Odonata				
Libellula depressa	14.6 ± 0.9	Weeds, adults	10	12/11/12, 55 m

Table 3. Average numbers of nodules in insects collected from fields in the Adana region in 2013. Values indicate numbers of discrete nodules ± SEM. Collection dates are in dd/mm/yy.

	Nodules/insect	Collection place, biological stages	Number of individuals	Collection date, altitude
Lepidoptera				
Geometrididae	13.3 ± 1.2	Alfalfa, larvae	10	04/02/13, 118 m
Papilio machaon	38.8 ± 2.3	Weeds, larvae	10	14/07/13, 84 m
Noctuidae	3.6 ± 0.9	Alfalfa, adults	10	30/06/13, 100 m
Autographa gamma	27.0 ± 8.1	Alfalfa, larvae	10	05/01/13, 120 m
Vanessa cardui	5.4 ± 1.2	Alfalfa, adults	10	05/01/13, 120 m
Vanessa cardui	14.3 ± 2.0	Alfalfa, adults	10	31/03/13, 56 m
Vanessa cardui	6.0 ± 1.0	Weeds, adults	10	07/04/13, 68 m
Vanessa cardui	19.8 ± 3.8	Alfalfa, larvae	10	12/05/13, 80 m
Vanessa cardui	6.2 ± 1.6	Weeds, adults	10	20/05/13, 60 m
Pieris brassicae	20.0 ± 2.6	Weeds, larvae	10	07/04/13, 75 m
Pieris brassicae	48.3 ± 14.3	Weeds, larvae	10	21/04/13, 80 m
Pieris brassicae	52.3 ± 10.2	Weeds, larvae	10	28/04/13, 130 m
Pieris brassicae	7.9 ± 1.9	Weeds, adults	10	20/05/13, 80 m
Pieris brassicae	13.0 ± 2.7	Alfalfa, adults	10	30/06/13, 100 m
Pieris brassicae	10.9 ± 3.1	Alfalfa, adults	10	14/07/13, 68 m
Pieris rapae	5.8 ± 1.0	Alfalfa, adults	10	20/03/13, 95 m
Pieris rapae	4.2 ± 0.9	Weeds, adults	10	07/04/13, 60 m
Pieris rapae	6.7 ± 2.0	Alfalfa, adults	10	21/07/13, 65 m
Pieris rapae	3.0 ± 1.5	Alfalfa, adults	10	11/08/13, 80 m
Pieris rapae	2.3 ± 0.6	Alfalfa, adults	10	18/08/13, 62 m
Hesperidae	2.8 ± 0.6	Weeds, adults	10	07/07/13, 170 m
Colias crocea	19.5 ± 4.5	Alfalfa, larvae	10	31/03/13, 57 m
Colias crocea	2.2 ± 0.7	Weeds, adults	10	07/04/13, 75 m
Colias crocea	3.1 ± 0.6	Weeds, adults	10	12/05/13, 70 m
Colias crocea	5.2 ± 1.6	Weeds, adults	10	20/05/13, 60 m
Colias crocea	8.5 ± 2.5	Alfalfa, adults	10	30/06/13, 100 m
Colias crocea	3.6 ± 2.8	Alfalfa, larvae	10	07/07/13, 90 m
Colias crocea	4.0 ± 1.0	Alfalfa, adults	10	14/07/13, 67 m
Colias crocea	3.2 ± 1.2	Alfalfa, adults	10	21/07/13, 65 m
Colias crocea	6.3 ± 2.9	Alfalfa, adults	10	11/08/13, 80 m
Colias crocea	2.0 ± 1.0	Alfalfa, adults	10	18/08/13, 62 m
Colias crocea	2.5 ± 1.5	Alfalfa, adults	10	25/08/13, 65 m
Satyridae	13.2 ± 5.6	Weeds, adults	10	23/06/13, 980 m
Aspitates sp.	19.2 ± 7.1	Weeds, larvae	10	27/05/13, 1000 m
Helicoverpa armigera	36.3 ± 5.2	Alfalfa, larvae	10	31/03/13, 58 m
Helicoverpa armigera	24.3 ± 2.5	Weeds, larvae	10	14/04/13, 75 m
Helicoverpa armigera	21.6 ± 3.2	Alfalfa, larvae	10	12/06/13, 60 m
Helicoverpa armigera	19.6 ± 1.4	Alfalfa, larvae	10	30/06/13, 100 m
Helicoverpa armigera	10.2 ± 2.2	Alfalfa, larvae	10	07/07/13, 90 m
Helicoverpa armigera	8.6 ± 1.6	Alfalfa, larvae	10	03/08/13, 63 m
Helicoverpa armigera	6.8 ± 2.3	Alfalfa, larvae	10	11/08/13, 500 m
Helicoverpa armigera	9.0 ± 1.8	Alfalfa, adults	10	18/08/13, 62 m
Helicoverpa armigera	9.0 ± 2.4	Alfalfa, larvae	10	01/09/13, 85 m
Polyommatus sp.	3.7 ± 1.8	Alfalfa, adults	10	21/07/13, 65 m
Amata sp.	31.1 ± 1.9	Weeds, larvae	10	03/06/13, 70 m

Table 3. (Continued).

Melonargia galethea	2.1 ± 1.0	Weeds, adults	10	20/05/13, 60 m
Geometridae	32.8 ± 5.3	Alfalfa, larvae	10	31/03/13, 53 m
Spodoptera exiqua	13.2 ± 2	Alfalfa, larvae	10	20/03/13, 95 m
Spodoptera exiqua	30.3 ± 7.2	Alfalfa, larvae	10	21/04/13, 80 m
Spodoptera littoralis	11.3 ± 3.1	Alfalfa, larvae	10	01/09/13, 80 m
Coleoptera				
Qulema melanapus	6.9 ± 0.6	Weeds, adults	10	04/02/13, 117 m
Adelia bipunctata	0.5 ± 0.5	Alfalfa, adults	10	04/02/13, 117 m
Adelia bipunctata	0.7 ± 0.3	Alfalfa, adults	10	11/08/13 505 m
Myriochila melancholica	17.9 ± 3.6	Soil, adults	10	03/08/13, 63 m
Myriochila melancholica	22.9 ± 4.6	Soil, adults	10	25/08/13, 65 m
Agriotes sp.	4.2 ± 0.8	Alfalfa, adults	10	21/04/13, 90 m
Julodis sp.	23.2 ± 2.6	Weeds, adults	10	14/04/13, 59 m
Julodis sp.	26.3 ± 5.1	Weeds, adults	10	28/04/13, 60 m
Omophlus proteus	9.5 ± 0.8	Weeds, adults	10	14/04/13, 67 m
Triponita hirta	7.7 ± 1.0	Weeds, adults	10	31/03/13, 56 m
Triponita hirta	8.7 ± 1.5	Weeds, adults	10	14/04/13, 58 m
Triponita hirta	12.8 ± 1.2	Weeds, adults	10	28/04/13, 70 m
Oxythyrea cinctella	19.3 ± 3.0	Weeds, adults	10	20/03/13 95 m
Oxythyrea cinctella	6.8 ± 2.4	Weeds, adults	10	20/05/13, 80 m
Oxythyrea cinctella	9.5 ± .3.5	Weeds, adults	10	14/07/13, 68 m
Coccinella semptempunctata	3.0 ± 1.0	Alfalfa, adults	10	05/01/13, 120 m
Coccinella semptempunctata	1.0 ± 0.3	Alfalfa, adults	10	04/02/13, 118 m
Coccinella semptempunctata	0.5 ± 0.5	Weeds, adults	10	20/03/13, 95 m
Coccinella semptempunctata	1.2 ± 0.3	Alfalfa, adults	10	20/03/13, 95 m
Coccinella semptempunctata	1.0 ± 0.6	Alfalfa, adults	10	31/03/13, 57 m
Coccinella semptempunctata	0.9 ± 0.4	Weeds, adults	10	14/04/13, 50 m
Coccinella semptempunctata	1.2 ± 0.3	Weeds, adults	10	28/04/13, 60 m
Coccinella semptempunctata	1.4 ± 0.5	Weeds, adults	10	05/05/13, 85 m
Coccinella semptempunctata	2.8 ± 0.9	Weeds, adults	10	12/05/13, 80 m
Coccinella semptempunctata	0.7 ± 0.3	Weeds, adults	10	03/06/13, 400 m
Coccinella semptempunctata	0.6 ± 0.2	Weeds, adults	10	12/06/13, 1000 m
Coccinella semptempunctata	0.0 ± 0.0	Weeds, adults	10	23/06/13, 980 m
Coccinella semptempunctata	0.5 ± 0.5	Alfalfa, adults	10	21/07/13, 70 m
Coccinella semptempunctata	0.6 ± 0.6	Alfalfa, adults	10	03/08/13, 63 m
Coccinella semptempunctata	1.3 ± 0.7	Alfalfa, adults	10	11/08/13, 505 m
Larinus latus	23.0 ± 2.5	Weeds, adults	10	14/04/13, 60 m
Larinus latus	38.2 ± 7.2	Weeds, adults	10	03/06/13, 1150 m
Larinus onopordi	34.0 ± 3.3	Weeds, adults	10	05/05/13, 85 m
Lixus sp.	10.2 ± 2.1	Weeds, adults	10	03/06/13, 1150 m
Cantharis spp.	4.1 ± 1.2	Weeds, adults	10	28/04/13, 60 m
Phyllopertha horticola	3.9 ± 0.8	Weeds, adults	10	14/04/13, 75 m
Phyllopertha horticola	18.3 ± 3.0	Weeds, adults	10	05/05/13, 80 m
Phyllopertha horticola	16.2 ± 4.3	Weeds, adults	10	03/06/13, 1150 m
Psyllioides sp.	1.5 ± 0.4	Weeds, larvae	10	05/05/13, 80 m
Anisoplia sp.	11.8 ± 1.9	Weeds, adults	10	14/04/13, 75 m
Anisoplia sp.	7.1 ± 1.8	Weeds, adults	10	05/05/13, 85 m
Nebria sp.	19.7 ± 3.8	Soil, adults	10	14/04/13, 74 m
Anisoplia austriaca	15.6 ± 2.1	Wheat, adults	10	27/05/13, 1000 m

Table 3. (Continued).

Clytra sp.	3.2 ± 0.9	Weeds, adults	10	23/06/13, 980 m
Labidostomis sp.	3.2 ± 0.8	Weeds, adults	10	20/05/13, 80 m
Gonioctena fornicata	0.8 ± 0.2	Alfalfa, adults	10	31/03/13, 58 m
Gonioctena fornicata	4.3 ± 1.2	Alfalfa, adults	10	21/04/13, 90 m
Gonioctena fornicata	2.9 ± 1.1	Weeds, adults	10	05/05/13, 85 m
Gonioctena fornicata	5.7 ± 1.8	Weeds, adults	10	27/05/13, 1000 m
Mylabris variabilis	12.1 ± 3.2	Weeds, adults	10	27/05/13, 1000 m
Coccinella bipunctata	1.2 ± 0.4	Weeds, adults	10	07/07/13, 170 m
Coccinella undecipunctata	0.2 ± 0.1	Alfalfa, adults	10	23/06/13, 65 m
Coccinella undecipunctata	0.6 ± 0.3	Alfalfa, adults	10	28/07/13,70 m
Coccinella undecipunctata	0.5 ± 0.2	Alfalfa, adults	10	01/09/13, 80 m
Lytta sp.	1.0 ± 0.4	Wheat, adults	10	20/03/13, 95 m
Hemiptera				
Eurygaster integriceps	105.8 ± 19.3	Soil, wintered adults	10	21/04/13, 80 m
Eurygaster integriceps	98.3 ± 16.3	Soil, wintered adults	10	28/04/13, 130 m
Eurygaster integriceps	11.1 ± 1.9	Weeds, new generation adults	10	12/06/13, 1000 m
Eurygaster integriceps	10.7 ± 1.9	Weeds, new generation adults	10	23/06/13, 980 m
Nezara viridula	6.0 ± 0.3	Alfalfa, adults	10	04/02/13, 118 m
Nezara viridula	7.2 ± 0.8	Alfalfa, adults	10	20/03/13, 95 m
Nezara viridula	10.3 ± 1.3	Weeds, adults	10	14/04/13, 57 m
Nezara viridula	12.3 ± 2.1	Alfalfa, adults	10	21/04/13, 80 m
Nezara viridula	11.2 ± 2	Alfalfa, nymphs	10	12/05/13, 80 m
Nezara viridula	10.0 ± 3.0	Weeds, nymphs	10	20/05/13, 80 m
Nezara viridula	12.0 ± 2.1	Weeds, adults	10	03/06/13, 400 m
Nezara viridula	13.9 ± 1.6	Alfalfa, adults	10	23/06/13, 65 m
Nezara viridula	11.3 ± 2.1	Alfalfa, adults	10	14/07/13, 67 m
Nezara viridula	5.6 ± 1.7	Alfalfa, nymphs	10	14/07/13, 67 m
Nezara viridula	4.3 ± 1.4	Alfalfa, nymphs	10	21/07/13, 70 m
Nezara viridula	5.5 ± 2.5	Alfalfa, adults	10	11/08/13, 80 m
Nezara viridula	9.0 ± 4.7	Alfalfa, adults	10	25/08/13, 64 m
Nezara viridula	0.2 ± 0.2	Alfalfa, nymphs	10	01/09/13, 85 m
Calocoris nemoralis	7.7 ± 2.3	Weeds, adults	10	31/03/13, 53 m
Eurydema ornatum	2.5 ± 0.5	Weeds, adults	10	20/03/13, 95 m
Eurydema ornatum	11.0 ± 3.2	Alfalfa, adults	10	14/07/13, 67 m
Eurydema ventrale	3.2 ± 1.2	Weeds, adults	10	28/07/13, 70 m
Eurydema ventrale	6.4 ± 2.2	Weeds, adults	10	11/08/13, 80 m
Eurydema ventrale	5.1 ± 0.9	Weeds, adults	10	18/08/13, 62 m
Eurydema ventrale	3.6 ± 0.9	Weeds, adults	10	25/08/13, 65 m
Eurydema ventrale	0.8 ± 0.3	Weeds, nymphs	10	25/08/13, 65 m
Eurydema ventrale	0.3 ± 0.2	Weeds, nymphs	10	01/09/13, 65 m
Klapperichicen viridissima	37.1 ± 2.2	Sycamore tree, adults	10	28/07/13, 70 m
Reduviidae	7.0 ± 1.6	Alfalfa, adults	10	30/06/13, 100 m
Reduviidae	10.0 ± 3.0	Weeds, adults	10	14/07/13, 65 m
Rhyncoris sp.	21.1 ± 4.6	Weeds, adults	10	12/05/13 1000 m
Rhyncoris sp.	22.8 ± 6.2	Weeds, adults	10	03/06/13, 1150 m
Rhyncoris sp.	3.0 ± 1.5	Weeds, adults	10	28/07/13, 70 m
Rhyncoris sp.	8.2 ± 1.7	Weeds, adults	10	25/08/13, 65 m
Apodiphus amygdali	16.3 ± 2.8	Sycamore tree, adults	10	18/08/13 47 m

Table 3. (Continued).

Miridae	5.6 ± 0.7	Weeds, adults	10	14/04/13, 57 m
Dolycoris baccarum	16.8 ± 1.7	Weeds, adults	10	14/04/13, 59 m
Dolycoris baccarum	31.8 ± 9.2	Alfalfa, adults	10	21/04/13, 95 m
Dolycoris baccarum	7.6 ± 3.0	Weeds, adults	10	12/06/13, 1000 m
Dolycoris baccarum	9.5 ± 3.5	Alfalfa, adults	10	30/06/13, 100 m
Dolycoris baccarum	2.8 ± 0.6	Alfalfa, adults	10	14/07/13, 67 m
Dolycoris baccarum	5.0 ± 2.1	Alfalfa, adults	10	21/07/13, 65 m
Carpocoris mediterranus	9.0 ± 2.0	Weeds, adults	10	20/03/13, 98 m
Carpocoris mediterranus	8.6 ± 1.3	Weeds, adults	10	07/04/13, 59 m
Carpocoris mediterranus	9.8 ± 1.6	Weeds, nymphs	10	28/04/13, 130 m
Carpocoris mediterranus	12.3 ± 3.8	Weeds, adults	10	03/08/13, 63 m
Carpocoris sp.	7.2 ± 1.3	Weeds, nymphs	10	27/05/13, 1000 m
Carpocoris sp.	19.0 ± 2.1	Alfalfa, adults	10	12/06/13, 60 m
Carpocoris sp.	17.0 ± 1.8	Alfalfa, adults	10	23/06/13, 65 m
Carpocoris sp.	6.8 ± 2.5	Alfalfa, adults	10	14/07/13, 68 m
Lygaeidae	4.0 ± 1.0	Weeds, adults	10	20/03/13, 95 m
Lygaeidae	0.6 ± 0.6	Weeds, adults	10	23/06/13, 980 m
Lygaeidae	2.7 ± 0.9	Weeds, adults	10	28/07/13, 70 m
Orthoptera				
Acrididae	68.7 ± 4.7	Weeds, adults	10	05/01/13, 120 m
Acrididae	73.9 ± 3.7	Weeds, adults	10	07/04/13, 75 m
Acrididae	68.6 ± 6.5	Weeds, adults	10	12/05/13, 80 m
Acrididae	86.5 ± 10.5	Weeds, adults	10	20/05/13, 80 m
Acrididae	115.5 ± 17.5	Alfalfa, adults	10	12/06/13, 60 m
Acrididae	53.3 ± 6.0	Weeds, nymphs	10	23/06/13, 980 m
Acrididae	85.0 ± 4.7	Alfalfa, adults	10	30/06/13, 100 m
Acrididae	63.8 ± 6.3	Soil, adults	10	07/07/13, 170 m
Acrididae	58.0 ± 18.4	Weeds, adults	10	14/07/13, 68 m
Acrididae	70.3 ± 5.7	Weeds, adults	10	03/08/13, 63 m
Acrididae	62.1 ± 5.7	Weeds, nymphs	10	11/08/13, 80 m
Acrididae	29.5 ± 5.7	Alfalfa, adults	10	11/08/13, 505 m
Acrididae	45.0 ± 5.8	Alfalfa, adults	10	18/08/13, 62 m
Poecilimon spp. (Tettigoniidae)	17.8 ± 3.6	Weeds, nymphs	10	20/03/13, 90 m
Poecilimon spp. (Tettigoniidae)	47.2 ± 3.0	Weeds, nymphs	10	07/04/13, 75 m
Poecilimon spp. (Tettigoniidae)	63.3 ± 5.0	Weeds, adults	10	20/05/13, 80 m
Poecilimon spp. (Tettigoniidae)	68.2 ± 9.2	Weeds, nymphs	10	27/05/13, 1000 m
Poecilimon spp. (Tettigoniidae)	40.8 ± 10.2	Weeds, nymphs	10	23/06/13, 980 m
Poecilimon spp. (Tettigoniidae)	57.7 ± 9.3	Alfalfa, adults	10	23/06/13, 65 m
Diptera				
Episyrphus balteatus	0.4 ± 0.2	Alfalfa, adults	10	04/02/13, 115 m
Eristalis tenax	20.0 ± 3.0	Weeds, adults	10	04/02/13, 117 m
Eristalis tenax	3.3 ± 0.6	Alfalfa, adults	10	20/03/13, 95 m
Eristalis tenax	8.4 ± 0.9	Weeds, adults	10	12/05/13, 70 m
Eristalis tenax	8.6 ± 2.1	Weeds, adults	10	27/05/13, 1000 m
Phasmida				
Gratidia sp.	24.0 ± 3.9	Weeds, adults	10	03/08/13, 63 m
Dermaptera				
Forficula sp.	5.0 ± 1.5	Weeds, adults	10	23/06/13, 980 m

Table 4. Single-factor ANOVA across species for collection site differences.

Collection sites in 2011	Nodules/insect[a]	Number of individuals
Plant material	27.0 ± 6.8b	70
Soil	120.3 ± 0.0a	10
Collection sites in 2012	Nodules/insect[a]	Number of individuals
Plant material	16.8 ± 2.1b	890
Soil	33.6 ± 11.9a	100
Collection sites in 2013	Nodules/insect[a]	Number of individuals
Plant material	10.2 ± 1.1b	1090
Soil	31.1 ± 10.9a	40

[a] Mean numbers of nodules in a column followed by different letters are significantly different for each year [($F_{(1,6)} = 23.63$, $P < 0.01$ for 2011), ($F_{(1,97)} = 5.27$, $P < 0.05$ for 2012), and ($F_{(1,111)} = 12.78$, $P < 0.001$ for 2013)].

Table 5. Single-factor ANOVA across species for insect order differences.

Insect orders in 2011	Nodules/insect[a]	Number of individuals
Lepidoptera	29.3 ± 7.5a	60
Hemiptera	66.7 ± 53.6a	20
Insect orders in 2012	Nodules/insect[a]	Number of individuals
Lepidoptera	15.9 ± 3.2b	180
Hemiptera	17.5 ± 4.0b	190
Coleoptera	14.9 ± 5.4b	290
Orthoptera	46.3 ± 7.3a	90
Insect orders in 2013	Nodules/insect[a]	Number of individuals
Lepidoptera	13.5 ± 1.8b	500
Hemiptera	7.9 ± 1.2b	530
Coleoptera	13.0 ± 2.6b	550
Orthoptera	61.9 ± 5.0a	190

[a] Mean numbers of nodules in a column followed by different letters are significantly different for each year [($F_{(1,6)} = 2.33$, $P = 0.1776$ for 2011), ($F_{(3,373)} = 4.84$, $P < 0.0001$ for 2012), and ($F_{(3,71)} = 64.31$, $P < 0.01$ for 2013)].

Manduca sexta, Dunn and Drake (1983) determined that following an injection of known numbers of bacterial cells, most bacterial cells were cleared from hemolymph circulation by nodulation in the first 2 h following infection. Later in the infection cycle, phagocytosis played a more significant role. Nodulation is seen as a complex process involving many steps, including the attachment of granulocytes to infecting bacterial cells, degranulation of granulocytes, attraction of plasmatocytes to the growing nodule, and the spreading of plasmatocytes around the nodule (Rowley and Ratcliffe, 1981). Dean et al. (2004) proposed an alternative model of nodulation that involves the action of a novel hemocyte form, which they named hyperphagocytic cells. According to their model, the novel hyperphagocytic cells are capable of attaching large numbers of bacterial cells, which become nuclei, for an ensuing sequence of cell actions that result in the formation of mature nodules. According to both models, the final step in nodulation is a melanization action driven by a cellular phenol oxidase. Finally, the darkened, melanized nodules attach to an internal organ or body wall, where they remain through the life of the insect.

Table 6. Single-factor ANOVA across species for biological stage differences.

Biological stages in 2011	Nodules/insect	Number of individuals
Adult	66.7 ± 53.6	20
Larvae	29.3 ± 7.5	60
Biological stages in 2012	Nodules/insect	Number of individuals
Adult	21.6 ± 3.8	520
Larvae	13.0 ± 2.7	220
Nymph	23.7 ± 8.1	60
Biological stages in 2013	Nodules/insect	Number of individuals
Adult	15.1 ± 1.8	1460
Larvae	21.6 ± 2.7	240
Nymph	22.6 ± 6.3	150

No significant differences were detected for each year [($F_{(1,6)}$ = 1.69, P = 0.2410 for 2011), ($F_{(2,77)}$ = 1.10, P = 0.3383 for 2012), and ($F_{(2,182)}$ = 1.59, P = 0.2069 for 2013)].

Because nodules are not cleared from insect hemocoels, they can be taken as a historical record of whether or not any particular insect has experienced a microbial infection. While the absence of nodules would not be positive proof that an insect is immunologically naïve, the presence of nodules indicates a past infection. Nodulation has been recorded following infections with bacteria (Miller et al., 1994), fungal spores (Dean et al., 2002; Lord et al., 2002), and some viral infections (Büyükgüzel et al., 2007; Durmuş et al., 2008). Moreover, in their work with several bacterial species, Howard et al. (1998) reported that some bacterial species evoked far more nodules than similar infections with other species. We infer that it is unlikely that a simple examination of nodules would reveal the nature of the infecting organism.

Using tobacco hornworms and larvae of the tenebrionid beetle *Zophobas atratus*, Howard et al. (1998) also found that nodulation intensity was related to the size of bacterial infection in a power rather than linear relationship ($y = 0.495 + 18.33X^{0.1558}$ and $y = 0.223 + 2.885X^{0.1343}$, respectively). These quantitative relationships emerged from analysis using one strain of one bacterial species. Nonetheless, it would appear that specimens with larger numbers of nodules had either experienced larger infections or had experienced multiple infections.

We noted an absence of clear patterns in natural microbial infections. As mentioned, we recorded far more nodules from insects associated with the soil than from insects collected from plants. This is true, for example, in sunn pest adults collected in April 2011 and April 2013. We noted that the new generation of sunn pests had very few nodules (approximately 10/adult) compared to older, overwintered adults (>105/individual), which is similar to the results reported by Tunaz and Stanley (2009). We recorded significantly more nodules from the orthopteran species than the lepidopteran, hemipteran, and coleopteran species, which is reasonable since orthopteran species were mostly collected from the soil. In general, insect orders and soil contact are probably the main associations with higher numbers of nodules. We infer from these observations that all insects are exposed to possible infection; however, the actual occurrence of a natural infection is a random event.

The specimens collected for this study appeared to be in good condition in the field. They were moving and consuming food, and on inspection their alimentary canals were filled. Specifically, the individuals that we examined exhibited the behavior and physical appearance of healthy animals. We take these observations to mean that the insects had experienced microbial infections, and by the time of our collections they had either checked the invasion or had recovered from the infections.

The ability to recover from infections in nature has profound biological and agricultural implications. Biologically, many microbes have evolved mechanisms to evade insect immune surveillance systems, allowing them to suppress their infection without stimulating host immune reactions. For example, the fungal insect pathogen, *Metarhizium anisopliae* produces a 60.4-kDa gene product, the MCL1 protein (Wang and St. Leger, 2006). This is a 3-domain protein with a central collagenous domain. This collagenous protein coats the hyphal bodies of the fungus and effectively hides the hyphal bodies from immune surveillance. Mutants disrupted in the *Mcl1* gene are rapidly attacked by hemocytes. Other microbes have evolved mechanisms to directly cripple insect immunity. The bacterium *Xenorhabdus nematophila*, for example, secretes factors that inhibit the eicosanoid signaling, which is crucial to launching cellular immune reactions (Stanley and Miller, 2006), and also secretes an antibiotic responsible for inhibiting phenol oxidase (Eleftherianos et al., 2007). These inhibitory actions render host insects entirely unable to activate immune effectors in the presence of infection. It would appear that insect immune systems exert selection forces on infecting microbes of sufficient power to influence evolution of mechanisms to avoid insect immunity.

The key agricultural implication of robust insect immunity is that the immune effectors can limit the usefulness and host ranges of microbial control agents. Many issues bear on the potential for increased use of biopesticides, including economics, political concerns, governmental roles, education (Lomer, 1999), and technical issues such as microbial product quality (Lacey et al., 2001). The data reported in this paper point to another important technical issue, namely our ability to fully understand and disable insect immune reactions.

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