***Autographa nigrisigna* looper (Lepidoptera: Noctuidae) excludes parasitoid egg using cuticular encystment induced by parasitoid ovarian fluid**

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Abstract

Autographa nigrisigna (Lepidoptera: Noctuidae) loopers form cuticular cysts on the dorsal site of the penultimate segment by parasitization of endoparasitoid *Campoletis chlorideae* (Hymenoptera: Ichneumonidae) and interrupt successful parasitization by excluding the parasitoid egg and larva in the hemocoel into the cyst. This study elucidated for the first time the difference in the exclusion rates with host age at the time of parasitization. All parasitized host loopers of D1L1 (Day 1, 1st instars) and D0L2 formed cuticular cysts and nearly 70% could exclude parasitoid eggs successfully; however, the number of loopers that could form cuticular cysts decreased with host aging at the time of parasitization. To elucidate the difference between the encapsulation system and cuticular encystment, it was examined whether the substances encapsulated are excluded by cuticular encystment. A small piece of antenna the same size as an egg used as a biotic foreign substance was absolutely encapsulated and was not excluded by encystment. Further, it was clarified whether parasitoid eggs with a different surface were encapsulated or excluded by cuticular encystment with or without ovarian fluid. UV eggs (with an intact surface and development ceased by UV treatment) were hard to encapsulate, but were excluded with encystment. Plasma eggs with a degenerate surface (treated with heating for 5 min at 60°C after 10-min immersion in plasma of *Pseudaletia separata*) were encapsulated and not excluded by cuticular encystment, although ovarian fluid increased the cyst formation rate. These results show that cuticular encystment functions to exclude parasitoid eggs incapable of encapsulation.

Key words: Cuticular encystment; encapsulation; Plusiinae; looper

INTRODUCTION

It is an important for living organisms to clear foreign organisms from inside the body. Lepidopteran larvae can exclude various organisms such fungi, bacteria or parasites that invade their hemocoel by means of phagocytosis, nodule formation or encapsulation as a defence system (Vinson, 1977; Gupta, 1989; Engström, 1992; Pathak, 1993; Pech and Strand, 1995; Ribeiro et al., 1996). Larger organisms than hemocytes invading the hemocoel are particularly excluded by a cellular defense system enclosing the hemocytes, like encapsulation (Salt, 1970).

Cuticular encystment is considered another type of defense system to clear away foreign substances such as endoparasitoid eggs from the hemocoel.

Arthur and Ewen (1975) reported that *Trichoplusia ni* (Hübner) looper formed an epicuticular cyst on the dorsum part of the penultimate segment when it was parasitized by a solitary endoparasitoid, *Banchus flavescens* Cresson. Cuticular encystment has been reported in eight species of Plusiinae loopers [*T. ni* (Hübner) (Arthur and Ewen, 1975), *Anagrapha falcifera* (Kirby) and *Autographa californica* (Speyer) (Ewen and Arthur, 1976), *A. nigrisigna* Walker, *Anadevidia peponis* (Fabricius), *T. intermixta* (Warren), *Acanthoplusia agnata* (Staudinger) and *Erythroplusia rutilifrons* (Walker) (Namba et al., 2004)], and appears to be common in Plusiinae species; however, the formation rate of the cuticular cyst varied according to host stages at the time of parasitization and the host species (Namba et al., 2004). There is no information

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about the efficacy of cuticular cyst formation in host developmental stages. It is well known that large foreign substances invading the hemocoel are encapsulated, but the question has arisen whether materials encapsulated in the hemocoel are excluded by cuticular encystment.

In this study, we firstly elucidated the difference in the exclusion rates with host age at the time of parasitization, and clarified the relationship between hemocyte response, such as encapsulation and exclusion by cuticular encystment, by using eggs with a different surface. Further, we examined the parasitoid factor causing cuticular encystment, such as an acid gland (venom), suggested by Ewen and Arthur (1976), in *T. ni* and *B. flavescens* association.

MATERIALS AND METHODS

Insect culture. Host looper *A. nigrisigna* were collected from cabbage fields in Nagakute, Aichi Prefecture in August 2004; however, some individuals were infected with *Nosema* sp. (Microsporididae). To establish a *Nosema*-free strain, *Nosema* infection was examined from each of the adult moths after egg collection. Eggs were collected from each paired moth in a vinyl bag (23×13 cm) with a cotton pad soaked in 2–3% sugar solution. Collected eggs were washed with 4–6% sodium hypochlorite according to Guy et al. (1985). *Nosema* infection was confirmed with the conventional method, that is, each whole body of the mother moth was crushed using a small clean mortar, and the smear was examined with Giemsa stain under a microscope (Sharan et al., 1992). Eggs oviposited by adult moths infected with *Nosema* were discarded. A selected *Nosema*-free strain was used as the host for experiments. Each parasitized or injected looper was reared individually on the artificial diet described previously (Kaneko, 1993; Namba et al., 2004) in small plastic Petri dishes (6 cm diameter, 1.5 cm high) under a long-day photoregime (16L:8D) at 25±1°C. *Campoletis chlorideae* (Uchida) (Hymenoptera: Ichneumonidae) is a solitary coinobiont endoparasitoid of many noctuid species (Lepidoptera: Noctuidae), including *Spodoptera litura* (Fabricius), *Helicoverpa armigera* (Hübner), *Helicoverpa assulta* Guenee, *Pseudaletia separata* (Walker), *Spodoptera exigua* (Hübner), *Agrotis ypsilon* (Rottemberg), *Anomis*

flava (Fabricius) and *Leucania loreyi* Duponchel (Sato, 1988; Yan and Wang, 2006). *C. chlorideae* was obtained from the host larvae, *S. litura*, *H. assulta*, *A. peponis* and *A. nigrisigna* collected in cabbage fields in Nagakute from September to November, 2003. The parasitoid colony was maintained in our laboratory by using 2nd instars of *P. separata* as a host. *C. chlorideae* parasitize younger hosts of *P. separata* successfully (Yan and Wang, 2006). After parasitization, *P. separata* larvae are reared on an artificial diet (Insecta® LF, Nihon Nohsan Co) under the same conditions as mentioned above. Newly emerged parasitoid wasps were kept in a plastic box (30×22×6 cm) for 3 days to ensure mating. Each plastic box was provided with a cotton pad soaked in 20–30% honey solution as food. Copulated adult wasps were maintained in a plastic tube (2.5 cm diameter, 9.5 cm long) with food until use.

Cuticular cyst formation rates of *A. nigrisigna* loopers parasitized at various different stages. *Autographa nigrisigna* loopers can pupate successfully by exclusion of parasitoid eggs. As it rarely happened that a parasitoid egg hatched after being confined in a cuticular cyst, it was regarded as exclusion of the egg. To clarify the relationship between cuticular cyst formation and successful parasitization, host loopers on different developmental stages were attacked by a *C. chlorideae* female 5–14 days post-emergence and the rates of cyst formation was examined. All host loopers attacked were reared individually to check for cuticular cyst formation until pupation or parasitoid emergence every day under a dissecting microscope. A cuticular cyst formed on the dorsal side of the penultimate segment is confirmed easily as exterior swelling under a dissecting microscope with low magnification.

Formation of a cuticular cyst on looper *Autographa nigrisigna* responding to various stimulations. In *A. nigrisigna*-*C. chlorideae* association, it is unknown which factors are effective for forming a cuticular cyst. To clarify the stimulation factor forming a cuticular cyst, four components, that is eggs, polydnavirus (PDV), ovarian fluid (OVF) and venom were taken from ovaries, and artificially injected into the looper. *C. chloridae* females have a typical ovary with a calyx region that produces PDV (Tian et al., 2007) and a poorly developed venom gland and reservoir like *C. sonorensis* (Nor-

ton et al., 1975; Webb and Strand, 2005). Lateral oviducts are filled with eggs, OVF and PDV. Ovaries were pulled out of the abdomen by clipping the ovipositor gently with fine forceps in physiological saline (PBS: 0.87% NaCl in 0.067 M phosphate buffer, pH 7.4). Venom reservoirs with filaments were separated from ovaries and placed in a dimple dish. Venom fluid was collected after spinning at 15,000 rpm for 10 min. Lateral oviducts after removing Dufour's gland were torn with fine forceps and spun for 10 min at 2,000 rpm to remove the cell debris and eggs. The resulting supernatant was centrifuged at 15,000 rpm for 10 min to separate PDV from OVF. The supernatant was adjusted to one female equivalent per microliter with PBS and used as ovarian fluid. The precipitate was used as PDV by adjusting to one female equivalent per microliter with PBS. Matured eggs were collected from lateral oviducts. Activated eggs (i.e., within 3 hours after oviposition) were retrieved from parasitized loopers. Both mature and activated eggs were centrifuged at 2,000 rpm for 10 min, and rinsed three times with PBS.

D1L2 and D0L3 loopers were used for material injection, because younger loopers were too small to inject materials artificially. Fourth and 5th instars were not suitable for this study judging from the results of parasitization data (Fig. 1). Each of three components (OVF, PDV and venom) was injected through a proleg of nonparasitized larva after narcotizing with CO₂ using a fine glass needle. Eggs were singly injected through a proleg in D0L3 loopers. D1L2 loopers were injected through a cervical location between the head and thorax because younger loopers before D1L2 were too small to inject eggs through a proleg successfully. The height (μm) of the cyst was measured 3–4 days after parasitoid attack when the cuticular cyst reached the maximum size.

Morphological observation of cuticular cyst.

To clarify the morphological difference in host loopers injected with various stimulants, 5 or 6 loopers in each case were fixed with 2.5% glutaraldehyde solution, post-fixed with Bouin's solution for 24 h, dehydrated and embedded in paraffin. Eight-micrometer sections were stained with Mayer's hematoxylin and 1% eosin Y solution (Sano, 1965). Further to examine amorphous materials secreted in a cyst, paraffin sections (20 μm thick) were double-stained with two kinds of

lectins, rhodamine succinylated wheat germ agglutinin (Rhodamin-s-WGA, Vector Lab., USA, 10–20 $\mu\text{g}/\text{ml}$ in PBS) and fluorescein tomato lectin (Fluorescein-LEL, Vector Lab. adjusted to the same concentration) for 2 h, then rinsed with PBS three times before observation using a confocal laser scanning microscope (LSM5 Pascal, Zeiss, Germany). WGA and LEL are lectins with binding sites specific for *N*-acetylglucosamine residues (Goldstein et al., 1975; Ward et al., 1985; Greven and Peters, 1986).

Response of loopers to artificially injected eggs with different surfaces. To examine the response of loopers to foreign substances, eggs after various treatments were injected into unparasitized and parasitized hosts and were observed as to whether they were encapsulated or excluded by cuticular encystment. To make eggs that ceased development but still possessed an intact surface, the activated eggs above were treated with ultraviolet (UV) light at 5 cm distance for 3 min, and were used as UV eggs. One percent SDS was reported to be effective to modify the parasitoid egg surface (Asgari and Schmidt, 1994). UV eggs were immersed in 1% SDS-PBS for 5 min; however, SDS eggs were not encapsulated in the host at least 24 h after injection ($n=12$, singly injected in a cervical place of D0L3). Heat treatment of eggs also was not effective to alternate the egg surface, because heating at 98°C for 5 min gave almost the same result as UV treatment alone (no encapsulation 62.5%, exclusion 37.5%, $N=8$). Further, to make eggs with a degenerated surface, UV eggs were first immersed in plasma of *P. separata* for 10 min at 25°C, following by heating for 5 min at 60°C, and used as plasma eggs. Cell-free hemolymph, as plasma, was adjusted as follows: hemolymph from day 3–4 last instars of *P. separata* after narcotization was collected on ice, 8% saturated 1-phenyl-2-thiourea (PTU) per microliter hemolymph in volume was immediately added, and then centrifuged to remove hemocytes at 2,000 rpm for 10 min at 4°C. Treated eggs were injected into a cervical location between the head and thorax with a fine glass needle-like injection of matured and activated eggs. A small piece of antenna was used as biotic foreign material. A piece of antenna from a *C. chloridae* female was cut to the same size as the parasitoid egg and heated for 5 min at 60°C before injection. The result (Table 1) that ovarian fluid

was most effective for cuticular cyst formation among the three components (OVF, PDV and venom) led us to confirm whether the encapsulation rate was enhanced instead of forming a cuticular cyst when no cyst was formed or whether another stimulant formed a cuticular cyst. With a dual injection, only one egg was injected per loopers within a few minutes following injection with OVF (3–4/10 female equivalent per loopers). Loopers were dissected in PTU-PBS (8% to saturated PTU in PBS) 3 days after injection to observe the encapsulation degree after confirming cuticular cyst formation under a dissecting microscope.

RESULTS

Cuticular cyst formation rates on *A. nigrisigna* loopers parasitized at various different stages

In early stages, such as D1L1 and D0L2, all loopers formed cuticular cysts (CF) and nearly 70% host loopers could pupate by exclusion of parasitoid egg or larva, but about 20% host loopers were killed successfully by parasitoid larvae egression (Fig. 1). Data were divided between loopers forming a cuticular cyst (CF) and those that failed to form a cuticular cyst (NCF) in the developmental stage of the host loopers at parasitization. The pupation rate of CF loopers decreased with the age of host loopers at the time of parasitization, while the number of NCF loopers increased gradually in 2nd and later instars (Fig. 1). Almost all D1L2 and D0L3 loopers in NCF were killed by successful parasitism. About half of all D0L4 loopers could form cysts (CF) but half failed to do so (NCF). More than 80% of parasitized D1L4 loopers could not form a cuticular cyst (NCF) and low successful parasitization was observed (Fig. 1). These results mean that *C. chloridae* failed to parasitize older host loopers like late 4th instars.

Formation of cuticular cyst on loopers *Auto-grapha nigrisigna* responding to various stimuli

Three components (OVF, PDV and venom) injected with the egg at oviposition, were examined to clarify the stimulation factor to form a cuticular cyst. OVF showed the strongest stimulant action to form a cuticular cyst among substances. The rate of cuticular cyst formation was 77.8% and 66.7% in D1L2 and D0L3, respectively (Table 1). Venom or PDV from *C. chloridae* did not form a cyst on

Fig. 1. Ratio of cuticular cyst formation on *A. nigrisigna* loopers attacked at various different stages. CF: loopers formed a cuticular cyst, NCF: loopers failed to form a cuticular cyst. Stage of each loopers attacked by *C. chloridae* is shown as day-instar (i.e. D1L1: day 1, 1st instar). Different letters in the same pattern of the same column (CF or NCF) indicate significant difference by chi-square independent test ($p < 0.05$) for respective values between two after one-way ANOVA.

loopers, similar to saline. A female wasp injects an egg with OVF, venom and PDV in the host at oviposition. Furthermore, we paid attention to the potential of eggs as a stimulant to form a cuticular cyst. Cyst formation in parasitized hosts as a positive control was about 80% in both D1L2 and D0L3. Injection of only matured and activated eggs showed that eggs worked as a stimulant in cyst formation, although they had a little less stimulant action than OVF (Table 1); however, cuticular cysts were similar in size to those in parasitized loopers.

The OVF dose response was acquired in D1L2 loopers (Fig. 2). One tenth-diluted OVF gave a 70% response in forming a cyst. Parasitization caused about 80% cyst formation in D1L2 and showed a similar rate to that of a one-tenth dose. Forty to 160 times dilution of one female equivalent gave about 20% cyst formation (Fig. 2).

Morphological observation of cuticular cyst

Epidermal cells became thickened and secreted cuticular-like material (clm) into the cyst space with OVF stimulation (Fig. 3A), contrary to no

Table 1. Rate of cuticular cyst formation on looper *Autographa nigrisigna* to various stimulation

Materials injected into unparasitized hosts	Developmental stage of loopers injected			
	D1L2 ^a		D0L3 ^a	
	Cyst formation rate (%)	Average height of cyst (μm)	Cyst formation rate (%)	Average height of cyst (μm)
Ovarian fluid	77.8 (28 ^b /36) a ^c	242 $\pm$ 33 a ^d	66.7 (18 ^b /27) a ^c	256 $\pm$ 67 a ^d
Polydnavirus	4.5 (2/44) b	—	6.7 (1/15) b	—
Venom	2.2 (1/45) b	—	0 (0/15)	—
PBS	7.1 (3/42) b	—	6.7 (1/15) b	—
Matured eggs ^e	69.2 (9/13) a	240 $\pm$ 89 a	50.0 (10/20) a	198 $\pm$ 49 a
Activated eggs ^f	73.5 (25/34) a	203 $\pm$ 58 a	58.3 (42/72) a	199 $\pm$ 70 a
Parasitized host	80.0 (40/45) a	221 $\pm$ 72 a	80.8 (21/26) a	203 $\pm$ 62 a

^a Injected volume was about 1/10 μl to day 1–2 instar (D1L2) and about 3/10 μl to day 0–3 instar (D0L3). Each sample was adjusted to one female equivalent per one μl .

^b Number of loopers that formed cuticular cysts in hosts injected with each material.

^c Different letters in the same column indicate statistical difference by contingency chi-square analysis ($p < 0.01$) for respective values except less than 5, by Fisher's exact test.

^d Same letter in the same column indicates non-significant difference by one-way ANOVA ($p < 0.01$).

^e Mature eggs were taken from lateral oviducts of *Camponotus chloridea*.

^f Activated eggs were taken from parasitized loopers within 3 h after oviposition. One egg was injected per a looper.

Fig. 2. Dose responses of *A. nigrisigna* looper to various concentrations of ovarian fluid from *C. chloridea*. Injection was performed on day 1, 2nd instar loopers. Rate of cyst formation was examined day 3 after injection. N indicates the number of loopers injected with each concentration of ovarian fluid. Para: parasitized control. Different letters in each bar indicate significant difference by one-way ANOVA ($p < 0.05$).

thickening of the epidermis in control samples (Fig. 3B). OVF stimulation increased the height of epidermal cells compared to those with normal parasitization (Fig. 3C). UV eggs also stimulated epidermal cells to secrete clm 72 h after injection (Fig. 3D). The thickened epidermal layer secreted cuticular-like material stained with fluorescein

tomato lectin (LEL) in the inter-space of cysts (Fig. 3E).

Response of host loopers to injected materials by encapsulation or cuticular encystment

To clarify the relationship between the encapsulation reaction and cuticular encystment, the response to parasitoid eggs was examined. UV eggs with an intact egg surface could avoid a host encapsulation reaction and about 30% UV eggs were excluded by cuticular encystment (Table 2). Plasma eggs with a degenerated surface were encapsulated at a rate of 40–60% regardless of OVF coexistence, but no egg was excluded by encystment. An antenna used as a positive control was absolutely encapsulated and was not excluded by encystment.

To examine the response against eggs with a different surface in the hemolymph of hosts regulated under normal parasitization, the same materials were injected into hosts within 10 min after parasitization. UV eggs and plasma eggs were easy to distinguish from normal eggs oviposited by females because of the different egg surface. More than one-half of the parasitized hosts formed cuticular cysts and excluded 13% of UV eggs and 15%

Table 2. Response of loopers to injected materials by encapsulation or cuticular encystment in day 0–3 instar loopers

Injected materials	Total no. of loopers injected [A]	Encapsulated			Not encapsulated (%)			No. of loopers forming cyst [F] [F/(D+E)] (%) ^c
		Total [(B+C)/A] (%)	Fully [B]	Partially ^a [C]	Total [(D+E)/A] (%)	No. of eggs unencapsulated in the hemocoel [D] [D/A] (%)	No. of eggs excluded by cyst [E] ^b [E/A] (%)	
Unparasitized host	With ovarian fluid ^d							
	UV egg ^e	24	2 (8.3)	1 ^h	22 (91.7)	15 (62.5) ac	7 (29.1)	15 (62.5) a
	Plasma egg ^f	20	11 (55)	3	9 (45.0)	9 (45.0) ab	0	16 (80.0) a
	Antenna ^g	15	15 (100)	0	0	0	0	12 (80.0) a
	Without ovarian fluid							
	UV egg	17	3 (17.6)	0	14 (82.4)	9 (52.9) abc	5 (29.4) a	7 (41.2) b
Parasitized host	Plasma egg	30	20 (66.7)	2	10 (33.3)	10 (33.3) b	0	2 (6.7) c
	Antenna	15	15 (100)	0	0	0	0	0
	UV egg	23	2 (8.7)	2	21 (91.3)	18 (78.3) c	3 (13.0) a	12 (52.2) ab
	Plasma egg	26	3 (11.5)	2	23 (88.5)	19 (73.1) ac	4 (15.4) a	15 (57.7) ab
	Antenna	16	16 (100)	0	0	0	0	11 (68.8) ab

^a Nearly half of egg surface was covered with hemocytes.^b Exclusion rate of injected materials by cuticular encystment.^c Rate of cuticular cyst formation in host loopers injected with materials.^d Ovarian fluid was removed from eggs and polydnavirus from lateral oviduct of female ovaries by centrifugation at 15,000 rpm for 10 min. Amount of ovarian fluid injected per looper was 3/10 female equivalent.^e UV egg was made from activated eggs exposed to germicidal ultraviolet for 3 min at 5 cm distance. Activated eggs were retrieved from parasitized 2nd instars 3 h after oviposition.^f Plasma egg was made from activated eggs incubated in cell-free hemolymph of non-parasitized day 3–6 instar of *P. separata* for 10 min, then heated at 60°C for 5 min prior to injection.^g A piece of *C. chloridae* antenna was cut to the same size as the egg and exposed to UV light, heated at 60°C for 5 min, and used as a positive control of biotic material.^h Different letters in the same column indicate statistical difference by contingency chi-square analysis ($p < 0.05$) for respective values except less than 5, by Fisher's exact test.

Fig. 3. Morphological change of epidermis on penultimate segment by injection of various materials. A: Host epidermal cells were thickened and secreted cuticular-like materials (clm) in inter-space of cyst 72 h after ovarian fluid (OVF) injection. B: Control of epidermal cells at the same developmental stage. C: Cuticular cyst of host looper 39 h after oviposition. *Campoletis chlorideae* egg (e) is excluded in the cuticular cyst. Epidermal cells are thickened and secrete clm. D: Injection of UV eggs also resulted in thickened epidermal cells and clm secretion 72 h after injection. E: Space in cuticular cyst was filled with clm stained with fluorescein tomato lectin (LEL) 72 h after oviposition. e: parasitoid egg. FB: fat body, ed: epidermis. Bar=50 μ m.

of plasma eggs by encystment without encapsulation.

DISCUSSION

Although it was reported that cuticular encystment worked as an exclusion mechanism in *Plusi-*

inae loopers (Arthur and Ewen, 1975; Ewen and Arthur, 1976; Namba et al., 2004), there was no information about the exclusion rate of parasitoid eggs with the aging of loopers at the time of parasitization. Our results show that younger loopers as late 1st and early 2nd instars are able to pupate by excluding parasitoid eggs during development even after parasitization; however, the pupation rate from late 2nd to late 3rd instars also decreased gradually. In particular, older loopers, such as 4th instars, were unsuitable for *C. chloridae* parasitization in spite of oviposition, because more than 80% of D1L4 loopers could not form a cuticular cyst and most pupated.

Ovarian fluid induced cuticular cyst formation in the penultimate segment of *A. nigrisigna*. On the other hand, venom fluid provided no stimulation to form a cuticular cyst, unlike in the *B. flavescens*-*T. ni* system (Ewen and Arthur, 1976). Although the cause of this difference remains unclarified, it seems reasonable that venom function transfers to that of ovarian fluid. Both mature and activated eggs act as stimulants to form a cyst just like OVF. Mature eggs were taken from lateral oviducts filled with OVF. It is considered that washing with saline may be not enough to remove OVF components from the egg surface. Although oviposited eggs seem to have their surface altered by the action of the host hemolymph, the altered surface could still stimulate epidermal cells to form a cyst. Davies and Vinson (1986) reported that in *Cardiochiles nigricipes*-*H. virescens* association, mature eggs obtained from lateral oviducts or from parasitized hosts within 1 h after oviposition are not encapsulated *in vitro*, or *in vivo* after 24 h, whereas all immature eggs located within the ovariole are encapsulated. A fibrous layer surrounding the egg surface seems to protect the egg from encapsulation. Asgari and Schmidt (1994) also reported a protective coat on the egg surface, which can be removed easily by SDS detergent. In the case of *C. chloridae*, SDS detergent had no effect on the degeneration of the egg surface. UV eggs can also escape from encapsulation of the host and stimulate the epidermal cells to form a cyst, probably due to the same intact surface as the activated eggs. The fact that about 50% of UV eggs were not encapsulated supports the finding that half of parasitized host loopers are killed by successful parasitism. If oviposited eggs develop without artificial aborting

by UV, parasitization will be successful at 50%. This means that the egg surface itself may act to avoid host encapsulation rather than coating material on the egg surface. The egg surface seems to stimulate epidermal cells. Materials injected into the host hemocoel were probably carried with backward blood-flow through the body cavity until they finally reached the penultimate segment which is opened around blood vessels. It is still unknown why only epidermal cells around the penultimate segment respond to OVF stimulation or UV eggs.

Plasma eggs injected into unparasitized loopers without ovarian fluid were encapsulated at a high rate and were difficult to exclude. Plasma eggs possess a degenerated surface from the effect of host hemolymph and heating; however, in parasitized loopers, the encapsulation rate decreased to 3.8%, although it became 11.5% if adding the rate of partially encapsulated eggs. Plasma eggs were hard to recognize as foreign substances in hemolymph conditioned with OVF and PDV plus venom. Recognition of foreign substances in parasitized hosts seems not to be simply explained by differences in the egg surface. The small pieces of antennae from female wasps used as biotic material were completely encapsulated as foreign substances even when the loopers were parasitized. None of the injected antenna was confined in the cyst. This shows that encapsulated foreign substances are not excluded by cuticular encystment. The encapsulated foreign substance probably did not move into the host hemocoel by attaching to some organs like the fat body or trachea.

Our results suggest that *A. nigrisigna* has evolved the cuticular encystment mechanism to exclude parasitoid eggs that are not encapsulated in the host hemocoel. This is especially true in younger instar loopers that do not develop sufficient encapsulation response to foreign substances; therefore, cuticular encystment may be effective to exclude foreign substances.

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